The Effect of Environmental and Anthropogenic Conditions on Shaping the Host-Microbiome of the Breadcrumb Sponge *Halichondria panicea*.

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Abstract

Marine sponges are vital to marine ecosystems, offering crucial habitats and functions, with their microbiome significantly contributing to nutrient recycling, biogeochemical activity, and host defense. The impact of the surrounding environment on marine sponge microbiomes has not been thoroughly explored, particularly in coastal areas with high pollution levels. To fill this gap, the sponge *Halichondria panicea* was selected in this study as an ideal model for coastal sponges because it is highly abundant in coastal areas around the world. This thesis presents a field study of the impact of environmental, biological, seasonal, and anthropogenic factors on *H. panicea*. Over the course of a year (2022), samples of the *H. panicea* microbial community were collected from three locations in the north-east of England, with a variety of bathing water quality levels, according to the UK's Environment Agency. Sponge, seawater, and attached seaweed samples were collected from the three locations and sequenced using metabarcoding of the 16S rRNA gene. The study emphasises the significant role of the seasons and anthropogenic activities in shaping the sponge bacterial community by affecting both composition and diversity. It also explores the acquisition of bacterial communities by *H. panicea* from its surroundings, revealing a significant proportion of the sponge microbiome is shared with seaweed, highlighting the interconnectedness of marine organisms in coastal environments. Additionally, the research investigates the relationship between the sponge's two colour morphs and its microbial community, suggesting that the yellow morph may be indicative of a sponge subjected to a certain stress. The study also includes the ex-situ cultivation of *H. panicea* to study the effect of different antifouling paints, revealing the negative impact of biocidal antifouling paint on sponge survival. Overall, the results of the thesis illustrate the complex interplay between environmental stressors and bacterial communities of the sponge *H. panicea* in coastal areas.

Dedication

To my wonderful family, Hadi, Ayat and Kawthar

Thank you for all the love, patience and support.

To my beloved Dad for his support and continuous prayers.

To the love of my life … Mom, who has always believed in me. May her soul rest in peace.

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Publications related to this thesis

Chapter 2 was published in October 2024 in the Aquatic Sciences journal.

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Declaration

I, Sakinah Alhaddad, hereby declare that I am the author of this thesis. All work described here in this thesis is my own, except where stated in the text. The work presented here has not been accepted in any previous application for a higher degree. All the sources of information have been consulted by myself and are acknowledged by means of references.

Sakinah Al-Haddad

April 2024

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Abbreviations

AMR - Antimicrobial resistant

ASV - Amplicon sequence variance

ASW – Sterile artificial seawater

DGGE - Denaturing gradient gel electrophoresis

EUMS – European Union Member States

FIB – Faecal indicator bacteria

FISH – Fluorescence in situ hybridisation

HMA - High microbial abundance

HTS – High throughput sequencing

IC - Ion chromatography

ICP-MS - Inductively coupled plasma mass spectrometry.

ICP-OES - Inductively coupled plasma optical emission spectroscopy

KEGG – Kyoto encyclopedia of genes and genomes

KISR – Kuwait Institute for Scientific Research

KO – KEGG Orthology

KΩ – Kiloohm

LMA – Low microbial abundance

MMT – Mixed mode of transmission

MTB – Magnetotactic bacteria

NGS – Next generation sequencing

NWD – Natural water discharge

OA – Ocean acidification

OTU – Operational taxonomic unit

PAR – Photosynthetic active radiation

PCR – Polymerase chain reaction

ppb – Part per billion

ppm – Part per million

ppt – Part per thousand

SSU – Small ribosomal subunit

TDS – Total dissolved solids

TRFLP – Terminal restriction fragment length polymorphism

UK – United Kingdom

US – United States

UV – Ultraviolet

Chapter 1. Introduction

1.1 Marine Sponges

Sponges, are traditionally viewed as the oldest extant animal phylum – the Porifera (Dohrmann & Wörheide, 2017; King & Rokas, 2017). They are multicellular marine animals with simple morphology, lacking nerves and digestive tract. Sponges filter seawater for food and acquire nutrients by phagocytosing captured bacteria and planktonic species (Enticknap *et al*., 2006; Levi & Levi, 1965). A notable property of sponges is their ability to regenerate, a phenomenon that appears to be driven by cell motility and unusual plasticity in differentiation. Since the nineteenth century, sponges have been of interest to cell biologists because of these characteristics (James-Clark, 1867; Saville-Kent, 1880; Wilson, 1907).

1.1.1 Sponge structure and physiology

As the genus name implies, the Porifera (pore bearing) bear a multitude of tiny openings (ostia) puncturing the adult and juvenile sponge bodies. The sponge body comprises a jelly-like substance (mesohyl), mainly collagen, reinforced by a dense network of collagen fibres. The mesohyl is surrounded by two major types of cells: choanocytes, the main proliferating cell type, which line the inner surface, and pinacocytes, which form the outer skin (Hentschel *et al*., 2012). Water passes through the ostia to enter chambers formed by choanocytes (Figure 1.1), characterised by an apical collar of narrow-spaced, rod-like microvilli surrounding a long flagellum. The beating of flagella boosts water flow through the chambers and then out through a large exhalant opening known as the osculum. Food particles (primarily bacteria) are captured by the collars of choanocytes as the water passes through the chambers (Leys & Eerkes-Medrano, 2006). Choanocyte proliferation in sponges is often accompanied by shedding, a process where non-apoptotic cells are lost. A sponge's rapid cell turnover supports tissue homeostasis, enabling sponges to continually regenerate their cell populations and remove damaged or aging cells, thus maintaining the tissue's health and functionality. The high turnover rate is a result of sponge tissues' dynamic nature, as they must adapt quickly to changes in environmental conditions while processing large volumes of water efficiently (Alexander *et al*., 2014). Pinacocytes are flat, thin cells that cover the sponge's exterior surface (Leys *et al*., 2009). They digest food particles that are too large to enter the ostia (Berquist, 1998; Ruppert *et al*., 2004) and anchor the base of the sponge

(Berquist, 1998). A large variety of cells may occur in the mesohyl between the pinacoderm and choanoderm depending on the sponge species (Simpson, 1984), including lophocytes, collencytes, rhabdiferous cells, oocytes, spermatocytes, sclerocytes, spongocytes, myocytes (muscle cells), grey cells and archaeocytes (or amoebocytes). Each cell type has its own function in the sponge. Lophocytes and collencytes are responsible for collagen secretion inside the mesohyl. Rhabdiferous cells produce polysaccharides that form the mesohyl. The spongocyte is a unique cell type only found in demosponges. It is responsible for producing collagen that is polymerised into spongin and stiffens the mesohyl. Archeocyte and sclerocyte cells, present in almost all sponge species, are well-studied cell types. They are amoeboid cells that move freely within the mesohyl (Ereskovsky, 2010). Archeocytes contain nuclei and are considered pluripotent stem cells; they feed on and remove debris that clogs the ostia (Funayama, 2010). Sclerocytes secrete spicules, sponge-like skeletal elements used as a defence against predators (Sethmann & Wörheide, 2008).

Sponges are classified into four classes based on the morphology and composition of the spicules. The Calcarea have calcareous skeletons (calcium carbonate spicules). The Hexactinellida are syncytial glass sponges that contain silicon dioxide as their skeleton. The Homoscleromorpha possess basement membranes and cell junctions resembling those of metazoans. They exhibit a massive or encrusting form, a very simple structure, as well as minimal variation in spicule size. The most common class is the Demospongiae, which comprises almost 90% of extant sponges, including all freshwater varieties, and they occupy the greatest diversity of habitats. Demosponges contain spongins and may or may not have spicules (Ereskovsky, 2010).

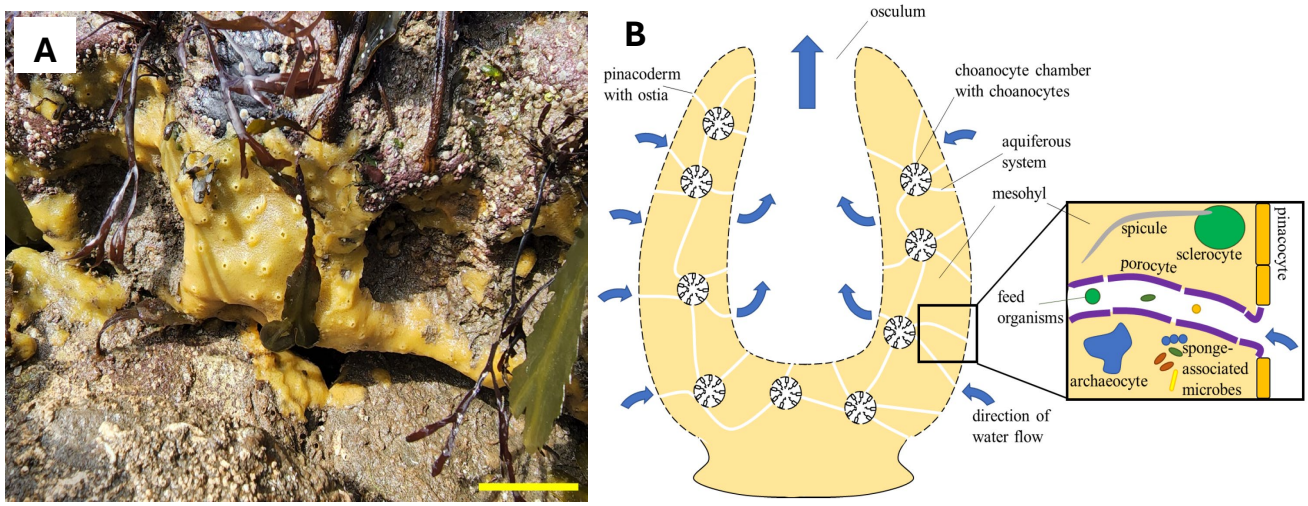


Figure 1. 1. A. The sponge *Halichondria panicea* (demosponge) in the marine environment, picture taken in January 2023 from Whitley Bay coast (the scale bar represents 13.2 cm). **B**. Diagram showing the typical demosponge anatomy and its internal structure enlarged (adapted from Knobloch, 2019). The arrows show the direction of water flow, which carries food particles, and the sponge aquiferous system.

1.1.2 The role of sponges in the marine ecosystem

Marine animals live in water containing numerous diverse microorganisms - bacteria, viruses, protozoa, archaea, fungi, and microalgae - that play a role in global biogeochemical cycles (Bolhuis & Cretoiu, 2016). Most marine microbes are found as biofilms (Ikuma *et al*., 2013) but can also be found in planktonic states, as well as in symbiotic relationships with other aquatic organisms (Apprill, 2017). Sponges play a significant role in influencing community structure and marine benthic ecosystem functioning (Figure 1.2). In fact, sponge-associated microbes mediate many of these functional roles (Pita *et al*., 2016) in three main categories. First is their impact on the substratum, including bioerosion, reef creation, substrate stabilisation, consolidation, and regeneration. Secondly, their association with other organisms facilitates primary and secondary production, provides microhabitat, enhances protection against predation, and increases survival success, range expansions and camouflage. Moreover, sponges act as settlement substrates, disrupting near-boundary and reef-level flow regimes; many are agents of biological disturbance

and release chemicals. Finally, they function in cycling nutrients, including carbon, silicon, and nitrogen, and in oxygen depletion (Bell, 2008).

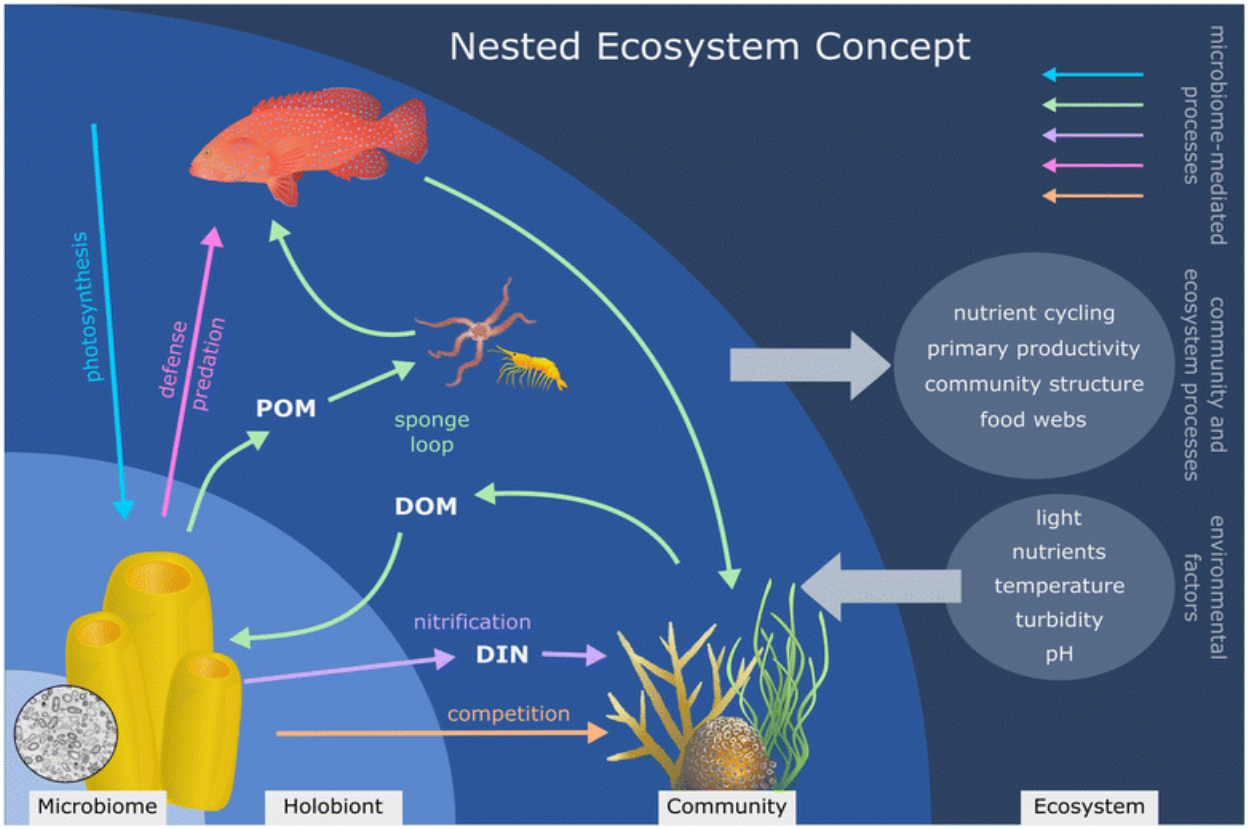


Figure 1. 2. Sponge functions within the marine ecosystem mediated by the microbiome. Microbiome functions (coloured arrows) contribute to holobiont functioning, which, in turn, affects community structure and ecosystems. The environment influences the microbiome, holobiont, community, and ecosystem processes at multiple scales. DOM: dissolved organic matter; POM: particulate organic matter; DIN: dissolved inorganic nitrogen (adapted from Pita *et al.*, 2018).

1.2 Sponge Microbiome

Sponges have no organs. Their health and function are dependent mainly on microorganisms in a symbiosis where both the host and the microbes benefit. Sponge-associated microsymbionts are the most diverse among marine invertebrates (Simister, *et al.*, 2011; Taylor, Radax, *et al.*, 2007). The complex network of interactions between symbiotic microbes and their host results in a distinct ecological unit known as a holobiont (Margulis & Fester, 1991; Simon *et al.*, 2019). During the sponge life cycle, the microbial component of the holobiont performs

essential functions for the sponge that are impossible for either partner to perform separately (McFall-Ngai *et al*., 2013), including immunity (Eberl, 2010), nutrition (Nicholson *et al*., 2012), defence (Flórez *et al*., 2015), and development (Koropatnick *et al*., 2004).

To avoid controversy regarding the members of a microbiome, it is crucial to clearly distinguish between the term microbiome and microbiota (Figure 1.3). Generally, microbiota refers to the collection of living microorganisms present in a defined medium, which includes bacteria, archaea, fungi, algae, and small protists (Marchesi & Ravel, 2015). It is debatable whether phages, viruses, plasmids, prions, viroids, and free DNA belong to the microbiota as they are not considered to be living microorganisms (Dupré & O'Malley, 2009). In the view of Whipps and coworkers (Whipps *et al*., 1988), however, a microbiome encompasses not only the community of microorganisms but also their "theatre of activity", which contains the full range of molecules they produce. In addition to their structural elements (nucleic acids, proteins, lipids, polysaccharides), metabolites (signalling molecules, toxins) and molecules produced by co-existing hosts are influenced by the surrounding environment. As a result, all mobile genetic elements, such as viruses, phages, extracellular DNA, and relics (DNA excreted from living and dead cells), should be considered microbiomes, not microbiota. Further, it is important to consider methodological aspects, especially when determining the differences between DNA and living organisms (Berg *et al*., 2020) because the presence of relic DNA can inflate observed prokaryotic and fungi diversity and lead to inaccurate estimations of relative abundances of taxa (Carini *et al*., 2016). There is also a tendency to confuse the term microbiome with metagenome. The latter refers to a collection of genomes and genes found in members of the microbiota (Marchesi & Ravel, 2015).

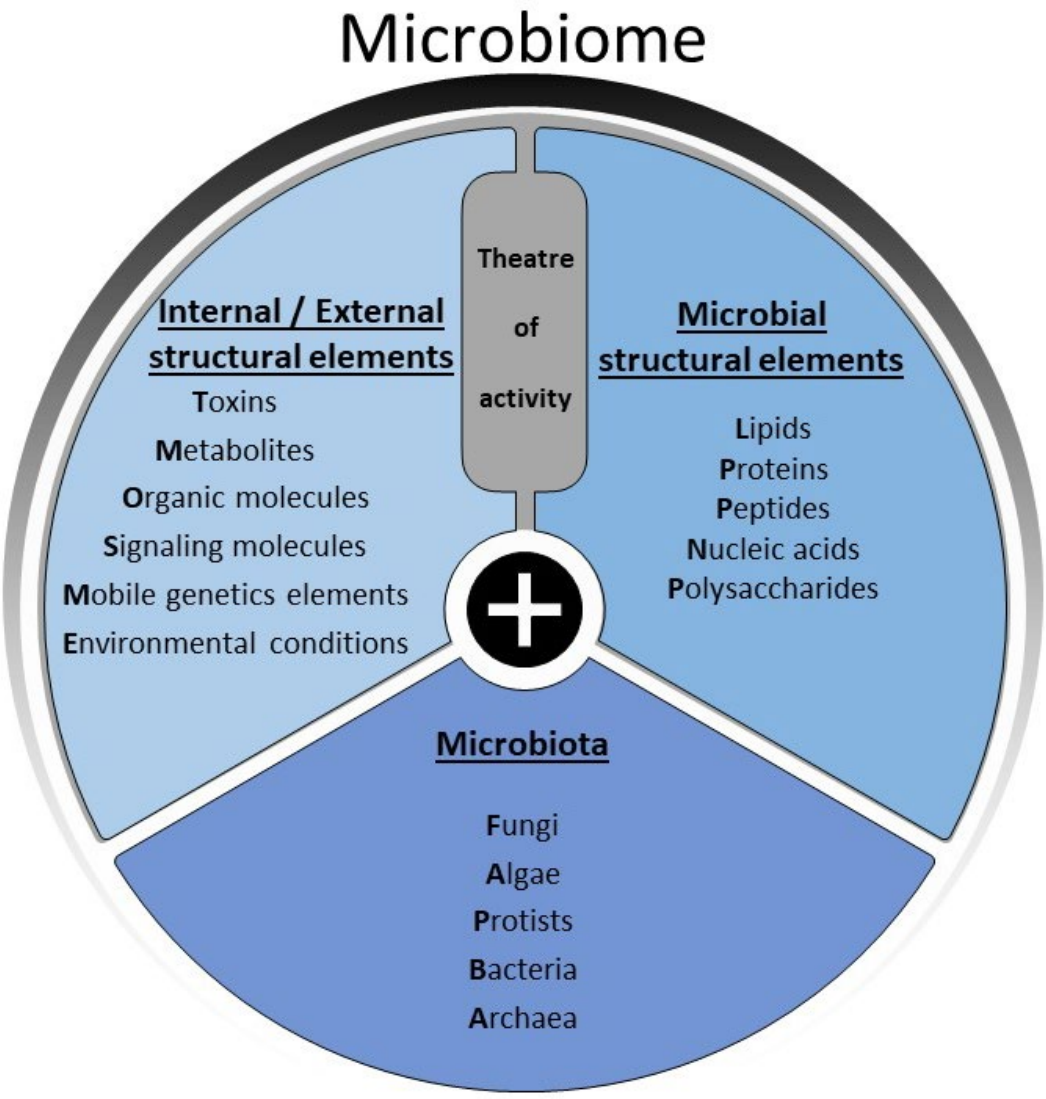


Figure 1. 3. A schematic highlighting the composition of the term microbiome containing both the microbiota (community of microorganisms) and their "theatre of activity" (structural elements, metabolites/signal molecules, and the surrounding environmental conditions) (modified from Fig. 2 in Berg *et al*., 2020).

Microbial communities in the sponge can be species-specific or generalist. Generalist microbes are found in the majority of sponge species from diverse geographic regions. In contrast, the specialists are abundant in particular species but rare or absent in most others (Thomas *et al*., 2016). The sponge acquires its microbiome through vertical transmission (microbial symbionts inherited from the parent sponge via reproductive stages), horizontal transmission (microbes from

the surrounding water enter the sponge during filter-feeding), or through mixed transmission mode (de Oliveira *et al*., 2020; Naim, 2015). The mixed mode of transmission (MMT) incorporates both vertical and horizontal transmission modes of the microbiome. The MMT of the microbiome is more common in sponges, but its frequency varies with the species and environments (de Oliveira *et al*., 2020).

The sponge-associated microbial community can comprise up to 40-60% of the sponge biomass (Gaikwad *et al*., 2016). In terms of the density of microbes in their tissues, sponges are classified into two groups: high microbial abundance (HMA) and low microbial abundance (LMA) sponges (Gloeckner *et al*., 2014; Hentschel *et al*., 2003). Studies on the sponge HMA and LMA dichotomy have a long history. Different techniques have been used to study the differences among sponge microbial communities, including transmission electron microscopy to visualise the density of the microbiome within sponge mesohyl matrix (Gloeckner *et al*., 2014; Maldonado *et al*., 2012), DAPI-counting (Gloeckner *et al*., 2014), gene sequencing, and machine learning algorithms (Moitinho-Silva, Steinert *et al*., 2017). Differences in microbial diversity between these two groups have been related to contrasting structural and physiological characteristics. More complex aquiferous systems and smaller choanocyte chambers are found in HMA sponges compared to LMA sponges (Blanquer *et al.*, 2013). Moreover, HMA sponges have a greater number of core bacterial groups that share a higher degree of overlap with seawater than LMA sponges (Erwin *et al*., 2015). HMA sponges contain large quantities and highly diverse microbial communities that produce defense-related secondary metabolites among other biochemical functions to enhance sponge function. LMA sponges, on the other hand, contain less microbes, are less diverse, and have decreased biochemical capacity when compared with HMA sponges in some biochemical pathways (Moitinho-Silva, Steinert *et al*., 2017; Pankey *et al*., 2022). Sponge biology and trophic ecology are impacted by these differences in microbial abundance (Lesser *et al*., 2022; Pankey *et al*., 2022). Other important differences exist between HMA and LMA sponges; HMA sponges have a higher mesohyl density and low choanocyte density, and a lower mass specific pumping rate, whereas LMA sponges have a lower mesohyl density, a higher choanocyte density, and a greater mass-specific pumping rate (Rix *et al*., 2020; Weisz *et al*., 2008). The difference between symbiotic phenotypes is associated with a general preference for DOM uptake by HMA sponges and POM uptake by LMA sponges (McMurray *et al*., 2018; Rix *et al*., 2020).

A growing interest in microbiome studies has led to a greater focus on the main microbiome community in specific hosts or habitats. The purpose of identifying a universal 'core microbiome' is to identify a component(s) that may be particularly crucial for host health and biological function (Parfrey *et al*., 2018; Shade & Handelsman, 2012; Turnbaugh *et al.,* 2007; Turnbaugh *et al.,* 2009). Therefore, microbiomes have been studied using co-occurrence analyses and experimental data to define core microbiota. Accordingly, the core microbiome is defined as a group of microbial taxa and their genomic and functional characteristics that frequently occur within a given habitat type (Custer *et al*., 2023; Neu *et al*., 2021). A concept like this is examined through taxon abundances and occupancy over multiple samples, as well as considering spatiotemporal variation (Hamady & Knight, 2009). In recent years, the term has evolved to encompass multiple definitions that often identify the core microbes based on their spatial distribution, temporal stability, ecological impact, and contribution to host function and health (Risely, 2020). The core taxonomic microbiome has provided insight into the microbial ecology of a diverse array of environments and hosts (Ainsworth *et al*., 2015; Henderson *et al*., 2015; Lundberg *et al*., 2012; Zaura *et al*., 2009), but their criteria for taxonomical quantification vary widely. The most common method is determining the proportion of samples that share a set of microbial taxa, the relative abundances of shared taxa between samples, or both. Similarly, the taxonomic level used to define the core may also differ, as it may be defined at the level of operational taxonomic units (OTUs), amplicon sequence variants (ASVs), phyla, or any taxonomic level (Neu *et al*., 2021).

A global project on the sponge microbiome has developed standards and curated databases that encompass the microbial diversity of sponges (Moitinho-Silva, Nielsen *et al*., 2017). Researchers have begun to reveal the functions of microbial communities and symbiont groups by combining novel approaches with state-of-the-art techniques (Kamke *et al*., 2013; Slaby *et al*., 2017; Thomas, Rusch, *et al*., 2010). The sponge-associated microbial ecosystem consists of complex ecological interaction networks encompassing hundreds or thousands of distinct microbial types or OTUs (Thompson *et al*., 2017). The definition of sponge OTUs varies among studies. The sequence identity thresholds for OTUs mostly range from 97-99% of the 16S rRNA gene (Kamke *et al*., 2010; Mysara *et al*., 2017; Moitinho-Silva, Steinert *et al*., 2017). Certain studies have increased the sequence identity to 100% (zero radius OTUs or ZOTUs) for accurate, biologically meaningful sequences, explaining metabolic and ecological qualities (Glasl *et al*., 2018; Moitinho-Silva, Nielsen *et al*., 2017). The results showed that clustering zero radius OTUs with 100% core

microbes present in sponge samples helped elucidate symbionts' horizontal transmission (Turon *et al*., 2018). Although the sponge microbiome plays an integral role in maintaining host health, survival, and function (McFall-Ngai *et al*., 2013), the mechanisms that lead to the organisation and structuring of microbiomes remain largely unknown (Lurgi *et al*., 2019).

1.3 Sequencing Methods and Metagenomic Analysis

Until a couple of decades ago, sponge microbial communities were only studied using electron microscopy (Vacelet & Donadey, 1977; Wilkinson, 1978; Rützler, 1990). The presence of unusual microorganisms and high levels of microbial diversity was noted in early works. However, molecular tools were needed to gain more precise insights into microbial community composition, including 16S rRNA gene libraries, fluorescence in situ hybridisation (FISH), denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP) (Hentschel *et al*., 2002; Li *et al*., 2007; Simamora *et al*., 2016). A 16S rRNA gene library constructed with universal bacterial polymerase chain reaction (PCR) primers revealed a phylogenetically complex yet highly sponge-specific microbial signature that differs distinctly from marine plankton (Fieseler *et al*., 2004; Hentschel *et al*., 2002). Starting from the early 2000s, modern sequencing technologies, referred to as next-generation sequencing (NGS) or high-throughput sequencing (HTS), have enabled a massive increase in sample throughput and deep coverage of microbial communities (Bourlat *et al*., 2013; Metzker, 2010), which has been used in many marine sponge studies (González-Acosta *et al.,* 2022; Pérez-Porro *et al*., 2013; Retnowati *et al*., 2021). In microbial ecology, these sequencing technologies enabled millions of sequences per run at a significantly lower cost and faster rate than previous sequencing technologies. Recently, HTS and NGS have focused on analysing bacterial hypervariable regions of 16S rRNA gene fragments directly from environmental samples (Bourlat *et al*., 2013). Therefore, they helped elucidate the microbial diversity of animal-associated and general ecosystem-associated microbiota, referred to as metataxonomics. Metataxonomics cannot directly infer the functional characteristics of microbial communities, whereas metagenomics enables genomic analysis of microorganisms in environmental samples (Hugenholtz & Tyson, 2008). NGS applied initially to clone library sequencing (Handelsman *et al.,* 1998; Handelsman, 2004), has now enabled the recovery of entire or partial genomes from environmental samples when coupled with advanced bioinformatic tools (Albertsen *et al*., 2013), enabling functional characterisation of microbial

communities. While these tools, as part of a 'multiomics' approach including metatranscriptomics, metabolomics, and metaproteomics have allowed researchers to elucidate gene expression, metabolite profiles, and protein complements in complex microbial assemblages (Gutleben *et al*., 2018), and have led to substantial documentation of the functional potential of microbial symbionts (Warnecke *et al*., 2007; Qin *et al*., 2010; Fan *et al*., 2012; Yoon *et al.*, 2015), there remain significant gaps in our knowledge. In particular, studying sponge-microbe symbiosis still requires hypothesis-driven, experimental research to move beyond theoretical models and confirm the putative physiology of symbionts and their specific metabolic pathways (Webster & Thomas, 2016).

Metabarcoding is a high-throughput sequencing technique that identifies and analyzes microbial communities by targeting specific genetic markers, such as the 16S rRNA gene. A metabarcoding is a useful approach for analysing microbial community structure, but its results must be interpreted in light of several biases (Abdelfattah *et al.*, 2018). PCR can result in over- or under-representation of certain taxa based on primer binding efficiency and template structure differences (Bonk *et al*., 2018; Bru *et al*., 2008). In addition, metabarcoding measures relative DNA abundance, not actual cell counts, which can be skewed by factors such as ribosomal RNA gene copy number, mitochondrial density, or DNA extraction efficiency (Duke & Burton, 2020). Sequence differences among closely related species and intragenomic sequence variation may also affect taxonomic resolution, creating artificial diversity (Estensmo *et al.*, 2021). Despite these limitations, metabarcoding is an effective tool for understanding microbial communities and their ecological roles, but it needs to be complemented with other approaches, such as quantitative PCR and metagenomics (Abdelfattah *et al.*, 2018).

1.4 Factors Affecting Sponge Survival

Global change is one of the most pressing challenges in conservation and applied ecology, and predicting the effects of multiple stressors is one of the most challenging tasks (Côté *et al*., 2016). Aquatic organisms are subjected to a growing number of stressors (physical and chemical). The heterogeneous nature of the stressors and their differing mechanisms of action can have additive, synergistic, or antagonistic effects on organism traits, such as affecting their metabolic and growth rates (Crain *et al*., 2008). Sponge-microbe associations are complex and influenced by various environmental factors (Cleary *et al*., 2013). Moreover, microbial community composition

can be affected by, for example, geographic location, salinity, temperature, oxygen, nutrients, pH, day length, biotic factors and pollution (Lozupone & Knight, 2007; Peters, 2015; Steele *et al*., 2011).

1.4.1 Temperature

The gradual warming of the ocean and the frequency of heat waves, as well as other phenomena associated with anthropogenic climate change, negatively impact benthic communities (Cooley *et al*., 2022; Smale *et al*., 2019). In sponges and other benthic organisms, environmental stress may disrupt or change holobiont functions, consequently affecting the entire ecosystem (Pita *et al*., 2018; Ramsby *et al*., 2018b; Rondon *et al*., 2020; Rubio-Portillo *et al*., 2016; Vargas *et al*., 2021). A rise in sea surface temperature can negatively affect some sponge species by impairing their growth and survival, resulting in necrosis and bleaching of their tissues (Bennett *et al*., 2017). Recently, mass mortality events in subarctic sponges have been reported in response to warming (Botté *et al*., 2023; Ereskovsky *et al*., 2019). Other sponge species, on the other hand, are more resistant to temperature changes (González-Aravena *et al*., 2019; Guzman & Conaco, 2016). Warming may also pose a challenge to the community of sponge-associated microbes, but studies have drawn conflicting conclusions. Some sponge species can remain stable with no noticeable effects of temperature on their microbiome (Cárdenas *et al*., 2019; Pineda *et al*., 2016; Simister, Taylor, Tsai, Fan, *et al*., 2012), while in other species, warming resulted in changes and even disruptions to their microbiomes (Castro-Fernández *et al*., 2023), which affected their health (Fan *et al*., 2013; Luter *et al*., 2012).

The microbiome of the bioeroding sponge *Cliona orientalis* shifted in composition after a 6 °C temperature increase, but when the temperature reached 32 °C, there was an even more significant change (Ramsby *et al*., 2018a). In the tropical sponge *Stylissa flabelliformis*, ocean warming weakened the sponge-microbe interactions, with less exchange of nutrients and phagocytosis evasion, which resulted in a lower number of stable symbionts (Botté *et al*., 2023). Consequently, sponge dysbiosis developed, characterised by an imbalance of microbiota that can lead to infections caused by opportunistic microbes (Egan & Gardiner, 2016). The dysbiosis caused toxic ammonia to accumulate, nutrient imbalances, and necrosis of host tissue (Botté *et al*., 2023). While the combined effect of warming and a pH decrease significantly changed the microbiome composition of the tropical sponge *Xestospongia muta* (Lesser *et al*., 2016).

Sponge communities are likely to be affected by various environmental factors, including temperature changes (Fan *et al*., 2013) and ecological stresses (Lamb & Watts, 2023). Changes in the surrounding environment may affect microbial community composition for species that demonstrate close ties with the associated seawater due to abiotic factors such as location or biotic factors like sponge morphology and high seawater filtering rates (Turon *et al*., 2018). Others whose close association with the surrounding water column is less (such as those in the subtidal or intertidal zones) can be impacted differently (Weigel & Erwin, 2016). Seasonal and extreme temperature fluctuations, as well as other abiotic factors, have been assessed to determine changes in the sponge microbiome (Cárdenas *et al*., 2019; Luter *et al*., 2014; Ramsby *et al*., 2018b; Webster, Cobb, *et al*., 2008; Wichels *et al.,* 2006), with some exhibiting high levels of stability (Cárdenas *et al*., 2019; Luter *et al*., 2014), while others showed shifts and disruptions among associated microbial communities. The stability of sponge microbiomes over geographical and seasonal changes has been studied previously, for example, in reefs off the coast of Florida. There were slight changes in bacterial taxa across seasons, including Alphaproteobacteria, Gammaproteobacteria and Cyanobacteria (White *et al*., 2012). These changes were similar to those observed in both *Hymeniacidon perlevis* and *Suberites massa* native UK sponges but were not significantly different across seasonal temperatures. The slight difference in the bacterial community compositions at the family level of *H. perlevis* and *Suberites massa* in response to seasonal changes is likely caused by transient or 'generalist' bacterial associations (Lamb & Watts, 2023). In addition, *Xestospongia muta* sponges from the Caribbean Sea showed significant differences in microbial communities between spring and autumn based on seasonal temperature (Villegas-Plazas *et al*., 2019).

Several studies have been conducted on the effects of temperature on the marine sponge *Halichondria panicea*, including filtration and pumping rate (Riisgård *et al*., 1993), reproductive cycle and larval release (Barthel, 1986; Witte *et al*., 1994), organic composition (Broadribb *et al*., 2021) and spicule production (Schönberg & Barthel, 1997). For example, a temperature increase from 6 to 12 °C increased the filtration rate of *H. panicea* by 4.3 ± 2.3 times (Riisgård *et al*., 1993), which results in high energy demand for filtering large volumes of seawater (Thomassen & Riisgård, 1995). The finding that positive growth of *H. panicea* only occurs in spring and early summer indicates that temperature is the main determining factor for sponge growth and is less correlated with food availability (Barthel, 1986). Synchronisation of oocyte maturation and larval

release was also less during winter and early spring compared to months with higher ambient temperatures (Witte *et al*., 1994). Moreover, the organic contents of *H. panicea* were observed to be affected by seasonal temperature. There was a higher proportion of inorganic tissue in the sponge during winter months, a correlation linked to increased wave action and a lower temperature, indicating either an increase in spicule size or a loss of organic material. A rapid drop in organic content was observed in some months, consistent with previously reported reproductive timings for this sponge, and likely corresponded to gamete release events. The increase in inorganic content is a secondary consequence of a reduced food supply during the winter months, which results in the sponges having to rely on organic reserves to meet metabolic demands. Consequently, sponges are likely to be stiffer and stronger, thus better able to handle higher wave action (Broadribb *et al*., 2021). Indeed, spicule production of *H. panicea* increased when the temperature increased in spring, being most pronounced after the release of larvae during summer. There was also a reduction in the mean spicule dimension, mainly in width, due to the high production of immature spicules during summer. A higher percentage of the mineral skeleton was observed in winter when wave forces were highest: 50% of dry weight versus 40% in summer (Schönberg & Barthel, 1997). Despite the extensive investigation of the effect of temperature on *H. panicea*, no studies have been conducted to determine the effect of seasonal temperature on the sponge's microbiome diversity and abundance.

1.4.2 pH

Ocean acidification (OA) is a serious problem that affects all marine ecosystems. The reduction in available carbonate ions can affect the ability of calcifying organisms to build and maintain calcium carbonate structures such as shells. Since some sponge species depend on the calcium carbonate saturation state to build their skeleton, these changes may affect their survival (Ribeiro *et al*., 2023; Webster, 2007). The resistance of some sponges to low pH conditions, however, has been demonstrated both experimentally (Duckworth *et al*., 2012) and through field research across different pH ranges, such as those in naturally acidified areas near CO_2 seeps (Morrow *et al*., 2015). The ability of certain species to adapt to OA may be supported by a stable microbiome with abundant phototrophic members (Posadas *et al*., 2022) or reflect their ability to restructure their associated microbiomes with new ones primarily through horizontal transmission (Goodwin *et al.,* 2014). Species with a greater diversity of microbes can develop functional

exuberance so that the holobiont can survive even when specific microbes are lost at low pH levels (Ribes *et al*., 2016). Changes in pH have a slight effect on sponge growth (McCullough, 2022), abundance (Duckworth & Peterson, 2013), spicule size and shape (McCullough, 2022; Ribeiro *et al*., 2021), microbial community (Botté *et al*., 2023), and metabolite concentrations (Botté *et al*., 2019) in some species, while others showed a significant decrease in abundance (Goodwin *et al.,* 2014), mortality and necrosis (Bates & Bell, 2018). For example, secondary metabolites of a coral reef sponge species responsible for reducing predation and fouling were slightly affected by decreased pH levels (Duckworth *et al.,* 2012; Januar *et al*., 2015a; Januar *et al*., 2015b). Moreover, the larvae of *H. panicea* are susceptible to OA (Rickborn *et al*., 2023).

1.4.3 Salinity

The concentration of ions in aquatic ecosystems is also changing. Salt input from anthropogenic sources salinises many freshwater ecosystems (Ondrasek & Rengel, 2021), while naturally saline ecosystems are being diluted by agricultural drainage (Velasco *et al*., 2018) and global warming-induced ice melt (Smyth & Elliott, 2016). Changes in salinity influence the ecology of different habitats based on the underlying physiology and tolerances of organisms and their ability to adapt to long- and short-term salinity fluctuations (Smyth & Elliott, 2016). The effects of acute salinity fluctuations (ranging from 36 psu to 25 psu) on reef communities were examined in a study involving representative HMA and LMA reef sponges. However, there were no visible signs of deterioration in health or differences in the composition or concentration of photopigments, nor were any changes detected in their microbial communities (Glasl *et al*., 2018). Furthermore, *Cymbastela concentrica* exhibited long-term tolerance to salinities ranging from 30.6 psu to 34.5 psu (Roberts *et al*., 2006). The marine sponge *Amphimedon compressa* showed increased microbial diversity (OTUs) in response to increases in anthropogenic salinity (Potens, 2016). Regarding *H. panicea*, exposure to low salinity water in Boknis Eck (salinity range: 14-16 ppt) and Tjärnö (salinity range: 25-30 ppt) did not cause a delay or slow down the reproductive cycle of the sponge compared to high salinity water at Helgoland (salinity range: 30-33 ppt) (Witte *et al*., 1994).

The impact of salt stress on bacterial cells' primary and secondary metabolism has been well documented (Andryukov *et al*., 2019; Wang *et al*., 2011). It has also been reported that the secondary metabolism of marine bacteria is influenced by adaptation to marine conditions (Bose

et al., 2015; de Carvalho & Fernandes, 2010; Shukla, 2016). The effect of different salinities on antibiotic production by sponge-derived *Salinispora* actinobacteria showed that at lower salinity (1%), the onset of growth was delayed. However, the antibiotic production yield (rifampicin) increased twofold when the salinity of the culture media increased to 3 percent (Ng *et al*., 2014). Supposedly, *mscL* gene loss, needed for osmotic adaptation, is the major reason *Salinispora* cannot survive when placed into a medium with low osmotic strength (Penn & Jensen, 2012). It is important to note that the tolerance of aquatic organisms to changing salinity is not solely dependent on salinity; joint effects of other stressors significantly impact the organism's performance (Velasco *et al.,* 2018).

1.4.4 Light intensity

Phototrophic marine sponges are highly sensitive to light attenuation compared to heterotrophic species and are, therefore, likely to be impacted by turbidity associated with dredging and natural sediment resuspension (Jones *et al*., 2016). The phototrophic sponges, *Cliona orientalis* and *Carteriospongia foliascens*, bleached after 28 days of exposure to very low light (<0.8 mol photons m^{-2} d^{-1}). When the same species were exposed to darkness, there were also shifts in their associated microbial communities (Pineda *et al*., 2016). Prokaryotic communities associated with *Cinachyrella kuekenthali* and *Xestospongia muta* sponges differed considerably between marine environments with low illumination (caves, deeper water) and high illumination (shallow water). The prokaryotic community composition of specimens collected from illuminated habitat was dominated by Alphaproteobacteria, Rhodothermia classes and Puniceispirillales, Rhodospirillales, Rhodobacterales, and Thalassobaculales orders. On the other hand, specimens sampled from a dimly lit habitat were associated with classes Dehalococcoidia, Spirochaetia, Entotheonellia, Nitrospiria, Schekmanbacteria, and Poribacteria, as well as orders Sneathiellales and Actinomarinales (Cleary *et al*., 2023). A cyanobacterial symbiont is a crucial component of phototrophic marine sponges, and light availability has been found to influence their abundance (Freeman & Easson, 2016). Using different intensities of light, the abundance of the symbiotic cyanobacterium *Synechococcus spongiarum* in the sponge host *Lendenfeldia chondrodes* was studied. Light-deficient conditions resulted in sponge growth being inhibited and cyanobacteria and pigments being expelled by the end of the experiment. In contrast, higher light conditions allowed sponges to grow quickly and cyanobacteria to multiply at a high rate. In addition,

photosynthetically active radiation caused cyanobacteria to accumulate lutein, a ultraviolet (UV) radiation-absorbing protein, to protect itself and the sponge host from UV damage (Curdt *et al*., 2022).

Some marine sponge species produce brominated alkaloids that exhibit cytotoxic, antiviral, antifungal and antibacterial activity produced as a defence mechanism. Light fluctuation affected the production of four main brominated alkaloids (Aero1, Aero2, Aply1 and Iso3) in *Aplysina aerophoba*. The abundance of the four alkaloids increased in both internal and external sponge layers after four months of ex-situ cultivation (Gerçe *et al*., 2009; Klöppel *et al*., 2008; Sacrista´n-Soriano *et al*., 2016). Sponges may also accumulate more toxic compounds in shaded habitats over well-illuminated habitats (Becerro *et al*., 1995; Ferretti *et al*., 2009). Moreover, transplanted *Dysidea granulosa* decreased secondary metabolite production in the absence of light (Becerro & Paul, 2004). Rather than a direct consequence of light, other factors (ex. predation or competition) associated with light may play an essential role in determining the abundance of the bioactive compounds (Sacrista´n-Soriano *et al*., 2016).

As marine sponges grow in coastal water, they are exposed to natural sunlight, including potentially damaging UV irradiation. *Dysidea* spp. (Dysideidae; Dictyoceratida), which is widely distributed in tropical and sub-tropical waters contains UV-absorbing metabolites (mycosporin-glycine, mycosporins, usujirene and palythene), either produced by the host or the symbiont, which act as an effective UV shield (Bandaranayake *et al*., 1996). A strong correlation exists between *Dysidea* sp. secondary metabolites and those found in cyanobacteria that are responsible for UV protection. Cyanobacteria are likely the main contributor to the high concentration of second metabolites within this sponge species (Agarwal *et al*., 2017; Becerro & Paul, 2004). A symbiotic *Streptomyces* strain (AQBWWS1) of the sponge *Callyspongia diffusa* produces carotenoid pigments such as lycopene under induced fluorescent light exposure, which could be used for mass production of pigments that could be incorporated into food as colourants and as a feed additive for ornamental fishes (Dharmaraj *et al*., 2009).

1.4.5 Other pollutants

Increasing urbanisation, industrialisation and boating activity translate into stress on marine organisms (Waters *et al*., 2016), including marine sponges (Marzinelli *et al*., 2018). Intensive

anthropogenic activities, such as population growth and industrial expansion, have increased heavy metal pollution in coastal areas in recent decades. Furthermore, heavy metals can bioaccumulate easily in aquatic environments due to their non-biodegradable properties (Khalef *et al*., 2022; Shaheen *et al.,* 2019). The high concentration of heavy metals in coastal waters causes the enrichment of heavy metals in marine organisms (Hao *et al.,* 2019). Heavy metal contamination has been associated with a variety of sources (Masindi & Muedi, 2018). For example, copper is the most common metal in coastal waters from both natural and anthropogenic sources, the latter including antifouling paints on boats' hulls (Biggs & D'Anna, 2012; Ytreberg *et al*., 2021). Although copper is an essential nutrient for marine organisms (Hall & Anderson, 1999), high concentrations are toxic (Rehman *et al*., 2019). Moreover, as many sponge species exist in fouling communities (Khalaman & Komendantov, 2011), they are targeted by antifouling biocides, including copper. The effect of heavy metals pollution varies among species. Some species showed higher tolerance to heavy metal pollution determined by a non-significant difference in their bacterial community (Gantt *et al*., 2017); conversely, other sponge species showed significant differences in bacterial diversity (Luter *et al*., 2012; Turon *et al*., 2019) and abundance (Tian *et al*., 2014), as well as spicule malformations and mortality (Calheira *et al*., 2020; Cebrian *et al*., 2006). This is mainly related to the sponge species and the levels of heavy metal contamination in the sampling sites.

Over the past decades, initiatives such as the Mussel Watch Program (e.g., Deudero *et al*., 2009; Fang *et al*., 2008; Goldberg *et al*., 1978; Rainbow, 1995) have extensively monitored the levels of heavy metals in marine invertebrates due to their toxicity and accumulative properties. Several other filter-feeding invertebrates have also been proposed as biomonitors of heavy metal contamination along shallow coastal areas, including tunicates (Bellante *et al*., 2016; Raftos & Radford, 2002), polychaetes (Said *et al*., 2017), barnacles (Reis *et al*., 2017), and sponges (Aljahdali & Alhassan, 2023; Cebrian *et al*., 2007; Orani *et al*., 2018). In order to determine if a species is an excellent marine biomonitor, most criteria include sedentary behaviour, ease of identification, abundance, long-living, availability around the year, the ability to tolerate environmental problems, and a strong net accumulation of metals (Boening, 1999; Rainbow, 1995).

Marine sponges are effective biomonitors of metals in coastal environments due to their prevalence, abundance in some ecosystems, long life spans, ease of sampling, high resistance to several pollutants, and the fact that they are sessile filter-feeders (Batista *et al*., 2014; de Mestre *et*

al., 2012). However, the bioaccumulation properties of trace metals within sponges depend on the considered element, geographical location, and sponge species. Moreover, bioaccumulation is more likely to be associated with sponge morphology or specific bacterial communities than with pumping rate (Orani *et al*., 2018). According to these criteria, some sponges like *H. panicea* (Hansen *et al*., 1995; Orani *et al*., 2018), *Hymeniacidon perlevis* (Gentric *et al*., 2016) and *Oscarella lobularis* (de Pao Mendonca *et al*., 2023) were considered good marine biomonitors for heavy metals pollution. Consequently, a study on *H. panicea* sponge demonstrated that after pre-exposure to Cu, Zn, and Cd for 14 days, it accumulated these metals from the surrounding medium. This sponge also tolerated high concentrations of metals up to 1000 µg/l. The accumulated zinc concentration in the sponge body was the highest among other exposed metals, ranging between 148-156 µg/g/day, suggesting zinc saturation at these higher levels (Hansen *et al.*, 1995). Another biomonitoring study using the coastal marine sponge *Sigmadocia fibulata* to detect metal pollution between inshore and offshore environment of the 'Gulf of Mannar', India, showed that the inshore sponges showed higher levels of metals accumulated compared to the offshore environment, where the bioaccumulation of metals in sponge tissue was in order of Fe>Al > Ni >Mn >Cu>Cr>Co >Ba >Zn >V>Cd >Pb. The higher metal accumulation in inshore sponges resulted in a substantial reduction in macromolecules (sugars, proteins, and lipids). This indicates that sponge health is affected by the higher metal pollution in the coastal environment from industrial, agricultural and sewage waste disposal (Rao *et al*., 2006). In addition to monitoring trace metals, marine sponges are good biomonitoring tools for organic and inorganic micropollutants and a filtering tool to improve water quality in moderately contaminated areas. The accumulation of organic and inorganic contaminants was assessed in a contaminated marina using Mediterranean sponges (*Petrosia ficiformis* and *Ircinia oros*). Both species contained higher concentrations of Zn and Cu after transfer to marinas, hinting that the metals may be present in higher concentrations in marinas due to antifouling paints. While the accumulation of zinc and copper in sponge fragments did not affect sponge health, this indicates that sponges can accumulate these metals. Moreover, both sponge species were more efficient at filtering organic and inorganic pollutants from seawater when attached to nets underneath the pontoon than when attached to bottom frames. A comparison between *I. oros* and *P. ficiformis* revealed that *P. ficiformis* displayed a more efficient metabolism, lower physiological stress levels, and a more stable associated microbiome (Bulleri *et al*., 2022).

The increased amount of nutrients and organic matter found in surface waters due to eutrophication may lead to coral reefs and nearshore environments being more vulnerable to OA (Webb *et al*., 2017). Temperature and nutrient levels may influence sponge-symbiont populations across broad (transoceanic, Giles *et al*., 2013; Schmitt *et al*., 2012) and narrow (a few kilometres, Turque *et al*., 2010) geographical distances. The effect of eutrophication was studied on many sponge species, resulting in a negative effect causing either reduced sponge (Beepat *et al*., 2020) weight or unstable sponge microbiome, which results in a diseased sponge (Pita *et al*., 2018; Webster & Taylor, 2012), while others showed tolerance (Baquiran & Conaco, 2018; Luter *et al*., 2014). In contrast, other sponge species are positively affected by eutrophication, which was reflected in increasing their abundance, growth and bioerosion rates (Holmes, 2000; Holmes *et al*., 2009; Southwell *et al*., 2008). Moreover, eutrophication negatively affects marine sponges indirectly, enhancing the growth of opportunistic microalgae and cyanobacteria to the point that their overgrowth creates unoxygenated environments that lead to sponge death (Turner *et al*., 2021; Wall *et al*., 2012; Zhao *et al*., 2018). In addition, eutrophication can cause the overgrowth of cyanobacterial mats on sponge bodies. This can exacerbate the disease and lead to sponge death from the resulting release of various biologically active compounds (Kaluzhnaya & Itskovich, 2017; Sorokovikova *et al*., 2020).

Rainfall and runoff from urban development have been associated with a marked deterioration in environmental waters, which can carry a variety of pollutants and pathogenic microorganisms (Monteiro *et al*., 2021). Stormwater runoff can contain pathogenic bacteria, viruses, and protozoa (Abia *et al*., 2016; Zhu *et al*., 2018). These organisms can be carried into environmental water bodies via sewer overflows, defective septic systems, agricultural runoff, wild animal defecation, and treated sewage discharges (Januário *et al*., 2019; Kucuksezgin *et al*., 2019; Ndione *et al*., 2022; Norat-Ramírez *et al*., 2019). Many studies are reporting the presence of faecal indicator bacteria (FIB) and enteric pathogens in stormwater (Aryal *et al*., 2021; Bernardin-Souibgui *et al*., 2018; Shahirnia, 2022; Xu *et al*., 2019), which eventually end up contaminating the marine environment (Steele *et al*., 2018). Microorganisms such as *Escherichia coli* and intestinal *Enterococcus* spp. are used to evaluate the microbial quality of water bodies (European Union, 2006; USEPA, 2000). Other studies have evaluated human pathogens in stormwater by including *Pseudomonas aeruginosa* (Januário *et al*., 2019), *Clostridium perfringens* (Schreiber *et al*., 2019), *Campylobacter* (Meng *et al*., 2018; Sidhu *et al*., 2012), *Salmonella* and *Shigella* spp.

(Sargunar, 2020). A high level of these indicators is found in the intestines of warm-blooded animals, and their presence in water bodies indicates faecal contamination and the existence of potential human pathogens (Ahmed *et al*., 2019). There is a substantial public health risk associated with exposure to waterborne organisms. Bathing waters are a reservoir and a pathway for the spread of antimicrobial resistant (AMR) organisms and contribute to the transmission of illness and disease (Farrell *et al*., 2021). To solve this issue, several countries, including the European Union Member States (EUMS) and other European countries, such as the United Kingdom (UK), the United States (US), Australia, and Canada, are working on controlling microbial contamination in recreational and bathing waters. As part of their efforts to protect public health, bathing water quality is constantly monitored at different bathing sites throughout the country (Environment Agency, 2022c; European Union, 2006; Health Canada, 2012; USEPA., 2012). Meanwhile, scientists were studying the possibility of using marine organisms to filter or remove these pathogens from the marine environment, and one of these considered animals was the sponges. Sponges are often characterised by relatively low pumping rates, yet their retention rates can be very high in well-developed aquiferous systems (McMurray *et al*., 2018; Rix *et al*., 2020). As a result of this ability, Porifera may influence or improve faecal contamination-associated microbial polluting assemblages in the marine environment (Claus *et al*., 1967). Therefore, sponges were used in integrated aquaculture for their ability to bioremediate faecal bacteria (Longo *et al*., 2016; Milanese *et al*., 2003; Zhang *et al*., 2010). In contrast, the accumulation of faecal bacteria in the sponge body negatively affects the sponge microbiome (Shore *et al*., 2021). Consequently, the imbalance of microorganisms in stressed tissues can lead to an enrichment of cell motility, chemotaxis, and virulence genes (Fan *et al*., 2013; Tian *et al*., 2014). It is as yet unclear how faecal coliform exposure affects marine sponge health, but some species may be relatively tolerant, consuming bacterial cells for nutrition (Chaves-Fonnegra *et al*., 2007).

The introduction of anthropogenic activities in seawater seriously threatens marine organisms (Pimiento *et al*., 2020), including sponges (Busch *et al*., 2020). The effect of nutrient load, heavy metals, pH, temperature and coliform bacteria have been studied intensively on different marine sponge species (Batista *et al*., 2018; Orani *et al*., 2018; Shore *et al*., 2021). However, a comprehensive study on the effect of these factors on the marine sponge microbiome, along with different environmental characteristics on-site and throughout the year, is yet to be

studied. As such, a study is required to understand better the main factor affecting sponge health through studying its microbiome community distribution.

1.5 The Marine Sponge *Halichondria panicea*

The sponge *Halichondria panicea* is known as the breadcrumb sponge. Its name refers to its crumb-like texture, as first described in 1766 by zoologist Peter Simon Pallas (Pallas, 1766). Its colour can range from green to creamy-yellow depending on its exposure to light and the presence of the symbiotic microalgae *Microspora ficulinae* (Christensen, 1985). Regarding shape, *H. panicea* ranges from thin sheets to massive forms, sometimes found growing extensively on the *Laminaria hyperborea* stipes (Ackers *et al*., 2007). The growth forms and the body shape of *H. panicea* are affected by both current regimes and wave forces (Barthel, 1991; Palumbi, 1984; Palumbi, 1986). *H. panicea* can inhabit a variety of niches but is commonly found in rock pools, under boulders, and under overhangs along the shoreline (Ackers *et al*., 2007). It is especially prevalent in areas exposed to waves or swept by the tide. In addition to widespread distribution along the shore, *H. panicea* is also known to inhabit deep waters up to 569m below the surface.

This viviparous sponge is readily cultured in laboratories as adults (Barthel & Theede, 1986; Knobloch *et al*., 2019a; Imhoff & Stöhr, 2003), larvae (Khalaman *et al*., 2011), primmorphs (Lavrov & Kosevich, 2018) and clonal explants (Kumala *et al*., 2017; Kumala & Canfield, 2018); and the spawning period of this temperate species is limited to a few weeks (Barthel & Detmer, 1990; Witte *et al*., 1994). *H. panicea* is cosmopolitan in its distribution (Erpenbeck *et al*., 2004; Hiscock, 2008). Therefore, *H. panicea* has been the subject of numerous ecological and biological studies over the last few decades and is among the most studied sponge species (Knobloch, 2019). As a model sponge, *H. panicea* has recently been used to investigate sponge-specific characteristics and processes, including the flagellar apparatus of sponge larvae and the types of adult cells (Sokolova *et al*., 2019), the sponge pump and the speed at which exhalent jets are formed (Goldstein *et al.,* 2019), the relationship between osculum dynamics, filtration and respiration activities (Kumala *et al*., 2017; Kumala & Canfield, 2018), the condition index based on seasonal food availability (Lüskow *et al*., 2019), primmorph formation (Sipkema *et al*., 2003) and heavy metal accumulation as a potential biomonitoring organism (Hansen *et al.,* 1995). In addition, the oxygen requirements of *H. panicea* were investigated to gain insight into the role of oxygen levels in the ocean and atmosphere in the emergence of animal life (Mills *et al*., 2014).

1.5.1 The mutualistic relationship between H. panicea and other marine organisms.

H. panicea was first studied in depth in the 1980s by Barthel (1986, 1988). He investigated the ecophysiology of *H. panicea*, assessing substrate specificity, growth rates, predatory activity, energy requirements and the sponge's role in coastal environments through field experiments and laboratory observations. In earlier ecological studies, *H. panicea* was associated with a rich and diverse fauna, including the scallop *Chlamys varia*, with which it exhibited commensal-protective mutualism (Forester, 1979), and the skeleton shrimp *Caprella linearis* displaying chemical attraction (Peattie & Hoare, 1981). Later, *Chondrus crispus*, a red seaweed, was discovered to form a mutualistic relationship with *H. panicea*, producing metabolites that inhibit barnacle settlement as a commensal-protective mutualism (Toth & Lindeborg, 2008). In addition to having a high regenerative capacity when grazed by nudibranchs (Knowlton & Highsmith, 2005), *H. panicea* can shed its outer cell layer regularly to remove fouling organisms through a process called tissue sloughing (Barthel & Wolfrath, 1989).

1.5.2 H. panicea host-specific symbiotic microbiome

A sponge model system could provide insight into microbiome stability, colonisation dynamics, and host-microbe interactions (Pita *et al*., 2016). The microbiomes of *H. panicea* from various geographical locations have been characterised by strain culture (Wichels *et al.,* 2006), 16S metabarcoding analysis (Naim *et al*., 2014; Thomas *et al*., 2016), and whole genome sequencing (Strehlow *et al*., 2022). Accordingly, *H. panicea* was characterised as a sponge with low microbial abundance and high variability in its bacterial diversity (Goldstein & Funch, 2022). Previously, electron microscope observations classified *H. panicea* as a LMA sponge due to its relatively low microorganism abundance in its mesohyl (Moitinho-Silva, Steinert *et al*., 2017). Recent advances in technology have of late confirmed this classification using the 16S rRNA FISH images through the low sponge-specific bacterial diversity, coupled with the low bacteria-to-sponge cell ratio (Knobloch *et al*., 2019b). The LMA sponge *H. panicea* has a monodominant culture of a single bacteria (25% – 80% relative abundance in 16S rRNA gene amplicons), which is recently described as *Candidatus Halichondribacter symbioticus* (Knobloch *et al*., 2019b). This Alphaproteobacterium exhibits several key features of a sponge-symbiont genome, including its ability to absorb ammonia, synthesise vitamin B12, and produce antimicrobial peptides (Knobloch

et al., 2019c). Studies on this Alphaproteobacteria from *H. panicea* show that it is host-specific and present in sponges from different geographical areas (Knobloch *et al*., 2019b; Rusanova *et al*., 2021; Wichels *et al*., 2006). The origin of this bacteria has yet to be studied. There is, however, a possibility that it has been transferred horizontally from the surrounding seawater or other marine organisms surrounding the sponge. Therefore, the complex microbiome of sponge *H. panicea* should be studied in greater detail in conjunction with other organisms like seaweed, barnacles, and others that live in its environment to determine the origin of its host-specific symbiotic microbiome.

1.5.3 The role of cyanobacteria in H. panicea

Photosynthetic organisms such as cyanobacteria are likely to be the most abundant on Earth, with the *Synechococcus*/*Prochlorococcus* clade being the most prominent in the oceans (Ulloa *et al.*, 2021). The presence of cyanobacteria has been confirmed living in association with marine sponges belonging to 22 orders and 60 families, including at least 53 Demospongiae, three Calcarea families, two Hexactinellida families, and two Homoscleromorpha families (Konstantinou *et al.,* 2018). All Porifera classes can host cyanobacteria, whereas only Demospongiae and Calcarea are known to live in association with cyanobacteria (Diaz *et al*., 2007; Regueiras *et al*., 2019; Thacker & Freeman, 2012). A number of these sponge-cyanobacteria associations are common in benthic organisms. Molecular studies have indicated that sponges and cyanobacteria interact mutually, in which the sponge host provides shelter and a surface for cyanobacteria, while the cyanobacterial symbionts supply sponges with food (by converting photosynthesis into nitrogen), chemical defence, and UV protection (Tan, 2023; Thacker & Freeman, 2012; Usher, 2008). Cyanobacterial symbionts can be obtained vertically through larvae, horizontally from the environment, or both (de Oliveira *et al*., 2020; Thacker & Freeman, 2012). In sponge hosts, cyanobacteria typically cause colouration, and changes in phycobiliprotein ratios can cause colours to change from yellow-green to red-brown in low light (Usher *et al*., 2004). In addition to their common appearance on sponge surfaces exposed to light, cyanobacteria may also be present within the sponge's inner core (Hentschel *et al*., 2006; Konstantinou *et al*., 2021). Cyanobacteria are extremely flexible in their photosynthetic apparatus, adapting both to the quantity and quality of light, enabling them to grow in a wide range of light conditions, from ocean depths that receive only 1% of surface radiation (Furnas & Crosbie, 1999) to full sunlight. The adaptation process involves not only changing the

relative ratio of phycobiliproteins but also changing the concentration and size of light-harvesting complexes (Bennett & Bogorad, 1973; Golubic *et al*., 1999; Moore & Chisholm, 1999; Wyman & Fay, 1986).

The colour morphs of *Halichondria panicea* have attracted much interest. There is evidence that microalgae are responsible for the green colour in, for example, *Microspora ficulinae* (Christensen, 1985) and *Desmodesmus* (Kravtsova *et al*., 2013). Other studies have implicated cyanobacteria as the main contributor to colour (Gorelova *et al.,* 2009). These studies were mainly focusing on a certain organism, either cyanobacteria or microalgae. Metabarcoding analysis of the 16S and 18S rRNA would help to identify the chlorophyll-containing microbial community responsible for the seasonal change in the colour of this sponge.

1.6 Research Aims and Thesis Outline

The importance of sponges to their environment is multifaceted. In various ecosystems, from tropical coral reefs to the deep sea, sponges provide habitat and food for many different types of organisms. The sponge symbionts are important to the functions of sponges in the marine ecosystem and play a crucial role in maintaining their health. Therefore, any disturbance in the sponge microbiome will affect the sponge's health and potentially the marine ecosystem. For that reason, the effect of different environmental conditions, including anthropogenic, on the survival of sponges in the marine ecosystem, has been discussed. This was done to identify the need for extensive studies on the combined effect of natural and anthropogenic stressors, which will help predict the consequences of future climate change with the increased human-based pollution on the survival of sponge populations through affecting their microbiome. Additionally, by predicting the biological response to these stressors, we may understand the possible means of enabling sponges to adapt to future conditions.

Therefore, this study aimed to investigate the sponge microbiome stability under different environmental and anthropogenic activities. The microbial diversity of the marine sponge *Halichondria panicea* from the north-east England coast (North Sea) under different bathing water quality was analysed using next-generation sequencing technology and to determine the combined effect of seasons and coastal pollution, including trace metals, nutrients and bacteria from run-off and sewage outflow on the members of its associated microbiota. Additionally, the objective was to investigate the origin of the most dominant symbiotic bacterial community of the sponge *H.*

panicea from its surrounding environment. Moreover, the objective was to investigate the role of the microbiome of *H. panicea* seasonal colour morphs. Finally, the objective was to elucidate the effect of antifouling paints on the sponge bacterial community.

Chapter 2 introduces the biodiversity of sponge-associated bacteria in sponge samples from three locations with different bathing water quality on the north-east England coast (North Sea) through deep-sequencing. The sponge microbial communities were compared between the tested locations using diversity analysis, a Venn diagram and community structure. An analysis of correlation was performed in order to identify the factors that most influence the sponge microbiome (top 10 bacterial phyla) - physical (temperature, pH, salinity, conductivity, total dissolved solids (TDS)), chemical (traces of metals and nutrients), or biological (faecal bacteria).

Chapter 3 seeks to gain insight into the horizontal transmission of the bacteria community of *H. panicea* sponge from the surrounding environment and uncover the origin of the host-specific bacterial taxon (OTU_1). As a result, seawater and seaweed samples attached to the sponge were investigated through metabarcoding analysis and indicator species analysis.

Chapter 4 mainly focuses on elucidating the microbial species responsible for the seasonal change in colour of *H. panicea*. This was achieved by metabarcoding the16S and 18S rRNA of the sponge samples for both colour morphs, as well as studying the differences in chlorophyll concentration and fluorescence between the two colour morphs.

Chapter 5 evaluates the best ex-situ cultivation conditions for *H. panicea* sponge in the lab for testing the effect of antifouling paint on sponge survival. Furthermore, it describes the effect of commercial antifouling paints on *H. panicea* survival by investigating its bacterial community. This was achieved by exposing the sponge samples to two different antifouling paints; biocidal and non-biocidal (environmentally friendly). The 16S rRNA metabarcoding analysis was used to reveal the differences in bacterial communities of sponges exposed to the two paints when compared to control sponges.

Chapter 6 concludes with how this thesis has contributed to the state-of-the-art knowledge of sponge microbiome. It also proposes new research directions to help understand the main stressors affecting sponges in a manner that could help sponges potentially adapt to future stresses.

Chapter 2. The Effect of Environmental and Anthropogenic Factors on the Microbiome of the Sponge, *Halichondria panicea*, at Three Coastal Sites in North-east England

2.1 Introduction

The microbiome community of sponges is a valuable tool for understanding the success or failure of the host. The disruption of their bacterial community has been reported to be a contributing factor to the extensive mass mortality of sponges (Webster & Thomas 2016) in the Mediterranean (Cebrian *et al*., 2011; Webster, Xavier, *et al*., 2008) and the Red Sea (Gao *et al*., 2014). While bacterial communities differ spatially and temporally in seawater (Cárdenas *et al*., 2018; Cleary *et al*., 2022; Glasl *et al*., 2017; Zeglin, 2015), sponge symbiotic bacterial communities do not change substantially across geographical and bathymetric ranges or over temperature, acidification, eutrophication or irradiance shifts (Erwin *et al*., 2012; Kandler *et al*., 2018; Luter *et al*., 2014; Pita *et al*., 2013; Strand *et al*., 2017). Studies have also examined the effects of different stressors on sponge bacterial communities, including sedimentation (Pita *et al*., 2018), micropollutants (Girard *et al*., 2021), nutrients (Luter *et al*., 2014; Simister, Taylor, Tsai, Fan, *et al*., 2012), heavy metals (Gantt *et al*., 2017; Tian *et al*., 2014) and crude oil (Luter *et al*., 2019). Typically, sponges exhibit high bacterial species diversity in well-preserved ecosystems, which decreases under anthropogenic pressure (Turon *et al*., 2019). Despite the extensive studies on sponge microbiome, a comprehensive study of the combined effect of seasonal variations and anthropogenic activities, including bathing water quality in coastal areas, has not been conducted yet.

The marine sponge *Halichondria panicea* is an LMA sponge (Knobloch *et al*., 2019b). *H. panicea* is globally abundant and easily accessible since it can be found in the intertidal zone. It is, therefore, a widely studied species in terms of its biology and ecophysiology (Mills *et al*., 2014; Riisgard *et al*., 2016). Interest in the microbial communities of this sponge has grown due to its complex relationships with unique microbial symbionts and the biotechnological potential of its secondary metabolites (Amelia *et al*., 2022; Dharamshi *et al*., 2022; Wang, 2006). The bacterial diversity of this sponge has been studied previously through both cultivation-dependent (Imhoff &

Stöhr, 2003; Schneemann *et al*., 2010; Steiner *et al*., 2024) and cultivation-independent (Naim *et al*., 2014; Steinert *et al*., 2017; Strehlow *et al*., 2022) methods. Metabarcoding studies on *H. panicea* aim to elucidate the entire microbial community of the sponge. Generally, studies on *H. panicea* either examined the V3-V4 region (Knobloch *et al*., 2019b; Moitinho-Silva, Steinert *et al*., 2017; Naim *et al*., 2014; Rusanova *et al*., 2021; Schmittmann *et al*., 2022) or the V1-V3 region (Jeong *et al*., 2013). As the V3-V4 region varies greatly among different bacteria, it is a good marker for distinguishing them in samples. Focusing on the V3-V4 region allows researchers to gain a comprehensive understanding of the microbial diversity (Castelino *et al*., 2017; Parulekar *et al*., 2017) associated with *H. panicea*. However, one study investigated sponge microbiomes using three primer sets for the full coverage of 16S rRNA genes (V1-V8 regions), which provides a more complete view of microbial diversity (Yang *et al*., 2019), although it is more complex and expensive.

Cultivating functional symbiotic bacteria to characterise their chemistry and environmental regulation has proved difficult (Knobloch *et al*., 2019a). Therefore, this study aimed to examine the stability of the sponge microbiome and resistance to perturbations through the metabarcoding analysis of the 16S rRNA gene (V3-V4 region). To do so, the *H. panicea* bacterial community was examined over four seasons at three different locations with different degrees of anthropogenic impact.

2.2 Methodology

2.2.1 Sponge external characteristics

Halichondria panicea varies in colour through the seasons and sometimes by location, meaning it can be difficult to distinguish. *H. panicea* occupies the intertidal zone to a depth of over 500 m (Burton, 1959; Vethaak *et al.,* 1982). The sponge is typically found in the intertidal area, attached to the underside of rocks and west-facing, away from the direct sunlight. Sampling sponges is best done when the tide is below one metre above the chart datum. *H. panicea* occurs mainly as a low encrusting form in wave-sheltered areas, but may reach 20 cm thick (Hiscock, 2008). The species can easily be confused with *H. bowerbanki* as they share the same external characteristics (bread-crumb-like texture), spicule types and lengths and are distributed widely along the coast throughout the year. However, they are distinguishable by the fact that *H. bowerbanki* lacks the exhalant

openings of *H. panicea*. In addition, *H. bowerbanki* ranges in colour from yellow-beige and, unlike *H. panicea*, is never green (Hiscock & Jones, 2007).

2.2.2 Sponge sampling locations

The sponge sampling was performed at three locations on the north-east England coast next to Northumbrian Water Ltd. storm water outfalls, with distances ranging from 100 to 300 meters from the drainage outlets. Sampling locations were selected according to bathing water quality, ranging from 'poor' to 'excellent' as rated by the Environment Agency. These ratings are based on *Escherichia coli* and intestinal *Enterococci* counts in these areas taken through the bathing season (May to September). The chosen sites were Cullercoats, Marsden Bay and Whitley Bay (Figure 2.1; Table 2.1). Since 2017, Marsden Bay and Whitley Bay have been consistently rated 'good' and 'excellent', respectively, while Cullercoats has been rated 'poor' since 2018.

Table 2. 1. Overview of the sampling of *H. panicea* at the three sites.

Site	**Coordinates**	**Natural drainage catchment**	**Sampling frequency**	**Bathing water quality**
Whitley Bay	55.064639, -1.450110	17 km^2	Every three months	Excellent
Cullercoats	55.035539, -1.431161	0.3 km^2	Every three months	Poor
Marsden Bay	54.983112, -1.378978	No	Every three months	Good-sufficient

Figure 2. 1. Map showing specific coordinates of the three sponge sampling locations. The map was created in the R program using the Stamen maps-ggmap package (Kahle & Wickham, 2013).

2.2.3 Sample collection

Sampling was done in January (Winter), April (Spring), July (Summer), and October (Autumn/Fall) during 2022. Triplicate biological sponge and seawater samples were collected from the intertidal area at each site, specifically from rock surfaces. Sponge samples were photographed prior to collection (Fig. 2). A sterile blunt knife was used to scrape a 3 cm2 piece of sponge tissue off the rock, which was transferred to a plastic bag with seawater from the surrounding environment. In addition, 3 L of surface seawater was collected in sterile containers at the exact locations as the sponge samples. Approximately 1 L of outfall drainage water was collected from two sites (Cullercoats and Whitley Bay) to test the type of contaminant carried by these waters. All samples were stored in an icebox for transport to the laboratory. Upon arriving in the laboratory, the sponge samples were cleaned of any attached marine organisms and soil particles, and they were washed three times with 35 ppt sterile artificial seawater to ensure removing remaining contaminants (Tropic Marin Classic Salt, Germany) and kept in sterile 50 mL Falcon™ tubes for

identification and DNA extraction. Seawater samples were filtered directly upon arrival at the lab, one litter of seawater samples was filtered in triplicate through a sterile cellulose nitrate 0.22 µm filter paper (Manta Diagnostics™). For microbial diversity analysis, samples were stored at -20 °C until DNA extraction was performed. The physical parameters of seawater at the sampling sites (water surrounding the sponge), namely, pH, salinity, temperature, total dissolved solids (TDS), resistivity and conductivity, were measured on-site using a multi-parameter pocket tester kit (Apera PC60-Z Smart, Shanghai San-Xin Instrumentation, China).

Figure 2. 2. *Halichondria panicea* sponge colony before sampling (Photo taken at Whitley Bay in April 2022). The scale bar represents 80 mm.

2.2.4 Chemical analysis of seawater samples

The chemistry of seawater samples was analysed by inductively coupled plasma mass spectrometry (ICP-MS) to detect the trace metals and ion chromatography (IC) for anion detection in seawater and land outfall water samples at the sampling sites. Thus, 25 mL of each water sample

was filtered through sterile 0.22 µm cellulose nitrate filters (Manta Diagnostics™), acidified using 0.5 mL trace grade nitric acid per 25 mL of sample and sent to the Newcastle University Science Agriculture and Engineering technical service department. For the trace metals analysis, all water samples were diluted 1/100 with gas before entering the ICP-MS column. For anion analysis, seawater samples were diluted 1/200, and land outfall water samples were diluted 1/50. Anions in seawater samples, such as nitrite, nitrate, ammonia, phosphate and silicate, were analysed using the ICP-MS first, however, some nutrients such as nitrate, nitrite, and phosphate were not detected in all the water samples. Therefore, these nutrients were measured again using a Skalar San++ Continuous Flow Analyser following the ROPME (2010) method at the Kuwait Institute for Scientific Research (KISR) for samples from April to October.

2.2.5 Sponge identification

Sponge identity was confirmed via spicule structure and external morphological traits. The protocol for spicule preparation described by Hooper (2000) was followed. First, a small sponge sample was digested using domestic bleach (containing <5% sodium hypochlorite) for 2-4 h until the sponge specimen was wholly digested. The bleach was carefully aspirated using a clean pipette, and the sample was washed with fresh sterile MilliQ water three times. The suspension was left to settle for 10 min between each washing stage to avoid aspirating the smaller spicules. One drop of the spicule suspension was placed on a clean glass slide and examined under the light microscope at 200x magnification. Spicule sizes were measured using the microscope stage graticule. Spicule form, size (length and width), and morphological characteristics were compared against the species description (Ackers *et al*.**,** 2007).

2.2.6 DNA extraction

To study the microbial diversity of the sponge samples, the 16S rRNA amplicon sequencing was analysed. Sponge DNA was extracted within one month of sampling using a DNA extraction kit (DNeasy PowerSoil Pro, Qiagen).

A small piece of the sponge, approximately 0.125-0.2 g, was added to the PowerBead Pro tube with 800 µL of solution CD1. The sample was mixed briefly, then vortexed horizontally using a 1.5-2 mL tube size vortex adapter (cat. no. 13000-V1-24) for 10-15 min (until the sponge tissue disintegrated) at maximum speed; this was followed by centrifugation at 15,000x *g* for 1 min. Next, the supernatant was transferred to a clean microcentrifuge tube (2 mL), and 200 µL of solution

CD2 was added, vortexed for 5 s, and centrifuged at 15,000x *g* for 1 min. The supernatant (up to 700 µL) was transferred to a clean microcentrifuge tube (2 mL), 600 µL of solution CD3 (saline solution) was added to it, and vortexed (5 s). Only 650 µL of the mixed solution (lysate) was transferred to an MB spin column, centrifuged at 15,000x g for 1 min, and the flow-through was discarded. This step was repeated 2-3 times until the whole lysate was processed. The MB spin column was then transferred to a new clean collection tube (2 mL), 500 µL of EA solution (wash buffer) was added, centrifuged at 15,000x *g* for 1 min, and the flow-through was discarded from the collection tube. Next, 500 µL of cold C5 solution (ethanol-based wash solution stored at -20 ℃) was added to the column, which was closed tightly and inverted gently to wash off any residual buffer that may have adhered to the edges of the column. The column was then incubated for 5 min at 4 ℃ before being centrifuged at 15,000x *g* for 1 min. After discarding the flow-through, an extra washing step with cold molecular grade 80% ethanol (stored at -20 ℃) was added to the column, which was closed tightly and inverted gently again to wash off any residual buffer that may be adhered to the edges of the column. The solution was again incubated in the column for 5 min at 4 ℃ before being centrifuged at 15,000x *g* for 1 min. After discarding the flow-through, a dry centrifugation step at 16,000x *g* for 2-3 min was done to ensure the removal of any ethanol traces from the DNA sample. Finally, the MB spin column was transferred to a new 1.5 mL Eppendorf tube to which 70 µL of C6 solution (elution buffer) was added to the centre of the filter membrane, centrifuged at 15,000x *g* for 1 min, the MB column was discarded, and the obtained DNA was stored at -20 ℃.

DNA was extracted from the seawater samples using a DNeasy PowerWater Kit (Qiagen). Due to the filtering step and potentially low yields of DNA, this kit was the most appropriate choice. The manufacturer guidelines for this kit were slightly modified to get a high-quality DNA yield. Moreover, an extra step of washing with cold ethanol was added at the end to ensure the removal of salt contamination in the extracted DNA sample. Thus, the cellulose nitrate 0.22 µm filter paper containing seawater microbiome was cut into small pieces using sterile forceps and scissors and placed in the 5 mL PowerWater DNA Bead Tube with 1000 µL of Solution PW1 (warm, incubated in the water bath at 55 ℃ to dissolve the precipitates). The DNA yield increased by cutting the filter paper into small pieces rather than rolling it as instructed in the manual. The tube was closed tightly, vortexed for 5 s, and vortexed horizontally using a vortex adaptor (cat. no. 13000-V1-15)

at maximum speed for 10 min instead of 5 min. The tube was then centrifuged at 4000x g for 1 min using a 15 mL tube rotor.

The supernatant was transferred into a new clean collection tube (2 mL) and centrifuged again at 13,000x *g* for 1 min to remove the remaining soil particles present in the supernatant that may affect the extraction method. The supernatant was transferred again to a new clean collection tube (2 mL), 200 µL of solution IRS was added to it and incubated at 4 ℃ for 5 min. After the incubation period, the tube was centrifuged at 13,000x *g* for 1 min, and avoiding the pellet, the supernatant was transferred to a new clean collection tube (2 mL). The supernatant was mixed (vortexed for 5 s) with 650 µL of PW3 solution (warm, incubated in the water bath at 55 ℃ to dissolve the precipitates). From that mixture, only 650 µL was loaded in the MB spin column and centrifuged at 13,000x *g* for 1 min. The flow-through was discarded, and this step was repeated until all the supernatant was processed. The MB spin column was transferred to a clean 2 mL collection tube; 650 µL of PW4 solution (shaken before use) was added and centrifuged at 13,000x *g* for 1 min. After discarding the flow-through, 650 µL of ethanol solution (stored at -20 ℃ before use) was added to the column, which was closed tightly and inverted gently to wash off any residual buffer that may have adhered to the column edges. The column was then incubated for 5 min at 4 ℃ before centrifuging at 13,000x *g* for 1 min. After discarding the flow-through, an extra washing step with cold molecular grade 80% ethanol (stored at -20 ℃) was added to the column, which was closed tightly and inverted gently again to wash off any residual buffer attached to the edges of the column. The column was incubated again for 5 min at 4 ℃ before centrifuging at 13,000x *g* for 1 min. After discarding the flow-through, a dry centrifugation step was done by centrifuging the column at maximum speed (15,000x *g*) for 2 min to remove any residual ethanol in the sample. The column was transferred to a new clean collection tube (2 mL), 70 µL of EB solution (elution buffer) was added to the centre of the filter membrane, centrifuged at 13,000x *g* for 1 min, the MB column was discarded, and the obtained DNA was stored at -20 ℃.

The quality and quantity of the extracted DNA samples were checked using a Nanodrop One Microvolume UV-Vis Spectrophotometer (Thermo Scientific). Samples were run on a 1% agarose gel, and the bands were visualised under UV light (Azure Biosystems™ c280).

2.2.7 DNA sequencing

The DNA samples were shipped to LC Sciences (Houston, USA) for sequencing. To study the V3-V4 region of the 16S rRNA gene of bacteria and archaea community, the primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used (Kozich *et al*., 2013). PCR was conducted with a 25 μL mixture containing 12.5 μL of Phusion® Hot start flex 2X Master Mix (New England Biolabs), 2.5 μL of each primer (10 μM), 50 ng of template DNA, and made up to 25 μL with double-distilled water. Thermal cycling was performed at 98 °C for 30 s, followed by 35 cycles at 98 °C for 10 s, 54 °C for 30 s, 72 °C for 45 s, and finally at 72 °C for 10 min. PCR products were isolated with 2% agarose gel, followed by purification with AMPure XT beads (Beckman Coulter Genomics, USA). The products were finally quantified by Qubit assay (Invitrogen, USA). After PCR, the products were analysed by Agilent 2100 Bioanalyser (Agilent, USA), and KAPA Library Quantification Kits (Kapa Biosciences, Woburn, MA, USA) were used to generate the sequencing library. Qualified libraries had concentrations > 2 nM. Sequencing was carried out on NovaSeq 6000 platform, 2x250 bp (NovaSeq 6000 SP Reagent Kit, 500 cycles) at LC Sciences. Raw reads with a length of 400–500 bp were generated, with a sequencing depth of approximately 50,000 reads per sample.

2.2.8 Bioinformatics

Data analysis was performed using DADA2 v.1.16 pipeline and R version 4.2.2. The DADA2 pipeline was implemented according to Benjamin Callahan's tutorial (https://benjjneb.github.io/dada2/tutorial.html), with some modifications to the filtering step. First, paired-end raw reads were barcoded, and the quality of raw data was checked before starting the downstream analyses by using the FastQC tool, which helped determine the sequence quality, GC content, primers, and adapters present in the raw data (Brown *et al*., 2017). The raw reads were subjected to quality filtering, denoising, and chimaera removal using the dada2 R package (version 1.26 for R version 4.2.2, Callahan *et al*., 2016). The primers were removed at the filtering step with the trimleft function with the length of each primer specified. The used filterAndTrim command is as follows: truncLen = c(240,240), maxN = 0, minLen = 100, maxEE = 5, trimLeft = 20, truncQ = 2. The relationship between the quality scores and the error rates was determined from the data, and the denoised amplicon sequence variants (ASVs) were inferred using the dada2 algorithm pool=”pseudo” command. After merging the sequences, the sequence table was constructed by

removing the sequences that are shorter than 390 bp, which are the results of non-specific priming. The resulting sequence table was subjected to the chimaera removal step using "removeBimeraDenovo" with method="consensus". As a final step, the aligned and filtered 16S rRNA gene V3/V4 sequences were assigned to taxonomy using SILVA v.138 (http://www.arb-silva.de/).

2.2.9 Bacterial community analysis

The next step of analysis was carried out using the R package Phyloseq, which is dedicated to the representation and analysis of microbiome data (version 1.42.0, McMurdie & Holmes, 2013). This package was used to combine the following data into one dataset: ASV table, taxa table, sampling data including any metadata collected, and phylogenetic tree. Next, data in the phyloseq object was cleaned from empty columns, Eukaryota, Chloroplast and Archaea, by using "subset_taxa" function. Following, data in the phyloseq object was subsetted using "subset_samples" according to sample type (either sponge or seawater). Next, "prune_taxa" was used to filter taxa to a total of above five (present in more than 5 samples) for seawater samples and sponge samples; the taxa number for seawater samples was reduced from 113,231 to 45,506; and from 113,231 to 22,417 for sponge samples. The community structure profiles for each sampling month were created by transforming the taxa counts to relative abundance using the "transform_sample_counts" function from the phyloseq package. Taxa were agglomerated using "tax_glom", and the tax table was melted using the "psmelt" function. The taxa data frames produced were visualised using the ggplot2 package (version 3.4.4; Wickham, 2016). To assess differences in the relative abundance of the top bacterial phyla across seasons and locations in sponges, Mann-Whitney U test was conducted using the rstatix package in R (version 0.7.2; Kassambara 2023). P-values were adjusted for multiple comparisons using the Benjamini-Hochberg (BH) method. To study the effect of bathing water quality on the sponge microbiome, four bacterial families containing faecal indicator bacterial genera were chosen: *Enterococcaceae, Enterobacteriaceae, Clostridiaceae* and *Pseudomonadaceae*. From the phyloseq package, the "subset_taxa" function was used to subset these four bacterial families from sponge samples phyloseq object. Approximately 274 bacterial taxa were obtained. The relative abundance of these bacterial families was estimated using the "tax_glom" and "psmelt" functions and visualised using ggplot2.

To prepare sponge samples for the next analysis (alpha and beta diversity analyses), they were rarefied using the "rarefy_even_depth" function to the minimum sequence depth of 35,038 reads based on 1,000 iterations of random subsampling.

2.2.10 Alpha and beta diversity analysis

Alpha and beta diversity tests are higher-level measures which are often used to describe the microbiome in a sample. Alpha diversity measures the microbiome diversity in a single sample (Lynch, 2023), while beta diversity measures the similarity or dissimilarity of two or more communities (Koleff *et al*., 2003). To obtain the alpha and beta diversity analysis for the sponge samples, a phyloseq package was used.

Observed richness, Shannon, and Inverse Simpson values were used for the alpha diversity analysis. To avoid any confusion with these data, each sample type data was analysed and modelled separately. The function "plot_richness" was used to estimate the difference in microbiome richness using Shannon and Simpson indices between locations in each season. According to Shapiro test, the diversity metrics (Observed, Chao, Shannon, and Inverse Simpson) were not normally distributed. Consequently, differences between sample groups within these metrics were investigated through non-parametric test (Kruskal-Wallis) using the "kruskal.test" function from the stats package in R. The results were then visualised using ggplot2.

Global (non-pairwise) and pairwise PERMANOVA testing was used for the beta diversity analysis based on the Bray-Curtis count-based distance metric with the adonis2 function to compare the bacterial community composition of ASVs between groups (seasons and locations), which was carried out using the vegan package (version 2.6.4, Dixon 2003; Oksanen et al. 2022). Moreover, the same PERMANOVA approach was applied to assess the impact of faecal indicator bacterial abundance on the variance in sponge microbial community composition among seasons. Furthermore, since the dataset was not normally distributed, the data were normalised through the rarefaction method to standardise sequence depth across samples. Then, ordination plots were produced based on dissimilarities with the nMDS (non-metric multidimensional scaling) method using "ordinate" and "plot_ordination" functions from phyloseq.

2.2.11 Venn diagram for the shared microbial community between different samples

VennDiagram and UpSetR packages were used to compare the bacterial genera shared by the two sample types (sponge and seawater) and among sampling locations and seasons. Venn diagrams for sponge taxa among sampling locations and different seasons were prepared by excluding the seawater taxa (ASVs) from the sponge samples. Afterwards, plots were generated using "get_vennlist", "get_upset", and "venn.diagram".

2.2.12 Correlation test for the effect of physical and chemical variables and faecal indicator bacteria on sponge top 10 most abundant bacterial phyla

Correlation tests were chosen to study the effects of environmental factors, such as chemical and physical parameters, on microbial community composition within the sponge. The microbiomeSeq package (Ssekagiri *et al*., 2017) was used in this analysis to estimate the effects of environmental variables on community structure using the “taxa.env.correlation” command under the Kendall method with a 0.05 p-value threshold. The output plots were visualised using ggplot2. The top 10 most abundant microbial phyla were chosen for this analysis, and the correlation tests were done according to sampling locations.

The effect of bathing water quality on the composition of the sponge's top 10 most abundant microbial phyla was also estimated using the correlation test. The bacterial families containing faecal indicator bacterial genera selected were Enterococcaceae, Enterobacteriaceae (Carrero-Colón *et al*., 2010; Olaolu *et al.*, 2014; Orel *et al*., 2022), Clostridiaceae (Wéry *et al*., 2010; Schreiber *et al*., 2019; Verduzco Garibay *et al*., 2021) and Pseudomonadaceae (Januário *et al*., 2019; Verduzco Garibay *et al*., 2021). These four faecal indicator bacterial families were chosen for this analysis to determine whether their presence in seawater samples affects the composition of the top 10 most abundant sponge microbial phyla. Based on the correlation coefficient (r) values, the relationships were classified into strong ($r > 0.5$), moderate ($r = 0.3$-0.5) and weak ($r < 0.3$). And the significant relationship was based on the P-values.

2.3 Results

H. panicea samples were identified through their morphological characteristics and microscopic examination of spicule shape and size. The spicules (Figure 2.3) were slightly curved, slender and sharp at both ends. Their length ranged from 100 to 480 µm.

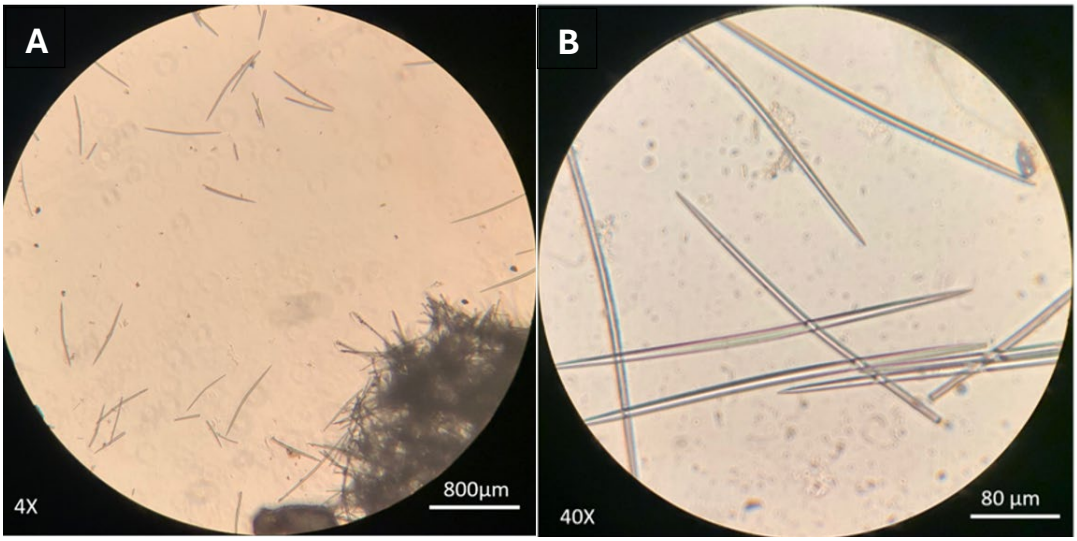


Figure 2. 3. *Halichondria panicea* spicule preparation and examination under the light microscope. A & B: lower magnification showing a bunch of *H. panicea* spicules at 4x and 40x magnification, respectively. The spicules measured between 159.50 - 246.13 µm in length.

2.3.1 Microbial analysis: Differences in bacterial communities between seasons and locations

Identifying the main factors affecting sponge health begins with understanding how the sponge microbiome differs between locations and seasons. Therefore, community structure, alpha and beta diversity analyses, along with Venn diagrams, were used to examine the differences between sponge microbiomes in each location and season in 2022.

The sponge bacterial community showed a distinctive shift in the abundance and diversity between seasons and locations (Figure 2.4, 2.5, 2.6, 2.7). The top 10 bacterial phyla were consistently found across all sponge samples, regardless of location or season. However, certain bacterial phyla significantly differ (according to Mann-Whitney test) between some locations (Appendix A- Figure A1) In general, the Proteobacteria phylum was predominant in all sponge samples and during all season accounting for 87% of the total bacterial community, followed by Firmicutes (3.40%), Bacteroidota (2.80%), Actinobacteriota (2.65%), Cyanobacteria (1.79%),

Planctomycetota (0.76%), Verrucomicrobiota (0.21%), Nitrospirota (0.20%), Acidobacteriota (0.19%) and Chloroflexi (0.13%). However, their relative abundance varies among seasons and locations (Appendix A - Table A1). Although sponge samples showed similar bacterial communities at the phylum level, differences were detected at the level of the top 20 genera. Most sponge microbial genera were found in all sampling locations, but the relative abundance of certain genera was location-dependent. The top 20 bacterial genera, in terms of abundance, from the three sampling locations and according to season, are presented in Figure 2.4. Specific genera such as *Escherichia-Shigella* and *HOC36_Unclassified* were most abundant in Cullercoats and Marsden Bay in April and July, respectively. Cullercoats and Marsden Bay reported mean relative abundances of Escherichia-Shigella in April of 0.81 and 0.52, respectively. While in July, HOC36_Unclassified was 0.79 and 3.05 in Cullercoats and Marsden Bay, respectively. Among other genera, *Enterococcus* and *Streptococcus* were abundant in the three sampling locations during April (Cullercoats= 2.07 and 1.16, Marsden Bay= 1.92 and 1.84, Whitley Bay= 2.42 and 1.78 respectively). *Escherichia-Shigella*, *Enterococcus* and *Streptococcus* genera belong to the faecal bacterial group that is recoverable from the gastrointestinal tracts of humans and animals. It was noted that Cullercoats, Marsden Bay, and Whitley Bay had a significant density of *Lactobacillus* and *Synechococcus* CC9902 genera in July. The density of the sponge microbial community was lowest in October at the three sampling locations when *Amylibacter* genera were most abundant in all samples more abundant in most of the samples (Cullercoats= 88.4, Marsden Bay= 60.3, Whitley Bay= 90.1). The relative composition of *Alphaproteobacteria_Unclassified* was highest in January samples and mostly at Cullercoats (4.60%).

There was a higher variability in the abundance of microbial genera across samples of Whitley Bay compared to the other locations, where some genera, such as *Arenicellaceae_Unclassified*, *Gammaproteobacteria_Unclassified* and *Herminiimonas*, only occurred at this location. Other genera were only found at Marsden Bay, including *Endozoicomonas*, *NS5 marine group,* and *SAR116 clade_Unclassified.*

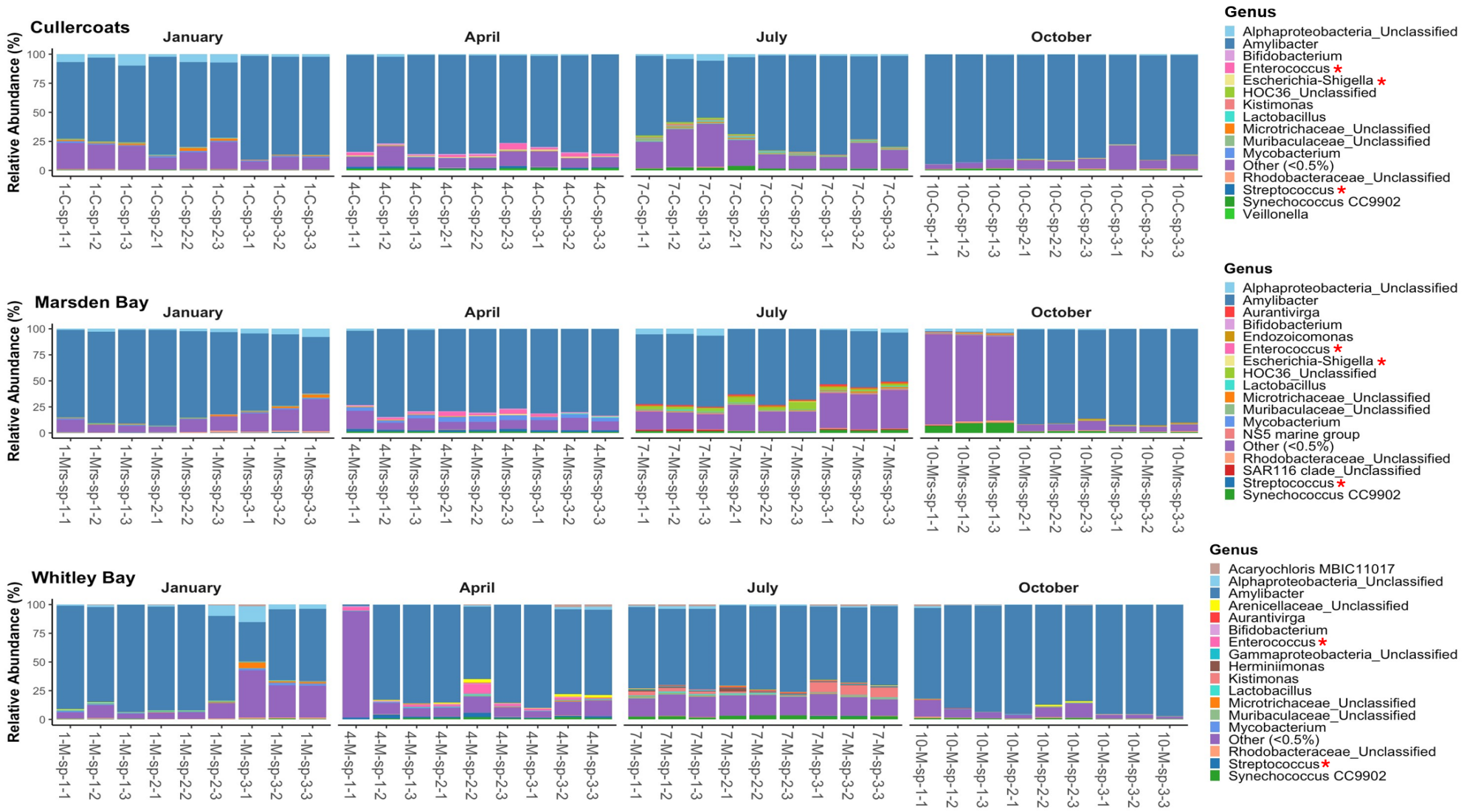


Figure 2. 4. The top 20 most abundant bacterial genera in sponge samples collected from Cullercoats, Whitley Bay and Marsden Bay in 2022. Genera marked with an asterisk (*) are recognised as faecal indicator bacteria, which are associated with poor bathing water quality. Sample codes indicate the sampling month (1= January, 4= April, 7= July, 10= October)- location (C= Cullercoats, M= Whitley Bay, Mrs= Marsden Bay)- sample type (sp = sponge)- sample number (biological replicate)- sample replicate (technical replicate).

The Venn diagrams in Figure 4 illustrate the distribution and overlap of bacterial genera among sponge samples, seawater, and different sampling locations across seasons. The shared bacterial genera between sponge and seawater samples are presented in Figure 2.5-A, showing that 1,002 genera are common between the two environments, while 181 genera are unique to the sponge and 179 are unique to seawater. This indicates a substantial overlap between the microbial communities of the sponge and the surrounding seawater, with a distinct set of sponge-specific genera. The shared bacterial genera among sponge samples (after excluding seawater taxa) collected from three different locations (Cullercoats, Marsden Bay, and Whitley Bay) are shown in Figure 4-B, revealing that 692 genera are present across all three locations, while each location also has a unique set of genera (44 at Cullercoats, 57 at Marsden Bay, and 73 at Whitley Bay), reflecting location-specific microbial diversity.

The seasonal distribution of shared bacterial genera at each location is displayed in Figure 2.5 (C, D, and E). At Whitley Bay (Figure 2.5-C), 165 genera are consistently shared across all four seasons (January, April, July, and October), with additional genera unique to each season, particularly in October. At Marsden Bay (Figure 2.5-D), 208 genera are present across all seasons, with a significant number of genera unique to specific seasons, especially October. At Cullercoats (Figure 2.5-E), 205 genera are consistently found throughout the year, while several genera are unique to individual seasons, with October showing the highest number of unique genera. Based on sampling months, the Venn diagrams reveal the distribution of bacterial genera among sponge samples, with October showing the highest number and the lowest in July. Overall, these plots demonstrate stable bacterial genera that persist throughout the year at each location, along with notable seasonal variations that contribute to distinct microbial profiles across different times and locations.

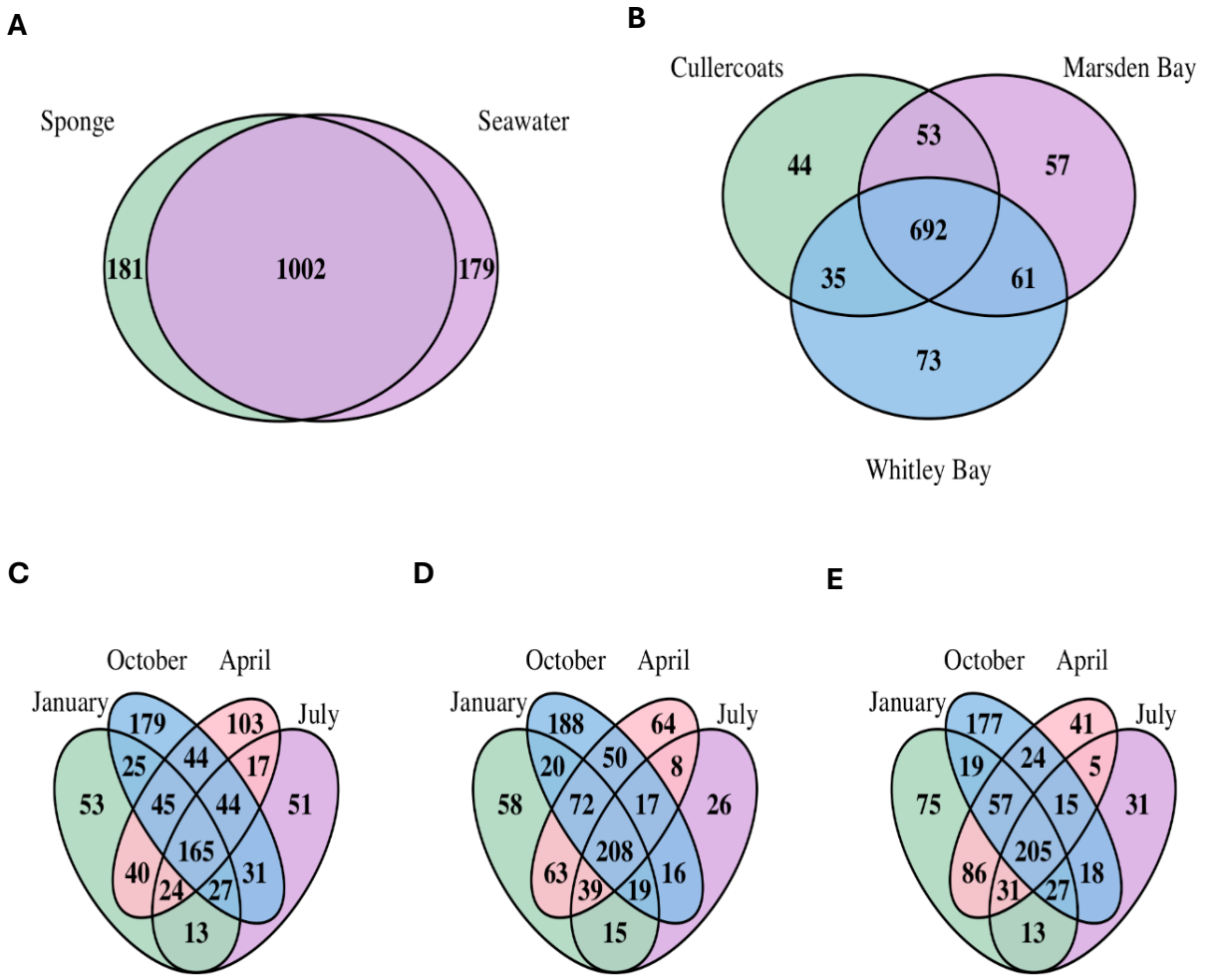


Figure 2. 5. The number of shared bacterial genera (**A**) between sponge and seawater samples and (**B**) among the three sampling locations (Cullercoats, Marsden Bay and Whitley Bay). Shared genera among sampling months in (**C**) Whitley Bay, (**D**) Marsden Bay and (**E**) Cullercoats.

The alpha diversity analysis of sponge samples from the three sampling locations in January, April, July and October is presented in Figure 2.6. The Kruskal-Wallis test revealed no statistically significant differences in bacterial diversity between locations across seasons ($P > 0.05$; Figure 2.6). However, when all locations were pooled together, significant differences in microbial diversity indices (Shannon, Simpson, Observed, and Chao1) were observed between different months or seasons (Appendix A- Figure A2). The Shannon and Simpson indices showed significantly higher diversity in July compared to other months ($P < 0.001$), while the Chao1 and Observed richness indices indicated notable differences, particularly between July and April ($P <$

0.001). In contrast, the alpha diversity differences between individual locations within the same season were not statistically significant ($P > 0.05$), suggesting that seasonal changes, rather than location-specific factors, have a more pronounced impact on the microbial community diversity of sponges (Figure 2.6).

For the beta diversity, Bray–Curtis distance using the global and pairwise PERMANOVA method between the ASVs of sponge samples revealed some differences among locations in only one season (Appendix A- Table A2). In January, sponge microbial composition differed significantly among the three sampling locations ($P = 0.001$). Whereas Whitley Bay showed a significant difference ($P < 0.05$) in sponge community structure when compared with Cullercoats and Marsden Bay. In contrast, April samples were not significantly different ($P = 0.735$). Finally, for July and October, there were no significant differences among the three sampling locations ($P > 0.05$). Upon examining the differences in microbial diversity of sponge samples among seasons, each location had a statistically significant difference ($P > 0.05$) between seasons (Figure 2.7- B, Appendix A- Table A3). Moreover, a significant seasonal difference ($P < 0.01$) and clear separate clustering of January samples was observed in ordination plots of beta diversity (Figure 2.7- A) with a 0.001 stress value. The distance between points represents the level of difference. Stress lower than 0.2 indicates that the nMDS analysis is reliable. The closer the samples in the graph, the higher their similarity.

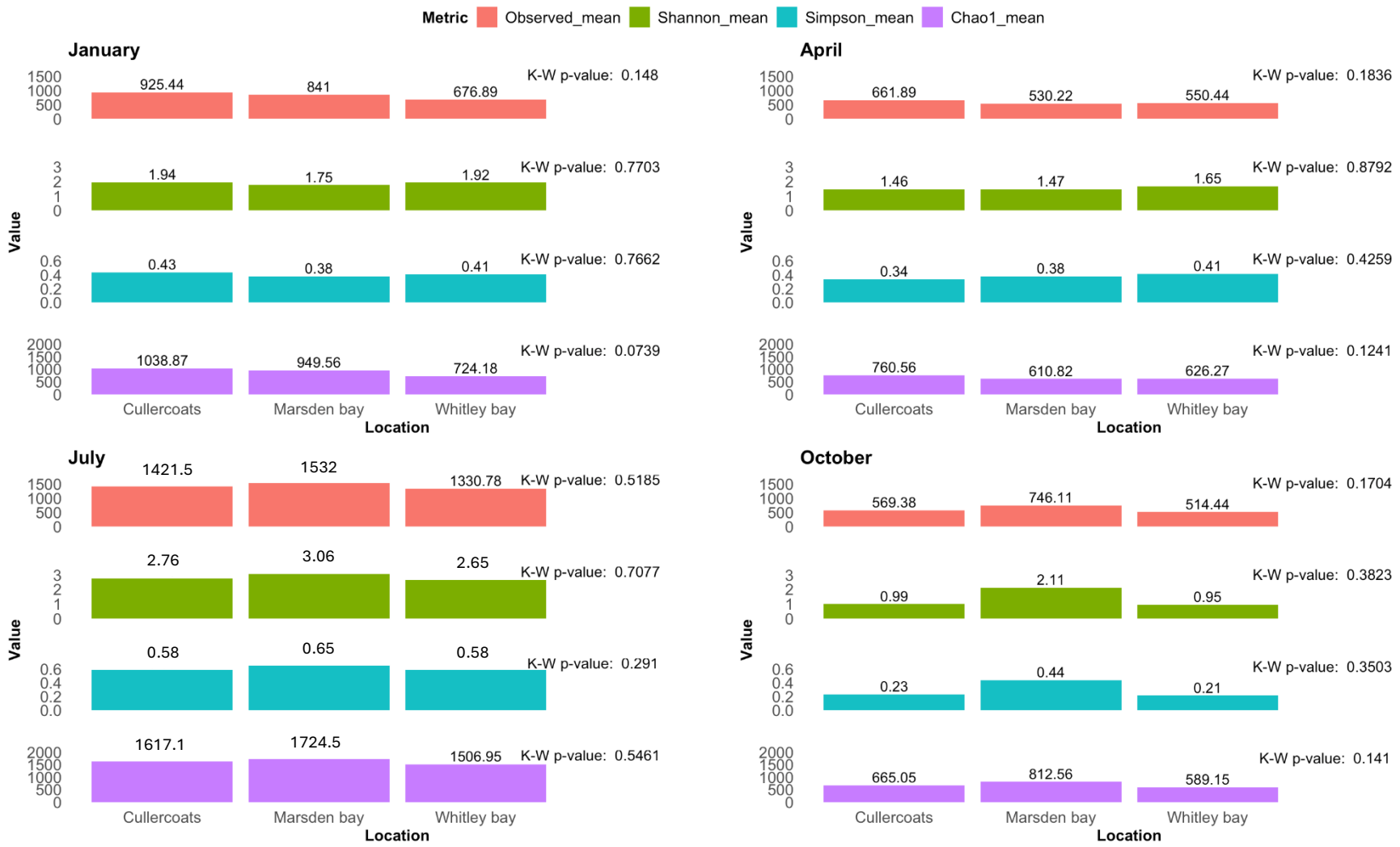


Figure 2. 6. Alpha diversity metrics (Observed, Shannon, Simpson, and Chao1 indices) for seasonal variation of sponge-associated bacterial communities across three locations: Cullercoats, Marsden Bay, and Whitley Bay. The mean values of these metrics are presented, accompanied by Kruskal-Wallis (K-W) p-values.

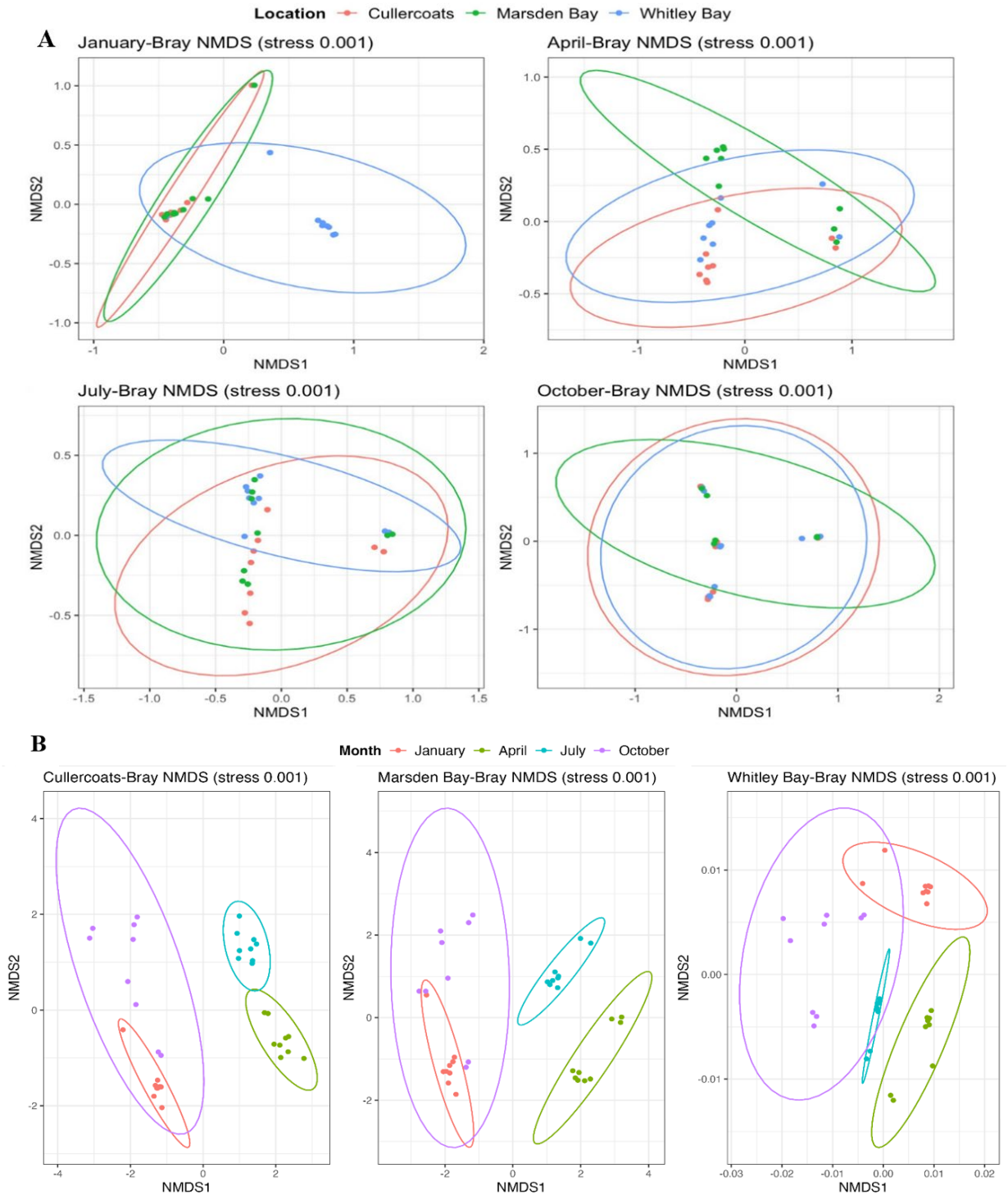


Figure 2. 7. Ordination plot of beta diversity (Bray-nMDS) for sponge bacterial composition. (**A**) according to season (January, April, July and October). (**B**) According to location. Each point in the graph represents one sample, and different colours represent different locations (**A**) or seasons (**B**).

As a result of the seasonal variability observed across sites in sponge microbial communities (Figures 2.4, 2.5, 2.6 and 2.7), the physical and chemical parameters, as well as bathing water quality, were analysed in relation to the abundance of the top ten dominant sponge bacterial phyla to identify potential environmental drivers of these community shifts.

2.3.2 Microbial analysis: Relation between chemical and physical properties of seawater and sponge bacterial community

The sponge bacterial community showed a distinctive shift in the abundance and diversity between seasons and locations (Figures 2.4, 2.5, 2.6 and 2.7), which could be explained by examining the physical and chemical properties of the surrounding seawater or the natural water discharge (NWD). In terms of physical properties, the seawater samples varied in temperature from 5.4-8.1 °C in January to 10-11 °C in April to 15-17 °C in July to 12.2-12.7 °C in October (Table 2.2). In contrast, the pH values ranged from 7.91 to 8.7. The pH of Whitley Bay seawater was slightly higher in April, July and October. In contrast, Cullercoats water samples only showed a slight pH increase in October. The salinity of seawater samples from the three sites ranged from 37.4 to 40.3 ppt, while the conductivity values ranged from 60.50 to 65 mS/cm. Furthermore, the TDS ranged from 30.2 to 32.5 ppt.

The NWD samples from the three sites had pH values ranging from 8.33 to 8.72, which was slightly higher than the seawater samples. The temperature of the NWD samples changed according to season but remained a few degrees higher than the seawater at the sampling sites. The salinity, conductivity and TDS properties of the NWD were very low. The salinity ranged from 0.3 to 0.57 ppt, conductivity was 0.53 to 1.13 mS/cm, and TDS ranged from 0.37 to 0.81 ppt throughout the year. Cullercoats NWD had higher salinity, conductivity and TDS than Whitley Bay NWD. Moreover, comparing the TDS with the salinity values showed that the NWD of both Cullercoats and Whitley Bay contained higher TDS values, meaning they had higher salt ions or organic pollutants. In contrast, the resistivity of Whitley Bay NWD was higher in January (1.6 KΩ), April (1.1 KΩ) and October (1.9 KΩ) compared to Cullercoats NWD waters (1.2 KΩ, 0.87 KΩ and 1.4 KΩ, respectively), which is directly related to the conductivity values (amount of dissolved salt in water).

Several of the physical parameters of seawater from the three sampling locations (Table 2.2) showed significant correlations with the abundance of the top 10 dominant sponge bacterial

phyla (Figure 2.8). A significant positive correlation was observed between temperature and the abundance of Cyanobacteria ($r = 0.3$, $P < 0.01$, moderate) and Bacteroidota ($r = 0.5$, $P < 0.01$, moderate). Conversely, Actinobacteriota showed a significant negative correlation with temperature at Cullercoats ($r = -0.4$, $P < 0.01$, moderate).

Additionally, there was a significant negative correlation between conductivity and salinity and the abundance of Fusobacteriota ($r = -0.6$, $P < 0.001$, strong) and Firmicutes ($r = -0.5$, $P < 0.001$, moderate). At Whitley Bay, a strong significant positive correlation ($r > 0.5$, $P < 0.001$) was observed between pH and the abundance of Cyanobacteria. In contrast, the correlation was moderate ($r = 0.4$-0.5, $P < 0.001$) for Firmicutes and Bacteroidota. Proteobacteria were more abundant at elevated pH ($r = 0.5$, $P < 0.001$, moderate) and conductivity ($r = 0.4$, $P < 0.01$, moderate) at Cullercoats. Conversely, Verrucomicrobiota at Cullercoats showed a significant moderate negative correlation with conductivity ($r = -0.5$, $P < 0.001$).

Table 2. 2. Physical parameters of water samples.

Month	Location	Sample type	pH	Temperature (°C)	Salinity (ppt)	Conductivity (mS/cm)	TDS (ppt)	Resistivity
January	Cullercoats	Seawater	8.29	5.4	39.70	63.60	32.00	>50 Ω
January	Cullercoats	NWD	8.33	10.8	0.40	0.81	0.58	1.2 KΩ
January	Whitley Bay	Seawater	7.91	8.1	39.70	63.90	32.00	>50 Ω
January	Whitley Bay	NWD	8.40	10.7	0.30	0.61	0.43	1.6 KΩ
January	Marsden Bay	Seawater	8.23	7.4	40.50	65.30	10.30	>50 Ω
April	Cullercoats	Seawater	8.39	10.0	38.60	64.30	32.00	>50 Ω
April	Cullercoats	NWD	8.72	11.3	0.57	1.13	0.81	0.87 KΩ
April	Whitley Bay	Seawater	8.60	11.0	37.40	62.50	31.60	>50 Ω
April	Whitley Bay	NWD	8.40	15.0	0.44	0.90	0.64	1.1 KΩ
April	Marsden Bay	Seawater	8.47	10.0	37.90	60.50	30.20	>50 Ω
July	Cullercoats	Seawater	8.33	15.8	38.40	61.30	30.90	>50 Ω
July	Cullercoats	NWD	8.45	21.7	0.47	0.95	0.66	1.1 KΩ
July	Whitley Bay	Seawater	8.70	17.0	37.90	62.70	31.20	>50 Ω
July	Whitley Bay	NWD	-	-	-	-	-	-
July	Marsden Bay	Seawater	8.44	15.0	38.50	61.90	31.20	>50 Ω
October	Cullercoats	Seawater	8.78	12.2	40.20	65.00	32.50	>50 Ω
October	Cullercoats	NWD	8.61	13.0	0.36	0.73	0.51	1.4 KΩ
October	Whitley Bay	Seawater	8.58	12.7	40.30	64.70	32.50	>50 Ω
October	Whitley Bay	NWD	8.33	12.5	0.26	0.53	0.37	1.9 KΩ
October	Marsden Bay	Seawater	8.74	12.5	39.9	64.60	32.3	>50 Ω

NWD= natural water discharge, **ppt=** part per thousand, **ppm=** part per million, **Ω**= ohm, **KΩ**= kiloohm, **-** = not measured.

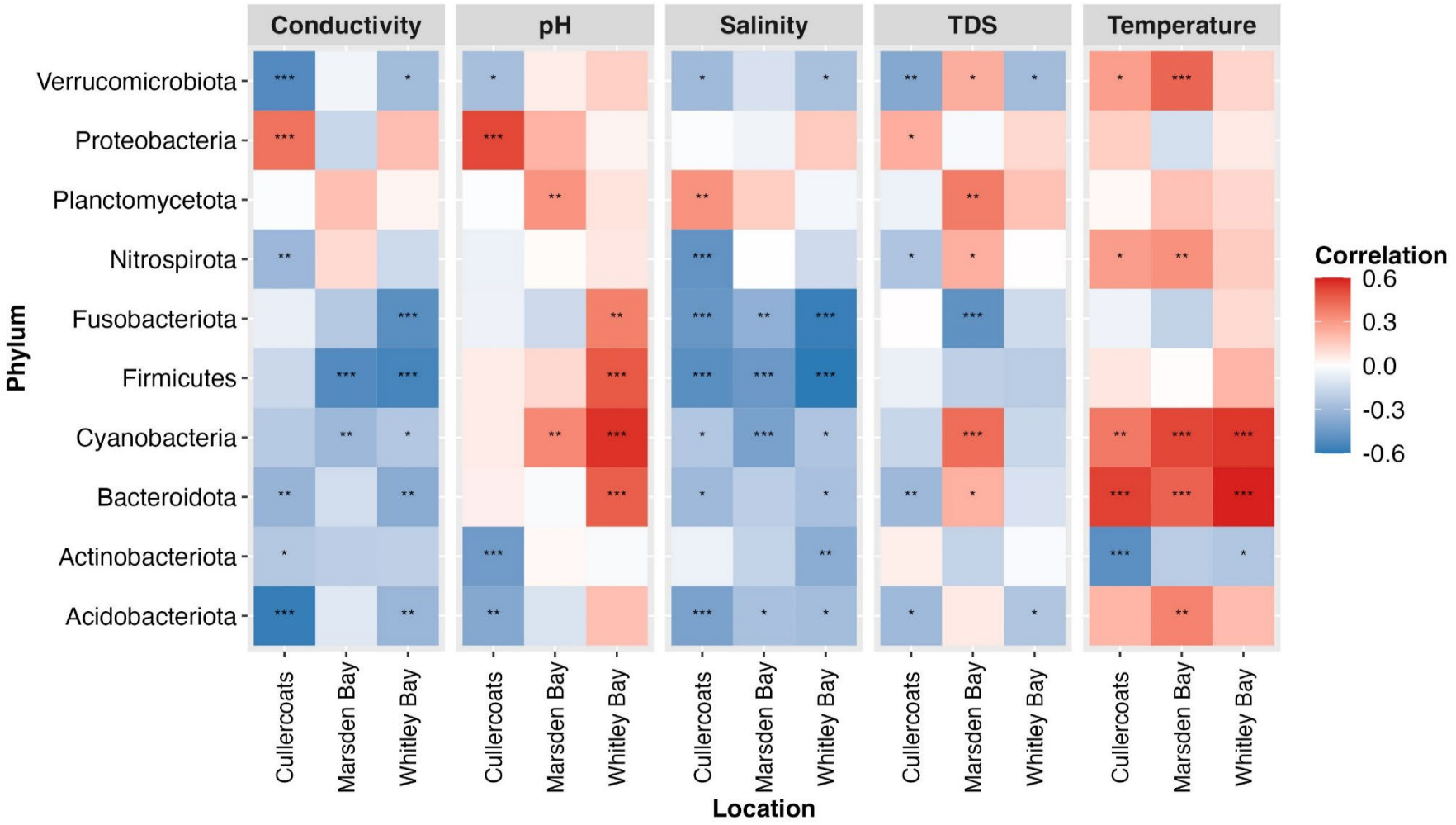


Figure 2. 8. Heatmap showing the correlation analysis between environmental factors and the abundance of the top 10 sponge microbiome phyla. Red indicates a positive correlation and blue indicates a negative correlation. Asterisks represent the significance of linear regression, *= $P < 0.05$, **= $P < 0.01$, *** =$P < 0.001$.

When looking at the chemical properties of the water samples, trace metals and nutrients vary among locations and seasons. The trace metals analysis of the seawater and water outfalls at the sampling locations in 2022 (January, April, July and October months) are presented in Table 2.3. Some of the metals exceeded the acceptable limits set by several Environmental agencies in Europe (Tovar-Sánchez et al., 2018), England (Environment Agency, 2014), Australia and New Zealand (Water Quality Australia, 2021).

The NWD samples showed elevated concentrations of aluminium (Al), manganese (Mn), iron (Fe) and copper (Cu) when compared to Australian and New Zealand and UK trace metal standard limits in the aquatic environment (Table 2.3). The Al concentration of NWD ranged from 0.63 to 31.45 µg/L, which is higher than Australian and New Zealand standards (0.5 µg/L). The January Al values also exceeded the standard limit suggested by Golding *et al*. (2015) (=24 µg/L), which is harmful to marine life. Mn concentration varied from 7.56 to 136.1 µg/L in the four sampling months, exceeding the standard limits of EPA (NSW EPA, 2000) with no limits suggested by UK, European, Australian and New Zealand agencies. According to the UK, Australia and New Zealand, the Fe and Cu toxicity limits are 1 and 2 µg/L for Fe and 3.76 and 2 µg/L for Cu, respectively, whereas the NWD samples exceeded both limits. In April, the Cullercoats NWD sample had a lead (Pb) value of 15.58 µg/L, which is far higher than Europe (7.2 µg/L) and Australia and New Zealand (4.4 µg/L) standards. Zinc (Zn) in the Whitley Bay NWD in April (51.63 µg/L) and the Cullercoats NWD in July (6.96 µg/L) also exceeded acceptable limits of 6.8 and 8 µg/L for Europe and Australia and New Zealand, respectively.

The seawater samples contained high levels of Al through January, April and October (compared to Australian and New Zealand standards, Table 2.3), except for Marsden Bay, which did not show any traces of Al in April but exceeded the limit (1.12 µg/L) in July. Cullercoats seawater exceeded the standard limits (Europe: 6.8 µg/L, Australia and New Zealand: 8 µg/L) for Zn in both months (13.87 and 18.62 µg/L, respectively), which in contrast was low in the NWD samples at the same location. Chromium (Cr) in Marsden Bay seawater (January) at 8.91 µg/L exceeded the mean long term dissolved concentration of the UK Environment Agency standards of (0.6 µg/L) but was lower than the safe limit determined by Australia and New Zealand (=10 µg/L). In April, the Fe concentration in seawater samples ranged from 6.31 to 6.40 µg/L, which exceeded the UK, Australia and New Zealand standards (1 and 2 µg/L, respectively). Among seawater samples, Marsden Bay in January had the highest Fe value (33.07 µg/L). Other metals

such as arsenic (As), strontium (Sr), cadmium (Cd), antimony (Sb) and barium (Ba) were within the standard limits of toxicant values (of Europe, UK, Australia and New Zealand environment agencies) in the marine environments in all water samples.

Seawater trace metal (Table 2.3) concentrations correlated significantly with the composition and diversity of the sponge microbiome, with some phyla appearing to be sensitive to trace metal levels in their surroundings (Figure 2.9). Fusobacteriota and Firmicutes showed a significant positive correlation with Cd, Fe, Pb and Zn ($r = -0.4$ to -0.6, $P < 0.001$, moderate to strong), while a negative correlation with Cu, Mn and Ni ($r = -0.4$ to -0.6, $P < 0.001$, moderate to strong). Aluminium correlates negatively with Cyanobacteria and Bacteroidota ($r = -0.4$ to -0.6, $P < 0.001$, moderate to strong). At Cullercoats, Actinobacteriota demonstrated a significant positive correlation with Al, Zn ($r > 0.5$, $P < 0.001$, strong), Cr and Pb ($r = 0.4$, $P < 0.01$, moderate) at the same location. Conversely, Actinobacteriota exhibited a strong significant negative correlation with Cu and Sr ($r > 0.5$, $P < 0.001$). At Marsden Bay, Firmicutes displayed a strong positive correlation with Cd and Zn ($r = 0.6$, $P < 0.001$), whereas a moderate to strong negative correlation was found with Al, Cu and Ni ($r = -0.3$ to -0.6, $P < 0.001$). Fusobacteriota were strongly positively correlated with Cd and Pb ($r = 0.6$, $P < 0.001$) and moderately negatively correlated with Cu and Mn ($r = -0.4$ to -0.5, $P < 0.001$). While in Whitley Bay, Bacteroidota showed a strong positive correlation with Ba ($r > 0.5$, $P < 0.001$) and a strong negative correlation with Al ($r = -0.6$, $P < 0.001$). Acidobacteriota demonstrated a moderate positive correlation with Cu ($r = -0.4$, $P < 0.001$) and a strong negative correlation with Cd ($r > -0.5$, $P < 0.001$).

Nine anions were measured in the water samples (seawater and water discharge). These anions were fluoride, chloride, nitrate, nitrite, bromide, sulphate, phosphate, ammonia and silicate (Table 2.4). The chloride concentration in seawater samples collected from Cullercoats and Whitley Bay exceeded the standard limit of natural chloride in seawater (18,980 ppm; (Standford University 2022) in January, July and October. The elevated chloride levels is are related to the higher levels of chloride in NWD during these months (Table 2.4). Nevertheless, Marsden Bay's chloride level in October was slightly above average. The bromide concentration was almost within the average seawater concentration (65 ppm) throughout the year, except for April, when all seawater samples experienced elevated bromide levels (117.76, 124.13, and 126.16 ppm for Cullercoats, Whitley and Marsden Bay, respectively) that exceeded the standard natural limit and EQS maximum allowable concentration of bromide in coastal waters (= 10 ppm). In April seawater

samples, the concentration of sulphate exceeded the average limit of sulphate in seawater, at 3014.14, 2903.63, and 3022.14 ppm in Cullercoats, Whitley and Marsden Bay, respectively.

The chloride concentration in the seawater samples collected from Cullercoats and Whitley Bay exceeded the standard limit of natural chloride in seawater (18,980 ppm; Stanford University, 2022) in January, July and October. The elevated chloride level is related to the input from water outfalls in both areas throughout the year, which comes from land runoff. Nevertheless, Marsden Bay's chloride level in October was slightly above average. The bromide concentration was almost within the average seawater concentration (65 ppm) throughout the year, except for April, when all seawater samples experienced elevated bromide levels (117.76, 124.13, and 126.16 ppm for Cullercoats, Whitley and Marsden Bay, respectively). In April seawater samples, the concentration of sulphate exceeded the average limit of sulphate in seawater, at 3014.14, 2903.63, and 3022.14 ppm in Cullercoats, Whitley and Marsden Bay, respectively.

April NWD samples experienced elevated ammonia and phosphate levels. Cullercoats and Whitley Bay NWD waters contained very high concentrations of ammonia and phosphate, exceeding the permissible limits in surface waters as specified by the Environment Agency (Environment Agency, 2022b; Johnson *et al*., 2007). Cullercoats and Whitley Bay NWD waters were highly contaminated with phosphate (April, July, and October samples) that exceeded the acceptable limit (0.1 mg/L) (Environment Agency, 2022b). There was, however, no effect of this increase on the levels of phosphate in seawater.

Based on the concentrations of nutrients in seawater samples at the sampling locations (Table 2.4), Cullercoats had the highest nutrient concentrations, including ammonia, bromide, nitrate, and silicate, which correlates with a high relative abundance of Proteobacteria ($r = 0.5$ to 0.6, $P < 0.001$, moderate to strong) (Figure 2.10, Appendix A- Figure A1). Actinobacteriota and Acidobacteriota showed a significant negative correlation with nitrate ($r = -0.3$ to -0.5, $P < 0.01$, moderate), nitrite ($r = -0.4$, $P < 0.05$, moderate), and silicate ($r = -0.3$ to -0.4, $P < 0.05$, moderate) at Cullercoats and Whitley Bay sites. Meanwhile, Firmicutes and Fusobacteriota were positively correlated with bromide ($r = 0.4$ to 0.5, $P < 0.01$, moderate) and sulfate ($r = 0.5$, $P < 0.001$, strong). Ammonia ($r = 0.3$, $P < 0.05$, moderate), phosphate ($r = 0.4$, $P < 0.01$, moderate), and sulfate ($r =$ 0.3-0.4, $P < 0.01$, moderate) showed significant positive correlations with cyanobacterial abundance across the three locations.

At Cullercoats, a strong positive correlation was observed between Proteobacteria and ammonia, bromide, nitrate, nitrite and silicate ($r = 0.5$ to 0.6, $P < 0.001$, moderate to strong). In contrast, a significant moderate negative correlation was found between Proteobacteria and chloride ($r = -0.4$, $P < 0.001$). Firmicutes at this location were positively correlated with sulphate ($r = 0.5$, $P < 0.001$, moderate), whereas Acidobacteriota, Actinobacteriota and Verrucomicrobiota exhibited a moderate negative correlation with nitrate ($r = -0.3$ to -0.5, $P < 0.001$). In Marsden Bay, a significant positive correlation was seen in bromide with Fusobacteriota and Firmicutes ($r > 0.5$, $P < 0.001$, strong). A moderate negative correlation was found in Acidobacteriota with nitrite and chloride ($r = -0.3$ to -0.5, $P < 0.01$ & 0.001, respectively). Cyanobacteria showed a moderate positive correlation with phosphate and sulphate ($r = 0.5$, $P < 0.001$). While in Whitley Bay, Actinobacteriota showed a significant negative correlation with nitrate, nitrite and phosphate ($r = 0.5$, $P < 0.001$, moderate). Conversely, Verrucomicrobiota had a significant negative correlation with nitrite and silicate ($r = 0.5$, $P < 0.001$, moderate).

Table 2. 3. Trace metal analysis of water samples.

Samples information			**Concentration (µg/L)**												
Month	**Location**	**Sample type**	**Al**	**Cr**	**Mn**	**Fe**	**Ni**	**Cu**	**Zn**	**As**	**Sr**	**Cd**	**Sb**	**Ba**	**Pb**
January	Cullercoats	Seawater	25.53	0.3	4.97	0	1.96	1.77	13.87	1.92	7112.76	0.01	0.12	7.65	0.23
January	Cullercoats	NWD	28.33	0.29	15.7	4.63	0.57	7.65	4.31	0.7	269.63	0	1.25	75.42	0.18
January	Whitley Bay	Seawater	22.49	0.45	12.56	0	2.10	2.73	2.08	1.72	6088.27	0.02	0.3	6.14	0.42
January	Whitley Bay	NWD	31.45	9.95	7.56	69.54	5.24	7.48	1.01	0.6	382.43	0.01	1.15	57.12	0.33
January	Marsden Bay	Seawater	18.63	8.91	2.94	33.07	3.42	2.39	0.14	1.21	4903.19	0	0.08	5.89	0.09
April	Cullercoats	Seawater	2.71	0.48	1.30	6.4	0.1	0.73	18.62	1.31	7081.67	0.03	2.28	8.09	1.26
April	Cullercoats	NWD	9.24	0.23	23.31	8.29	0.37	3.22	3.78	0.58	490.24	0.01	1.66	35.91	15.58
April	Whitley Bay	Seawater	5.22	0.31	2.36	6.31	0.1	0.64	12.95	0.72	7114.66	0.06	3.06	8.15	2.89
April	Whitley Bay	NWD	19.11	0.28	7.60	25.7	1.37	7.4	51.63	0.91	765.80	0.06	2.39	47.33	2.85
April	Marsden Bay	Seawater	0	0.14	0.56	6.38	0	0.74	6.82	1.97	7510.22	0.1	1.45	6.57	1.78
July	Cullercoats	Seawater	0	0.18	17.75	0	0.61	2.42	4.33	2.77	8498.42	0.05	0.24	8.26	0
July	Cullercoats	NWD	0.63	0.16	25.39	0	2.69	3.39	6.96	1.72	612.82	0	0.62	32.05	0
July	Whitley	Seawater	0	0.14	11.95	0	1.08	2.81	2.12	1.88	8268.35	0	0.14	9.84	0
July	Whitley	NWD	-	-	-	-	-	-	-	-	-	-	-	-	-
July	Marsden	Seawater	1.12	0.17	16.15	0	0	0.96	3.36	0.78	4363.72	0.03	0.08	4.88	0
October	Cullercoats	Seawater	1.98	0.16	10.28	0	1.51	2.45	2.56	1.78	8894.59	0	0.16	7.93	0
October	Cullercoats	NWD	3.28	0	136.1	0	1.11	5.70	1.29	0.83	231.67	0	0.5	65.64	0

October	Whitley	Seawater	3.33	0	27.74	0	0.93	2.56	4.984	3.31	8522.28	0.07	0.28	8.84	0
October	Whitley	NWD	1.87	0.18	14.65	0	1.64	6.43	4.77	1.0	313.50	0.03	0.72	44.76	0
October	Marsden	Seawater	1.00	0.08	8.17	0	1.04	2.57	3.23	2.31	8360.21	0	0.22	8.0	0
The Criterion Maximum Concentration			24[c]	0.6/ 32[d]	2.0[a]	1[d]	20[b]	3.76[d]	6.8[d]	25[d]	-	0.2[b]	-	-	7.2[b]
Standard limit (Australian and New Zealand)-2000-2021 water quality guidelines 99% limit			0.5	10	-	2.0	7.0	2.0	8	2.3	-	0.2	270	-	4.4
Average concentration in seawater (Stanford University)			190	-	1	2	0.05	9	1.4	2.4	13000	-	-	5	0.5

a: NSW EPA 2000
b: Tovar-Sánchez *et al*., 2018
c: Golding *et al*., 2015
d: Mean dissolved concentration in saltwater, long term= 0.6 µg/l, short term= 32 µg/l. Environment Agency, 2014

Table 2. 4. Nutrient analysis of water samples.

Samples information			**Concentration (ppm)**								
Month	Location	Sample type	Fluoride (F^-)	Chloride (Cl^-)	Nitrite (NO_2^-)	Bromide (Br^-)	Sulphate (SO_4^{-2})	Nitrate (NO_3^-)	Phosphate (PO_4^-)	Ammonia (NH_3^-)	Silicate (SiO_3^{2-})
January	Cullercoats	Seawater	0	22319.8	0	43.01	2448.6	0	0	-	-
January	Cullercoats	NWD	0.36	2769.87	0	0	35.29	0	0	-	-
January	Whitley Bay	Seawater	0	18708.5	0	39.64	2345.05	0	0	-	-
January	Whitley Bay	NWD	0	2869.71	0	0	87.43	0	0	-	-
January	Marsden Bay	Seawater	0	14637.2	0	29.51	1629.31	0	0	-	-
April	Cullercoats	Seawater	0	18501.64	0.02	117.76	3014.14	0.05	0.06	0.03	0.43
April	Cullercoats	NWD	0	749.06	0.02	31.83	225.91	0.11	0.29	0.10	1.23
April	Whitley Bay	Seawater	0	17974.65	0.00	124.13	2903.63	0.02	0.05	0.01	0.31
April	Whitley Bay	NWD	0	1350.00	0.17	33.33	270.63	1.17	0.35	0.09	15.52
April	Marsden Bay	Seawater	0	18312.54	0.02	126.162	3022.14	0.06	0.06	0.01	0.39
July	Cullercoats	Seawater	0	21584.21	0	60.05	2503.02	0.02	0.07	0.02	0.27
July	Cullercoats	NWD	0	1835.33	0.11	15.31	356.17	0.38	2.45	0.04	3.59
July	Whitley	Seawater	0	21689.67	0	44.71	2520.83	0.02	0.05	0.02	0.16
July	Whitley	NWD	-	-	-	-	-	-	-	-	-
July	Marsden	Seawater	0	12850.08	0	61.26	2247.26	0.02	0.04	0.02	0.18
October	Cullercoats	Seawater	0	21444.86	0.05	75.07	2478.70	0.11	0.06	0.02	0.44
October	Cullercoats	NWD	0	417.12	0.10	5.92	97.43	0.32	1.18	0.12	16.57
October	Whitley	Seawater	0	21300.97	0.04	52.50	2459.91	0.09	0.06	0.03	0.39

October	Whitley	NWD	0	157.23	0.13	11.76	154.53	0.55	0.68	0.02	10.47
October	Marsden	Seawater	0	20095.11	0.07	22.83	2312.59	0.15	0.09	0.02	0.45
Average concentration in seawater (Stanford University)			1	18980	0.05	65	2649	0.7	0.1	0.05	4
UK Environment Agency (acceptable limit in surface waters)			1	250	0.5	-	400	10	0.1	0.02	-

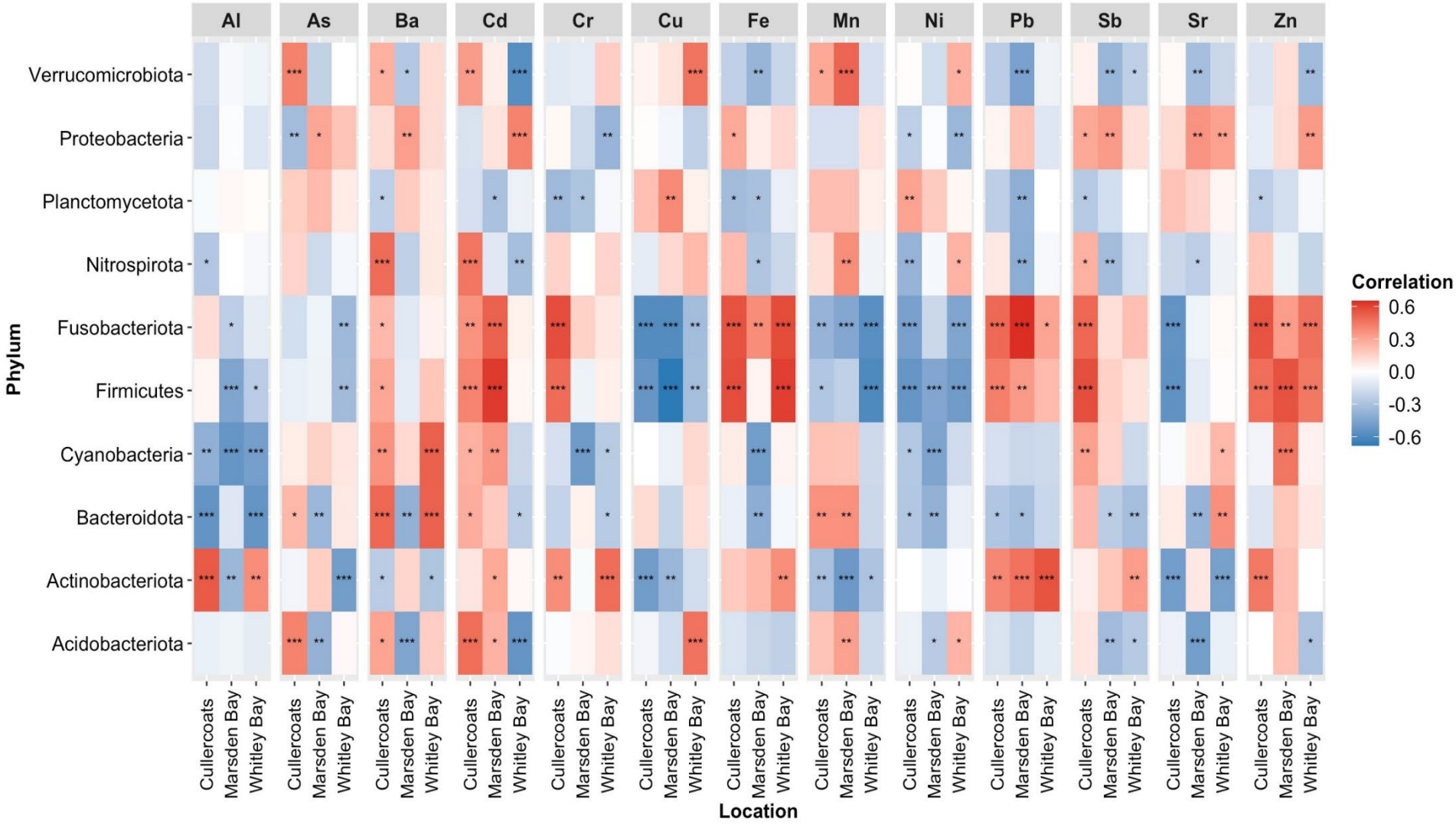


Figure 2. 9. Heatmap of correlation analysis between trace metals and abundance of the top 10 sponge microbiome phyla. Red indicates a positive correlation and blue indicates a negative correlation. Asterisks represent significance of linear regression, *= $P < 0.05$, **= $P < 0.01$, *** =$P < 0.001$.

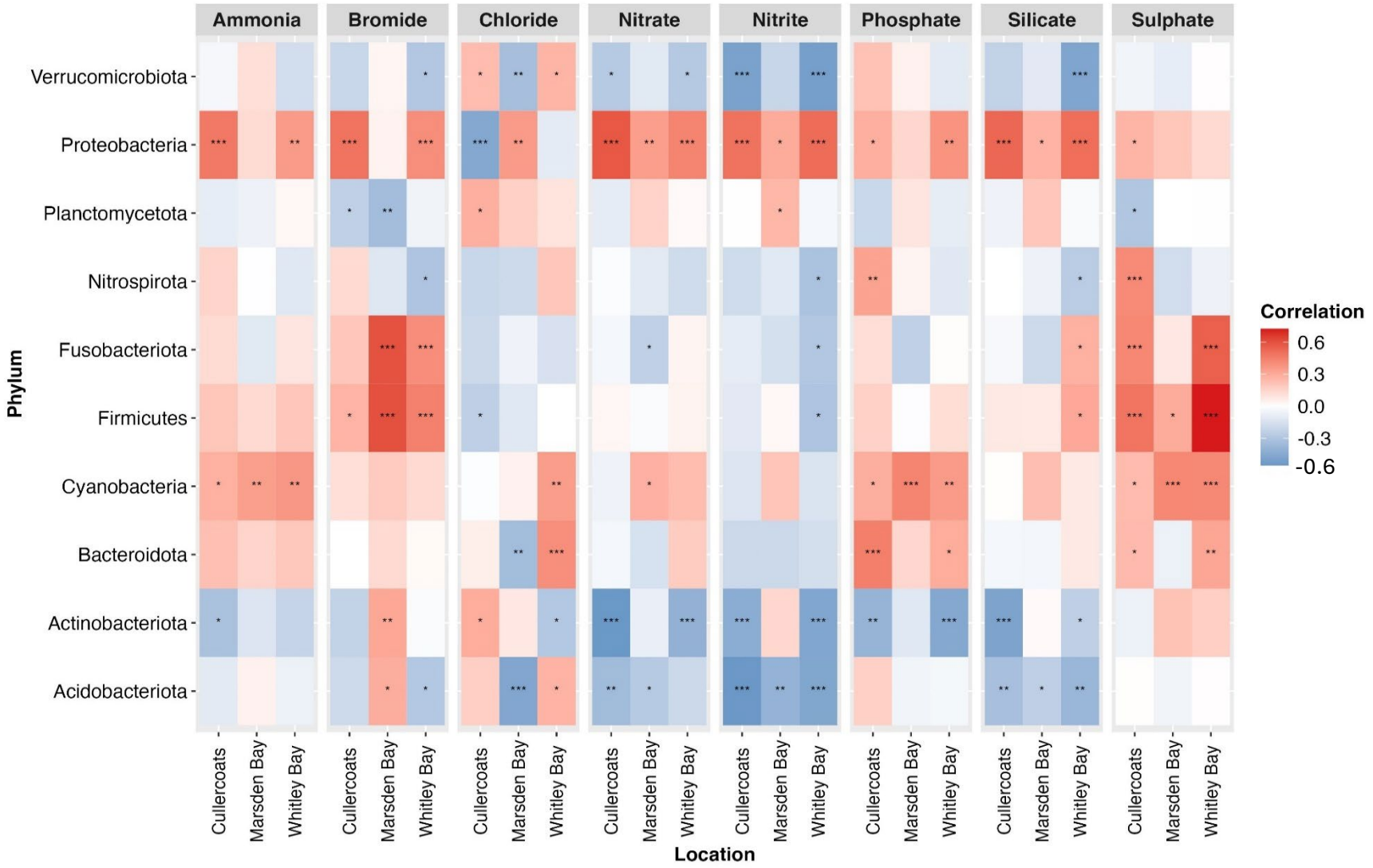


Figure 2. 10. Heatmap of correlation analysis between the analysed nutrients and abundance of the top 10 sponge microbiome phyla. Red indicates a positive correlation and blue indicates a negative correlation. Asterisks represent significance of linear regression, *= $P < 0.05$, **= $P < 0.01$, *** =$P < 0.001$.

2.3.3 Microbial analysis: Effect of bathing water quality on sponge bacterial community

The relative abundance of faecal bacteria from selected genera in seawater samples was similarly reflected in the relative abundance of these bacteria in sponge samples (Figure 2.11). This relationship was most evident in the April samples. In April, the mean relative abundance of *Enterococcus* and *Escherichia-Shigella* was the highest in seawater (69.5 and 24.5, respectively) and consequently in sponges (64.3 and 29.9, respectively). Similarly, October seawater samples showed a clear mean relative abundance of *Escherichia-Shigella* in Cullercoats (21.7) and Marsden Bay (49.6), while Whitley Bay showed a higher abundance of *Pseudomonas* (39.3) and different *Clostridium sensu stricto* species (59.8). Consequently, this was reflected in the higher abundance of *Escherichia-Shigella* in sponges in Cullercoats (27.1) and Marsden Bay (25.2), and *Clostridium sensu stricto* species in Whitley Bay (58.2) in October. While *Pseudomonas* was not detected in Whitley Bay sponge samples. Faecal genera from other sampling months were also abundant but at a lower number than those from April and October.

Faecal indicator bacterial families in sponge samples negatively affect the diversity of the sponge bacterial community. The PERMANOVA analysis showed a significant effect ($P = 0.001$) of Enterococcaceae on sponge bacterial diversity (Appendix A- Table A5). Meanwhile, the other families (*Pseudomonadaceae*, *Enterobacteriaceae* and *Clostridiaceae*) had no significant impact ($P > 0.05$) on the sponge bacterial community. The influence of faecal indicator bacterial families in sponges on the variance of sponge bacterial community composition among seasons is presented in Appendix A- Table A6. According to the pairwise PERMANOVA analysis, the *Enterococcaceae* family had the greatest impact on sponge bacterial diversity, especially in April compared to other months.

Bathing water quality significantly impacted the *H. panicea* microbiome (Figure 2.12). The presence of *Enterobacteriaceae* and *Enterococcaceae* faecal bacterial families in seawater samples was strongly and positively correlated with the abundance of Firmicutes ($r = 0.6$, $P < 0.001$) and Fusobacteriota ($r = 0.6$, $P < 0.001$) in the sponge microbiome, which was clearly observed in April (Appendix A- Table A4). *Enterobacteriaceae* showed a positive significant correlation with Cyanobacteria ($r = 0.3$, $P < 0.01$, moderate). The *Pseudomonadaceae* family in seawater samples showed a moderate positive correlation with the abundance of Cyanobacteria and Bacteroidota ($r = 0.4$, $P < 0.001$) in the sponge samples. In contrast, *Pseudomonadaceae* exhibited a significant negative correlation with Actinobacteriota ($r = -0.4$, $P < 0.001$, moderate). *Clostridiaceae* faecal bacteria were

negatively correlated with the abundance of Nitrospirota in the sponge microbiome ($r = -0.3$, $P < 0.01$, moderate) while showing a weak negative correlation with Firmicutes ($r > -0.3$, $P < 0.05$).

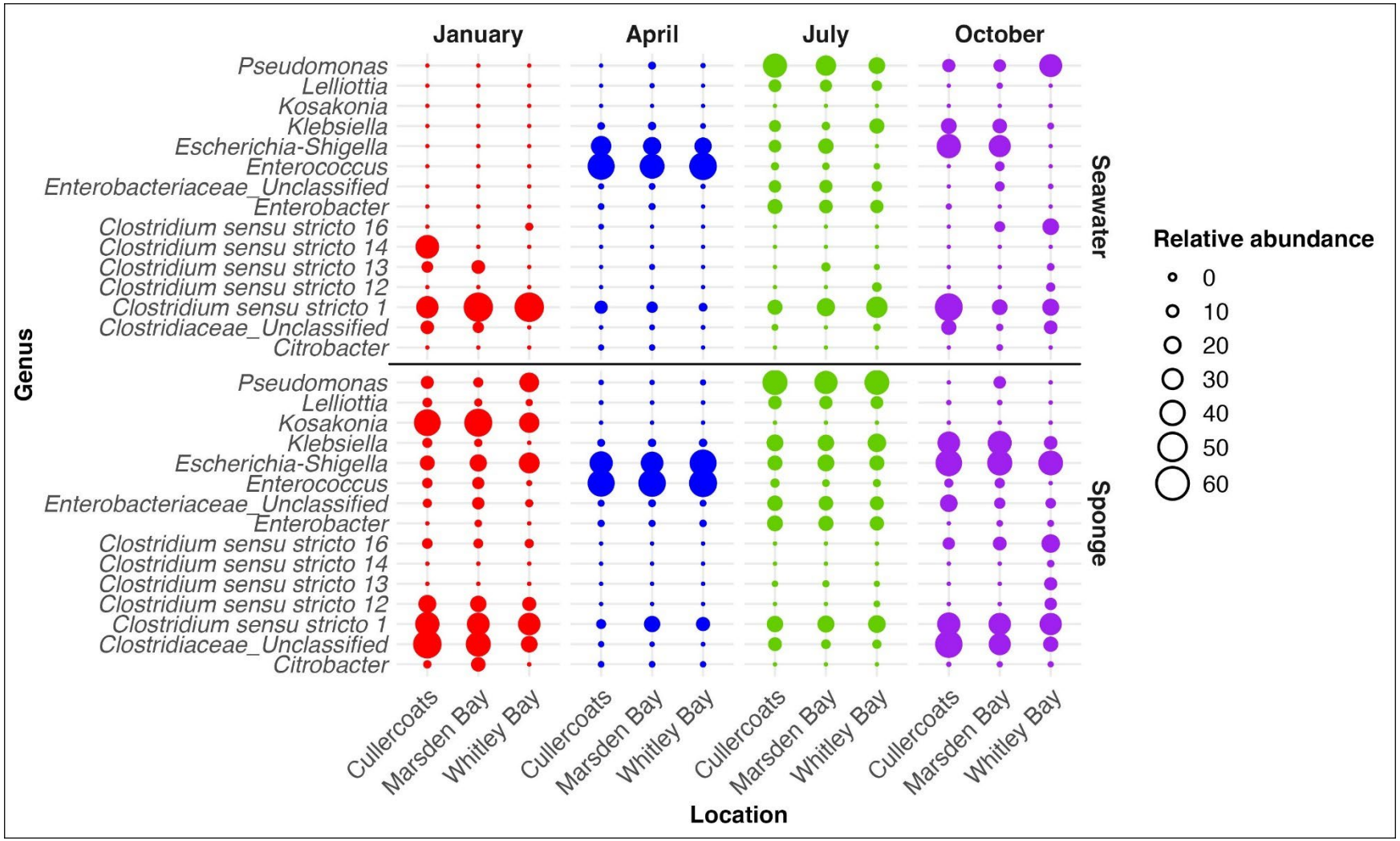


Figure 2. 11. The relative abundance of the most dominant faecal indicator bacterial genera belonging to *Enterococcus, Enterobacteriaceae, Clostridiaceae* and *Pseudomonadaceae* families in seawater (top plot) and sponge (bottom plot) samples collected from Cullercoats, Marsden Bay and Whitley Bay during 4 seasons in 2022.

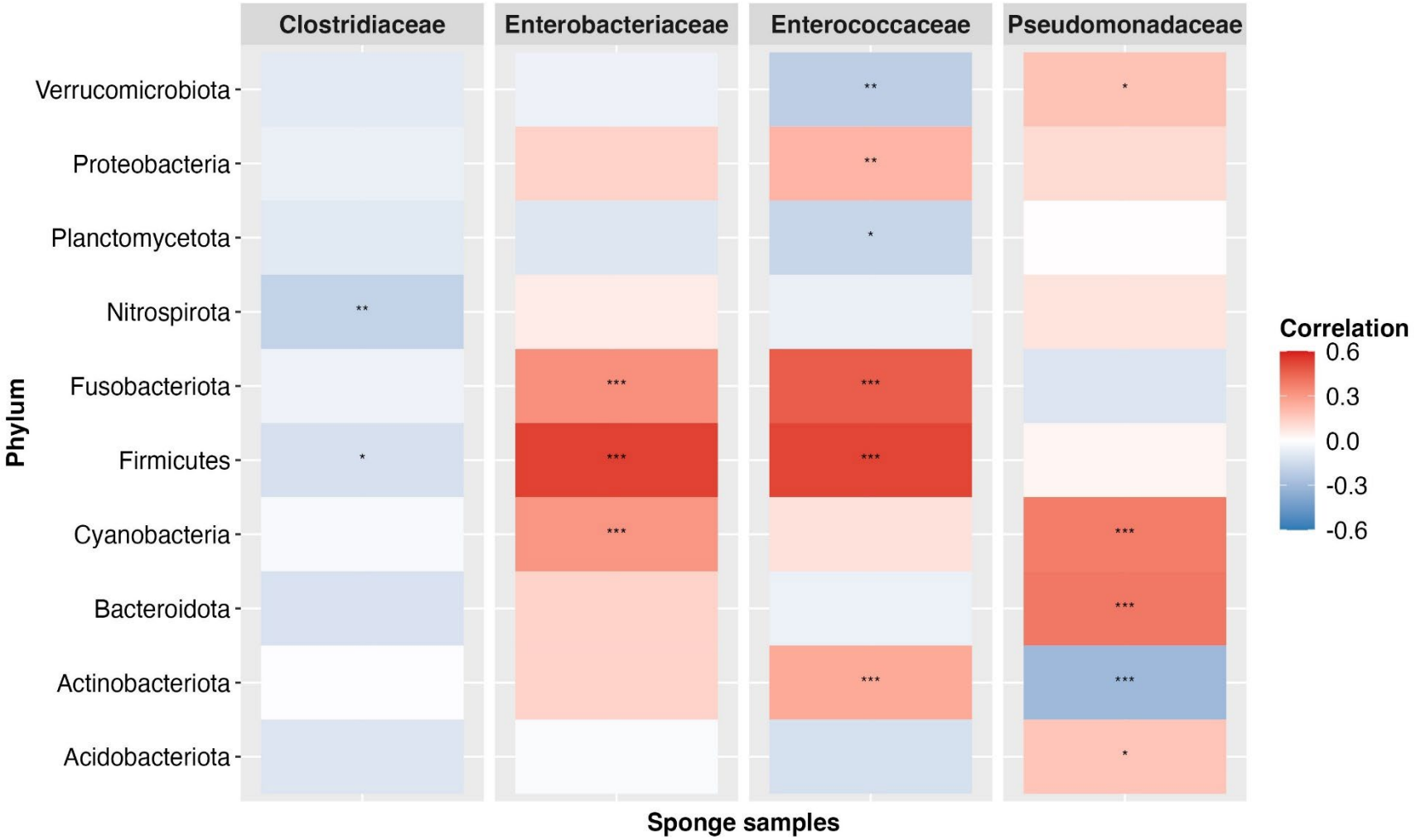


Figure 2. 12. Heatmap of correlation analysis between the abundance of faecal indicator bacterial families (*Clostridiaceae, Enterobacteriaceae, Enterococcaceae* and *Pseudomonadaceae*) and the abundance of the top 10 sponge microbiome phyla. The red indicates a positive correlation, and the blue indicates a negative correlation. Asterisks represent significance of linear regression, *= $P < 0.05$, **= $P < 0.01$, *** =$P < 0.001$.

2.4 Discussion

The sponge microbiome is highly affected by its surrounding environment (de Freitas *et al*., 2023; Lamb & Watts, 2023). This was clearly observed through the analysis of the V3-V4 region of the 16S rRNA of the model sponge, *Halichondria panicea*, from locations with different bathing water quality and through different seasons. The Environment Agency has categorised the bathing water quality according to the abundance of enteric bacteria such as *Escherichia coli* and intestinal *Enterococci* counts at the selected sites. However, the sponge samples did not show a significant difference in their microbiome community among sampling locations, except for January (Appendix A- Table A2). Moreover, the highest sponge bacterial relative abundance and lower diversity were observed in all samples in April and October, which was linked to faecal bacteria present in seawater in both months (Figure 2.12). The higher levels of faecal bacteria in both months (April and October) could be linked to more rainfall than other sampling months (as recorded by the weather station at Newcastle International Airport in 2022, cited in (Weather Spark, 2024), thus resulting in more runoff and wastewater contamination in coastal areas (Shore *et al*., 2021). Since *H. panicea* is known as an LMA sponge (Goldstein & Funch 2022; Moitinho-Silva, Steinert *et al*., 2017) that acquires most of its microbiome from its surrounding environment (Figure 2.5-A; Gantt *et al*., 2019; Moitinho-Silva, Steinert *et al*., 2017), the faecal bacteria were presumably transferred from seawater to the sponge tissue (Cartwright *et al*., 2024; Faiqoh, 2020) as shown in the relative abundance of these bacteria in sponge samples in April and October (Figure 2.11). The presence of faecal bacteria within sponge tissue (Figure 2.12) decreased bacterial diversity in both months (Appendix A- Table A5 and A6), which suggests that these bacterial groups compete with the main (the highest abundance) sponge microbiome in relation to abundance. This finding is also supported by other research showing that the diversity of sponge microbiomes in polluted environments is less compared to relatively pristine areas (Turon *et al.*, 2019). Furthermore, the higher abundance of *Escherichia-Shigella* and *Enterococcus* genera in seawater samples during both months indicates sewage contamination since these genera are commonly found in the faeces of humans and animals (Li *et al*., 2021; Shi *et al*., 2023). In general, faecal coliform exposure to marine sponges has not been well studied (Shore *et al*., 2021), but some sponges may be relatively tolerant, using bacterial cells as a source of nutrition (Chaves-Fonnegra *et al*., 2007).

Faecal waste contains organic matter and nutrients like sulphate, potassium, phosphate, and ammonium (Andreev *et al*., 2017; Azeez *et al*., 2023), the discharge of which into the environment

is viewed as a nutrient load. According to nutrient analysis data (Table 2.4), the nutrient levels (nitrate, phosphate, sulphate, bromide, and ammonia) in April NWD samples were the highest, followed by October, consistent with the elevated levels of faecal bacteria (Figure 2.11). The elevated sulfate levels in natural water drainage in April could be due to human activities. These emissions are primarily caused by industrial effluents (Jenzri *et al*., 2022; Tejada-Tovar *et al*., 2021), wetlands drainage (University of Newcastle, 2023), coal mining (Banks & Boyce, 2023) and fertiliser leaching from agricultural soils (Kidd *et al*., 2017) that could be washed up with water runoffs. While the source of phosphate could be sewage effluent, which accounts for about 60 to 70% of the total phosphate input to rivers and surface waters in England, agriculture contributes around 25%, but it is expected to increase to 30 to 35% by 2025 (Murdoch, 2022). For ammonia, anthropogenic sources, such as sewage treatment, industrial effluents, and runoff from agricultural land, are the main contributors to elevated levels in surface waters (Jadon *et al*., 2022; Johnson *et al*., 2007; Quan *et al*., 2022). In contrast, the bromide level was higher in April seawater samples than in the corresponding NWD. Bromide-containing compounds such as methyl bromide and ethylene dibromide are commonly used in agriculture as general pesticides (Lovas *et al*., 2023; Neal *et al*., 2007; Praharaj & Nara 2023), so could have been introduced via earlier discharge events, or they may be introduced to the seawater from the atmosphere (Quivet *et al*., 2022), as precipitate to the ocean in the form of rain, snow, or cloud water (Coleman *et al*., 2005). Overall, these contaminants are likely introduced to Whitley Bay and Cullercoats seawater through discharge outfalls, whereas the faecal bacteria contamination at Marsden Bay is assumed to be predominantly influenced by the River Tyne.

A change in seawater nutrients can impact the microbiome community in terms of composition and diversity. In nutrient-rich waters, chemoautotrophic bacteria, which produce their food by converting ammonia, nitrate, nitrite, ferrous iron, hydrogen sulfide, and other inorganic compounds into energy (Engel, 2019; Fuhrmann, 2021), are more abundant. The chemoautotrophic properties of Proteobacteria have been linked to the oxidation of sulfur, methane, and hydrogen, sulfate reduction, and denitrification of nitrogen (Zhou *et al*., 2020). Furthermore, in polluted environments, Proteobacteria may harbour pathogens like *E. coli, Salmonella, Shigella, Campylobacter, Vibrio*, and *Helicobacter* that can cause serious health issues (Moon *et al.*, 2018; Morato *et al*., 2009). The abundance of Proteobacteria and Firmicutes (the top two bacterial phyla in *H. panicea* sponge microbiome) was positively and significantly correlated with the elevated levels of nutrients in seawater samples (Figure 2.10, Table 4). The highest abundance of

Proteobacteria was observed in October, when nitrate and nitrite concentrations were also the highest. However, higher levels of sulfate and bromide in April seawater correlate with the abundance of Firmicutes (Table 2.4, Appendix A- Table A1). Previous studies found that Proteobacteria and Firmicutes abundance correlated with anthropogenic activities such as the discharge of organic matter and nutrients from sewage outfalls (Paliaga *et al*., 2017; Waterworth *et al*., 2020), which disrupted the sponge microbiome's natural balance (Shore *et al*., 2021). This resulted in an increase in Proteobacteria and a reduction in beneficial symbionts (Waterworth *et al*., 2020). It is also known that Proteobacteria contain nitrogen cycling members, which explains why their increased abundance depends on the availability of nitrogen in the environment (Suh *et al*., 2015).

The elevated levels of certain trace metals were positively correlated with the composition of the top bacterial phyla of the sponge microbiome. It has previously been reported that field studies of sponges show that heavy metal concentrations are correlated with sponge microbial community structure (e.g., Bauvais *et al*., 2015; Lo Giudice *et al*., 2024). According to Gantt *et al*., (2017), only a slight variation in the abundance and diversity of certain bacteria was observed in associated microbial communities of *Crambe crambe* sponges in both natural and heavy metal-impacted areas. According to this finding, not all sponge-associated microbial communities are negatively affected by pollution. (Gantt *et al*., 2017). The results of our study support these findings since most of the bacteria communities were shared between locations, and only Fusobacteriodota and Firmicutes had a positive correlation with Zn, Ba, Cd, Cr, Fe, Sb and Pb abundance in Cullercoats in April when these metals were at their highest concentrations. The high metal content in seawater samples likely resulted from wastewater input in the coastal waters through the water outfalls. Whitley Bay had the highest metal concentration in its NWD samples because of the natural drainage catchment surrounding this area, which covers approximately 17 km^2 from primarily suburban areas. In contrast, the coast at Cullercoats is exposed to an urban natural drainage catchment area of approximately 0.3 km^2 (Environment Agency, 2022c). Marsden Bay had no natural surface water draining or anything close to it; however, certain metals exceeded the natural level of seawater. This increase may come from the River Tyne input. The River Tyne is highly contaminated with heavy metals from the uplands, mining, and metal-rich sediments (Gibbons & Hall 2022; GOV.UK 2020; Hudson-Edwards 2003). Furthermore, anthropogenic activities such as releasing wastewater effluents and solid waste associated with industrial processes may source elevated metal concentrations in the tested locations (Lantzy & Mackenzie

1979; Mishra *et al*., 2023). The Environment Agency also pointed out that the abandoned metal mining activities are the reason for the elevated metal concentrations such as Cd, Pb, Fe and Zn in Tyne River waters (Hudson-Edwards *et al*., 2008). The elevated levels of certain metals and ions in the Newcastle coastal waters may be attributed to the illegal/uncontrolled disposal of sewage that has recently been discovered throughout the north-east of England. There are about 61 pipes without permits found in the north-east, which pump raw sewage into the waterways (Gibbons & Hall, 2022).

Seasonal variations in sponge microbial community composition have been reported in different regions including the Mediterranean Sea (Erwin *et al*., 2015), Caribbean Sea (Villegas-Plazas *et al*., 2019; White *et al*., 2012), New Zealand (Anderson *et al*., 2010) and Red Sea (Kamel *et al*., 2022). In agreement with the aforementioned studies, this study likely found differential composition in sponge bacteria as a result of seasonal fluctuations. The abundance and diversity of the bacterial population of *H. panicea* showed a significant difference among seasons (Figure 2.4, 2.6, 2.7 & Appendix A- Table A3). In October sponge samples showed the highest relative abundance of bacterial community accompanied by the lowest diversity, while in July they had the highest diversity and lowest microbial abundance compared to other months. This is highly correlated with the temperature variations. In July, the higher temperature was highly correlated with the abundance of Cyanobacteria and Bacteroidota during the summer season (July) (Figure 2.8, Appendix A- Table A1). The abundance of cyanobacteria in summer is also highly correlated with the increased sunlight that is needed for their photosynthesis (Luo *et al*., 2022; Paerl, 2014; Wilkinson & Vacelet, 1979), while the abundance of symbiotic Bacteroidota was correlated with the higher temperature and nutrient levels in summer (Glasl *et al*., 2020).

2.5 Conclusion and Future Study

In conclusion, the bacterial community of the sponge *Halichondria panicea* in north-east England is highly affected by its surrounding environment (seawater). Seawater chemistry, season, nutrient pollution, and bathing water quality significantly impacted the abundance of the sponge bacterial community. Although the three tested locations differ in their bathing water quality, the respective sponge samples shared most of their sponge bacterial community (692 genera). As shown in April samples, the faecal bacteria compete with the sponge bacterial community by shifting the diversity and composition of the sponge's bacterial community. These bacterial groups may affect the functionality and health of the sponge. Further analysis should be considered to

check the functions of the top 10 bacterial phyla in the sponge community through shotgun metagenomics.

Chapter 3. The Horizontal Transmission of *Halichondria panicea* Sponge Microbiome

3.1 Introduction

A sponge's microbial community can be both abundant and diverse, with many microorganisms exclusive to specific sponge species (Lafi *et al*., 2009; Taylor, Radax, *et al*., 2007). Bacteria and algae are among the microorganisms with which sponges form symbiotic relationships. Approximately 50% of sponge biomass comprises symbionts (Hentschel *et al*., 2006).

The concept of symbiotic relationships is based on a close ecological association between two species, which may be mutually beneficial or could benefit one organism at the expense of the other (Oulhen *et al*., 2016). In the case of sponges, microorganisms may benefit from nutrients and a sheltered surface for growth, while sponges may benefit from nutrients and metabolites provided by microorganism metabolism (Hentschel *et al*., 2012; Hudspith *et al*., 2021; Thomas, Rusch, *et al*., 2010). The stability of symbioses can be achieved by both horizontal and vertical transmission. Symbioses, which are non-continuous throughout the life cycle of the host, can be horizontally transmitted from the environment. Conversely, vertical transmission occurs when symbioses are maintained during the host's life cycle and across generations (Bright *et al*., 2010, McFall-Ngai, 2002; Vrijenhoek, 2010). In sponges, vertical and horizontal transmission are both essential for sustaining a symbiotic lifestyle (Carrier *et al*., 2022). Furthermore, the properties of these symbiont communities vary between species and are classified as low microbial abundance (LMA) or high microbial abundance (HMA) sponges. The microbial populations of LMA sponges are approximately the same as those of the surrounding seawater, while those of HMA sponges are several orders of magnitude more abundant (Gloeckner *et al*., 2014; Moitinho-Silva, Steinert *et al*., 2017). Generally, HMA sponges are enriched in Acidobacteria, Chloroflexi, and Poribacteria, whereas LMA sponges are enriched in Cyanobacteria and Proteobacteria. Additionally, HMA sponge symbionts primarily carry out autotrophic and heterotrophic metabolism, whereas LMA sponge hosts perform these processes primarily (Bayer *et al*., 2014; Rix *et al*., 2020).

Sponges have phylogenetically related microbial communities that differ from those of benthic and planktonic organisms (Alex & Antunes, 2015; Simister, *et al*., 2011; Taylor, Radax, *et al.,* 2007; Webster *et al*., 2010). Studies conducted with deep sequencing confirm that bacteria

previously found only in sponges also exist in seawater, although at a low level (Taylor *et al*., 2013). In the past decade, sponge-bacterial symbioses have sparked new research into their ecological and evolutionary implications (Webster & Taylor, 2012). Most studies have focused on the vertical and horizontal transmission of sponge microbiome from the surrounding environment, especially from seawater and sediments (Busch *et al*., 2022; Oliveira *et al*., 2020). However, there is a paucity of knowledge regarding the bacterial communities shared with seaweed and other organisms attached to marine sponges.

The aim of this study was to determine the diversity and origin of the microbial communities in *Halichondria panicea*, a globally common coastal marine sponge. To address host-specificity questions and identify intra- and inter-species commonalities and variation, the microbiome of the sponge, attached seaweeds and the seawater were studied.

3.2 Methodology

3.2.1 Sponge sampling locations

Sponge samples were collected at three locations on the north-east England coast next to Northumbrian Water Ltd storm water outfalls. The chosen sites were Cullercoats Bay, Marsden Bay and Whitley Bay.

3.2.2 Sample collection

A total of thirty-six sponge samples were collected for this study, of which twenty-three were collected with their attached seaweeds. Twelve seawater samples were also collected from sampling sites. Sampling was done in January (Winter), April (Spring), July (Summer), and October (Autumn/Fall) during 2022. Most of the attached seaweed was red, though four samples were brown. The sampling method used was described in detail in Chapter 2. A list of sample codes is presented in Appendix B- Table B1, along with further information about sampling locations and months.

3.3.3 Sponge and seaweed identification

The sponge sample's identity was confirmed via spicule structure and external morphological traits. The followed protocol for spicule preparation was described by Hooper (2000). The protocol was described in detail in Chapter 2.

Seaweed samples were identified according to their habitat and morphological characteristics. Samples were examined under a stereomicroscope (Motic SFC-11C, Motic®) and compared against the species descriptions in Bunker *et al.* (2017). The collected seaweed pictures and names are presented in Appendix C- Table C1.

3.3.4 DNA extraction

The DNA of the collected seawater samples was extracted using a DNA extraction kit (DNeasy PowerWater kit, Qiagen). The manufacturer guidelines were slightly modified to maximise DNA yield. DNA extraction of seawater samples was described in detail in Chapter 2. To study the microbial diversity of the sponge and seaweed samples, 16S rRNA amplicon sequencing was analysed. Sponge DNA was extracted within a month of sampling. The DNA was extracted using a DNA extraction kit (DNeasy PowerSoil Pro, Qiagen), for which the manufacturer guidelines were slightly modified for each sample type to maximise DNA yield.

A small piece of the sponge, approximately 0.125- 0.2 g, was added to a PowerBead Pro tube with 800 µL of solution CD1. The mixture was briefly mixed, then vortexed horizontally using a 1.5-2 mL tube size vortex adapter (cat. no. 13000-V1-24) for 10-15 minutes (until the sponge tissue disintegrated) at maximum speed; this was followed by centrifugation at 15,000x g for a minute. Next, the supernatant was transferred to a clean microcentrifuge tube (2 mL), and 200 µL of solution CD2 was added, vortexed for 5 seconds, and centrifuged at 15,000x g for 1 minute. The supernatant (up to 700 µL) was transferred to a clean microcentrifuge tube (2 mL), 600 µL of solution CD3 (saline solution) was added, and vortexed (5 sec) to mix. Only 650 µl of the mixed solution (lysate) was transferred to a MB spin column, centrifuged at 15,000x g for 1 minute, and the flow-through discarded. This step was repeated 2-3 times until the whole lysate was processed. The MB spin column was then transferred to a new clean collection tube (2 mL), 500 µL of EA solution (wash buffer) was added, centrifuged at 15,000x g for 1 minute, and the flow-through was discarded from the collection tube. Next, 500 µL of cold C5 solution (ethanol-based wash solution stored at -20 ℃) was added to the column, which was closed tightly and inverted gently to remove any residual buffer that may have adhered to the edges of the column. The column was then incubated for 5 minutes at 4 ℃ and centrifuged at 15,000x g for 1 minute. After discarding the flow-through, an extra washing step with cold molecular grade 80% ethanol (stored at -20 ℃) was added to the column, which was closed tightly and inverted gently again to wash off any residual

buffer that may have adhered to the edges of the column. The solution was again incubated in the column for 5 minutes at 4 ℃ before being centrifuged at 15,000x g for 1 minute. After discarding the flow-through, a dry centrifugation step at 16,000x g for 2-3 minutes was done to ensure the removal of any ethanol traces from the DNA sample. Finally, the MB spin column was transferred to a new 1.5 mL Eppendorf tube to which 70 µL of C6 solution (elution buffer) was added to the centre of the filter membrane, centrifuged at 15,000x g for 1 minute, the MB column was discarded, and the obtained DNA was stored at -20℃.

For the seaweed samples, a small piece of approximately 0.125- 0.2 g of the thallus was ground using a sterile (autoclaved) mortar and pestle with added 1000 µL of sterile DNA-free water (DEPC-Treated Water, Invitrogen™) to obtain a seaweed suspension. Then, in the PowerBead Pro Tube, 300 µL of the suspension was added to 800 µL of CD1 solution. For the brown seaweed, an extra step was added to the extraction protocol for a higher DNA yield. Since brown seaweeds are highly rich in polysaccharides and polyphenols, which bind firmly to the DNA and inhibit its extraction by forming a gelatinous layer, an extra incubation step at 65 ℃ for 10 minutes was performed on the samples after adding CD1 solution and before homogenising. The following steps were then performed as for the sponge samples.

3.3.5 DNA sequencing

The DNA samples were shipped to LC Sciences (Houston, USA) for sequencing of the V3-V4 region of the 16S rRNA gene of the bacteria and archaea communities. The primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify bacteria 16S rRNA gene V3–V4 hypervariable regions. The amplification, purification and library preparation kits and protocols used were described in detail in Chapter 2.

3.3.6 Bioinformatics

The bioinformatics pipeline for the collected samples (sponge, seawater and seaweed) raw data was performed in the R program using the dada2 package (version 1.26 for R version 4.2.2). The pipeline was described in detail in Chapter 2.

In order to confirm the host specificity of the most dominant bacterial sequence in *H. panicea* sponge, 16S rRNA (V3-V4 region) raw data of *H. panicea* and surrounding seawater were obtained from other studies like Knobloch *et al*., 2019b and Rusanova *et al*., 2021. In Knobloch

study, *H. panicea* was collected from Iceland and extracted DNA was deposited in GeneBank (BioProject ID= PRJNA495906), where only two sponges (SRR8040133 & SRR8040134) and two seawater (SRR8040374 & SRR8040375) samples raw data were selected for this analysis. While in Rusanova's study *H. panicea* was collected from the White Sea and the extracted DNA of the sponge samples was deposited in GeneBank under the BioProject PRJNA781598, where two sponge samples (SRR17015070 & SRR17015083) samples and one seawater (SRR17015076) sample raw data were selected for this study. Representative samples of sponge, seawater and seaweeds (total= 56) were selected from our study, *H. panicea* sponge ex-situ cultivation samples from Chapter 5 (after 14 days of ex-situ cultivation in artificial seawater) and sponge and seawater sequences from Knobloch and Rusanova studies (the selected samples are presented in Table 3.1). The raw data were combined in one file and analysed together following dada2 pipeline. The followed protocol was similar to the previous analysis, except that the used filterAndTrim command was adjusted according to the nature of the combined data, as follows: truncLen = c(240,240), maxN = 0, minLen = 100, maxEE = 5, trimLeft = 20, truncQ = 2. The relationship between the quality scores and the error rates was determined from the data, and the denoised ASVs were inferred using the DADA2 algorithm pool= FALSE command. In order to infer the relatedness of ASVs, sequences were aligned, measured distance and clustered into 99% OTUs using the DECIPHER package (version 2.26.0, Wright, 2016), and assigned to ASVs taxa using merge_taxa_vec from speedyseq package (version 0.5.3.9021, McLaren, 2020).

3.3.7 Phyloseq analysis and community structure

The cleaned and filtered microbiome sequence data (ASV and OTU tables) were analysed using the R package Phyloseq (McMurdie & Holmes, 2013). This package combined the following data into a single dataset: ASV/OTU table, taxa table, sampling data, associated metadata, and phylogenetic trees. Two phyloseq objects were prepared for this study, one for the ASVs of the collected samples (sponge, seawater and seaweeds) from Newcastle (ps), and the other one for the combined OTUs (ps0). Next, data in the phyloseq objects were cleaned from empty columns, Eukaryota, Chloroplast and Archaea, by using “subset_taxa” function. Then, "prune_taxa" was used to filter taxa for taxa_sums >0 present in all sample types (seawater, sponge, and seaweed). The taxa number for all samples was reduced from 114,587 to 113,230 for ps and from 15940 to 15740 for ps0. For further analysis, “subset_samples” function was used to create phyloseq object for each sample type. Then, "prune_taxa" was used again to filter taxa for taxa_sums >5 present in

the sponge (ps.sp), seawater (ps.sw) and seaweed (ps.alg) samples. Thus, the final taxa numbers were 22,417, 19,328 and 22,654 for sponge, seawater and seaweed samples, respectively. The community structure profile for each sample type was created by transforming the taxa counts to relative abundance using “transform_sample_counts” function from phyloseq package. Taxa were agglomerated using “tax_glom” and tax table was melted using “psmelt” functions. The produced taxa data frames were visualised using ggplot2 package (Wickham, 2016) as pie charts for top bacterial phyla and bar plots for top genera.

To prepare samples for the beta diversity analyses, the phyloseq object containing all samples (sponge, seawater and seaweed) was normalized through proportion (relative abundance) using “transform_sample_counts” function.

3.3.8 Beta diversity analysis and modelling

To obtain beta diversity analysis for the sponge, seawater and seaweed samples, the phyloseq package was also used. Following rarefaction, ordination plots were produced based on the Bray-NMDS (non-metric multidimensional scaling) method using “ordinate” and “plot_ordination” functions from the phyloseq package.

3.3.9 Venn diagram for the shared microbial community between different samples

A Venn diagram illustrates the similarities and differences between groups in a graphical form. Therefore, VennDiagram and UpSetR packages were used to compare the microbial communities (ASVs) shared by the three sample types (sponge, seaweed and seawater) and between sponge with attached seaweed (brown and red colour). This was done using the functions "get_vennlist", "get_upset", and "venn.diagram".

3.3.10 Analysis of combined data (OTU Table): Abundance bar plots, Indicator species analysis, and Function prediction

The most predominant OTU (OTU1) in all samples (sponge, seaweed, and seawater) was tested for its relative abundance. Using ggplot2, bar plots were created to show the relative abundance of OTU1 versus other OTUs in sponge samples (wild vs. ex-situ) and among different sample types (seawater and seaweed).

Indicator species analysis is performed to study the difference in OTUs among samples, and especially to indicate the source of the most dominant OTU (OTU1) in *H. panicea* sponge. Indicator species analysis was tested using “multipatt” function from indicspecies r package (version 1.7.14, de Cáceres & Legendre, 2009).

TAX4FUN was used in this study to predict the functional genes of the most dominant bacterial sequence in sponge samples (OTU_1). Bacterial functions were predicted using TAX4FUN2 package in R (version 1.1.5, Wemheuer *et al*., 2020). The TAX4FUN output files were generated by uploading OTU’s fasta and OTU abundance table files created in R. The "funRefBlast" function was employed to compare OTUs with the 16S reference sequences using BLAST, followed by the calculation of functional predictions through the "makeFunctionalPrediction" function, with a similarity threshold set at 90%. Human-related pathways were excluded from the produced pathways table and the top 10 most dominant pathways were visualised using ggplot2.

Table 3. 1. Sponge, seawater and seaweed samples used to create the OTU table including representative samples from this study and some from previous studies on *H. panicea*.

Sample code	Source	Reference
1-C-sp-1-1	Sponge	This study
1-M-sp-1-1	Sponge	This study
1-M-sp-2-1	Sponge	This study
1-M-sp-3-1	Sponge	This study
1-Mrs-sp-1-1	Sponge	This study
10-C-sp-2-1	Sponge	This study
10-C-sp-3-1	Sponge	This study
10-M-sp-1-1	Sponge	This study
10-M-sp-2-1	Sponge	This study
10-M-sp-3-1	Sponge	This study
10-Mrs-sp-2-1	Sponge	This study
4-C-sp-1-1	Sponge	This study
4-C-sp-3-1	Sponge	This study
4-M-sp-2-1	Sponge	This study
4-Mrs-sp-1-1	Sponge	This study
4-Mrs-sp-2-1	Sponge	This study
4-Mrs-sp-3-1	Sponge	This study
7-C-sp-1-1	Sponge	This study
7-C-sp-3-1	Sponge	This study
7-M-sp-1-1	Sponge	This study
7-Mrs-sp-1-1	Sponge	This study
7-Mrs-sp-3-1	Sponge	This study
D14-B1	Sponge	Chapter 5
D14-B2	Sponge	Chapter 5
D14-B3	Sponge	Chapter 5
1-C-alg-1-1	Seaweed	This study
1-M-alg-1-1	Seaweed	This study
1-M-alg-2-1	Seaweed	This study
1-M-alg-3-1	Seaweed	This study
1-Mrs-alg-1-1	Seaweed	This study
10-C-alg-2-1	Seaweed	This study
10-C-alg-3-1	Seaweed	This study
10-M-alg-1-1	Seaweed	This study
10-M-alg-2-1	Seaweed	This study
10-M-alg-3-1	Seaweed	This study
10-Mrs-alg-2-1	Seaweed	This study

Sample code	Source	Reference
4-C-alg-1-1	Seaweed	This study
4-C-alg-3-1	Seaweed	This study
4-M-alg-2-1	Seaweed	This study
4-Mrs-alg-1-1	Seaweed	This study
4-Mrs-alg-2-1	Seaweed	This study
4-Mrs-alg-3-1	Seaweed	This study
7-C-alg-1-1	Seaweed	This study
7-C-alg-3-1	Seaweed	This study
7-M-alg-1-1	Seaweed	This study
7-Mrs-alg-1-1	Seaweed	This study
7-Mrs-alg-3-1	Seaweed	This study
1-C-SW-1	Seawater	This study
1-M-SW-1	Seawater	This study
1-Mrs-SW-1	Seawater	This study
4-C-SW-1	Seawater	This study
4-M-SW-1	Seawater	This study
4-Mrs-SW-1	Seawater	This study
7-C-SW-1	Seawater	This study
7-M-SW-1	Seawater	This study
7-Mrs-SW-1	Seawater	This study
10-C-SW-1	Seawater	This study
10-M-SW-1	Seawater	This study
10-Mrs-SW-1	Seawater	This study
K-iceland-sp-1 (SRR8040133)	Sponge	Knobloch *et al*., 2019b
K-iceland-sp-2 (SRR8040134)	Sponge	Knobloch *et al*., 2019b
K-iceland-SW-1 (SRR8040374)	Seawater	Knobloch *et al*., 2019b
K-iceland-SW-2 (SRR8040375)	Seawater	Knobloch *et al*., 2019b
R-Whitesea-sp-1 (SRR17015070)	Sponge	Rusanova *et al*., 2021
R-Whitesea-sp-2 (SRR17015083)	Sponge	Rusanova *et al*., 2021
R-Whitesea-SW-1 (SRR17015076)	Seawater	Rusanova *et al*., 2021

3.3 Results

3.4.1 Microbial community structure

The top 10 phyla in the collected samples (seawater, sponge and seaweed) are presented in Figure 3.1. The Proteobacteria phylum was the most prevalent in all samples. For the bacteria phyla, Actinobacteriota, Bacteroidota, Cyanobacteria, Firmicutes, Planctomycetota, and Verrucomicrobiota dominated all samples. In contrast, there were certain bacterial phyla that were only found in sponge samples, such as Desulfobacterota and Nitrospirota. Furthermore, sponge and seawater samples were the only ones that contained Campilobacterota. Some bacteria were only associated with certain seaweed colours. For example, Deinococcota was only found in red seaweed, whereas Patescibacteria was only found in brown seaweed. The brown seaweed data represent the top 10 bacterial phyla in *Fucus vesiculosus* only since it was the only brown seaweed found and collected attached to *H. panicea* (Appendix C). The Acidobacteriota was detected in both red and brown seaweeds but was absent/less abundant in other samples (sponges and seawater).

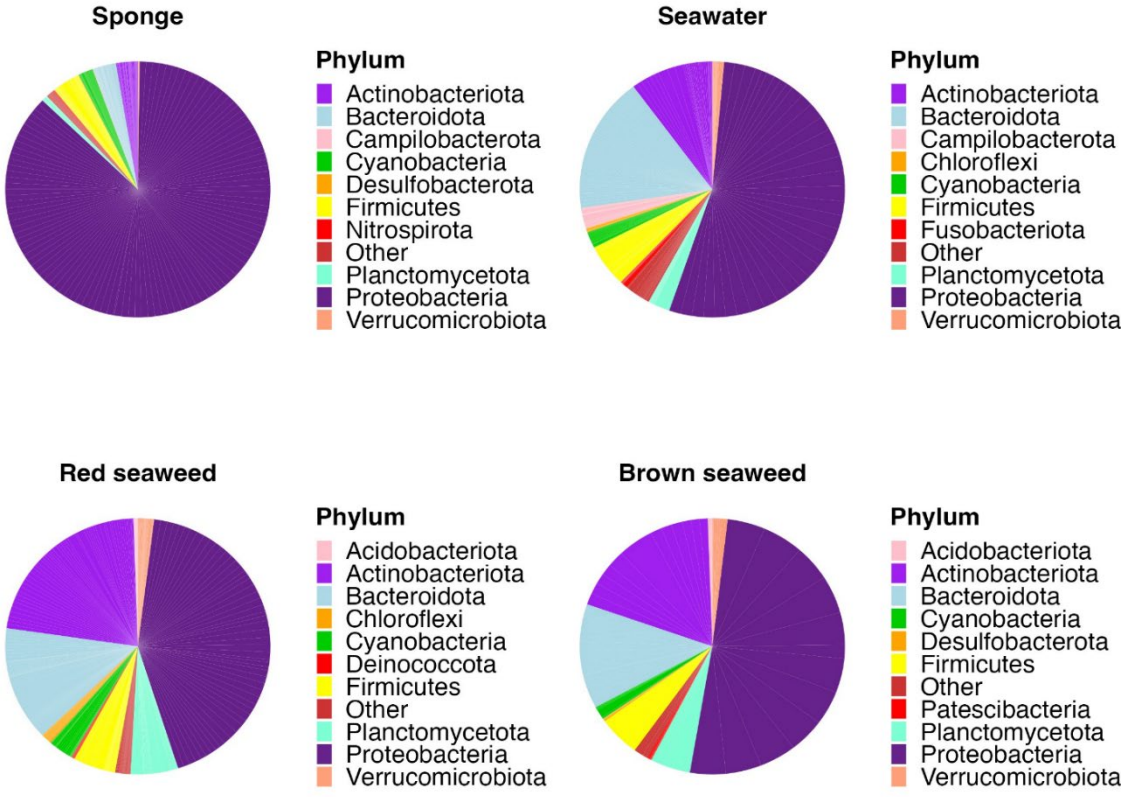


Figure 3. 1. The relative abundance of the top 10 bacterial Phyla in all samples collected from Cullercoats, Whitley Bay and Marsden Bay in 2022.

The top 20 bacterial genera in the collected samples are presented in Figures 3.2, 3.3 and 3.4. For the sponge samples, the top 10 genera (Figure 3.2) were *Amylibacter* (75%)> *Alphaproteobacteria_Unclassified* (1.90%)> *Synechococcus CC9902* (1.30%)> *Terasakiellaceae_Unclassified* (0.77%)> *Mycobacterium* (0.64%)> *Gammaproteobacteria_Unclassified* (0.58%)> *Faecalibacterium* (0.56%)> *Enterococcus* (0.54%)> *Microtrichaceae_Unclassified* (0.48%)> *Streptococcus* (0.47%). Specific genera associated with seaweed such as *Alphaproteobacteria_Unclassified, Gammaproteobacteria_Unclassified, Acaryochloris* MBIC 11017*, Microtrichaceae_Unclassified, Mycobacterium* and *Streptococcus.* were only shared with sponge samples. Other genera like *Enterococcus* and *Synechococcus CC9902* were found in both sponge and seawater samples in higher abundance. *Amylibacter* was among the bacterial taxa that were shared between the three sample types (sponge, seaweed and seawater), however, the relative abundance of *Amylibacter* was the highest in sponge samples (75%) compared to seaweed and seawater samples.

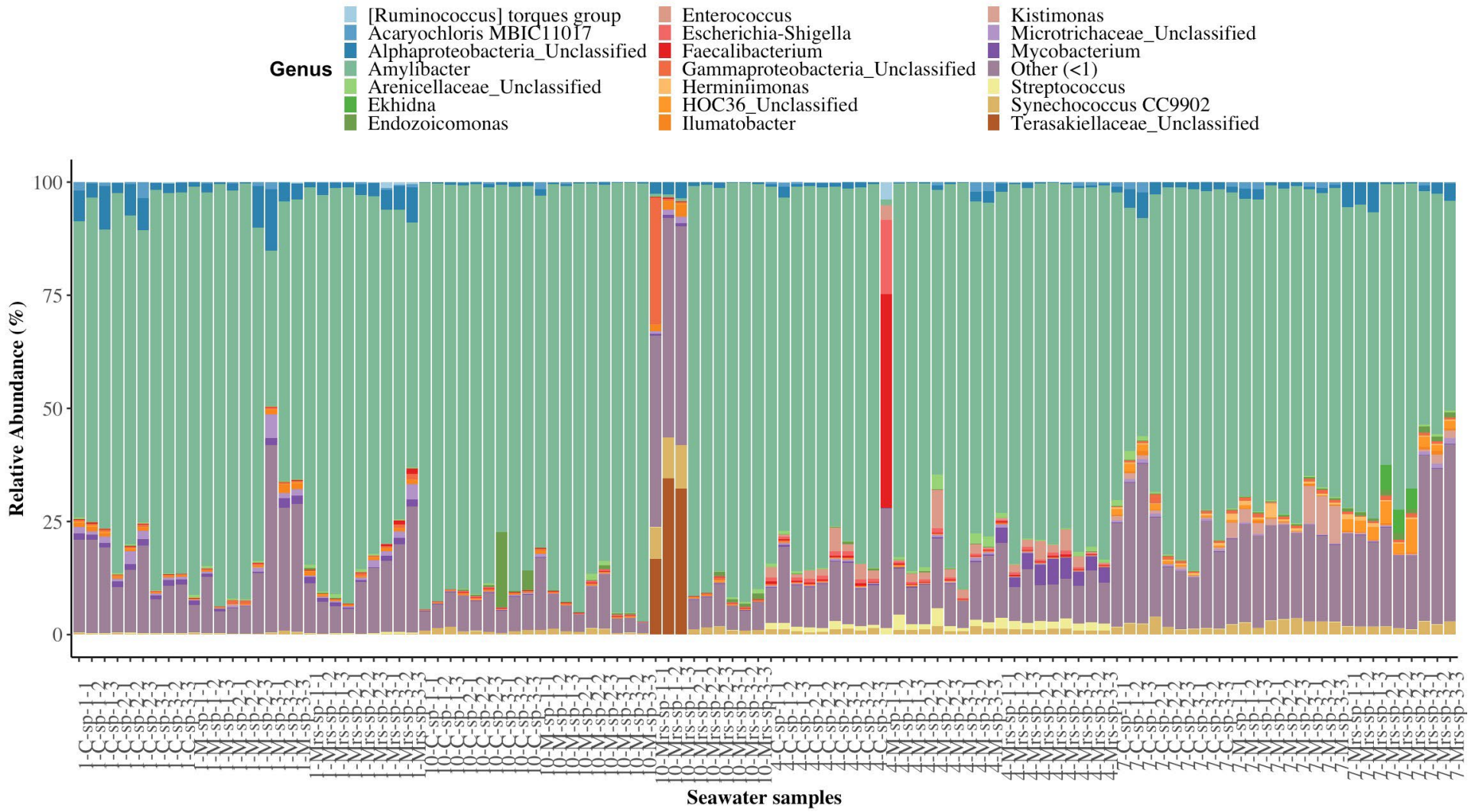


Figure 3. 2. The top 20 bacterial genera in sponge samples collected from Cullercoats, Whitley Bay and Marsden Bay through different seasons. Sample codes are listed in Appendix B with additional information regarding sampling sites and months.

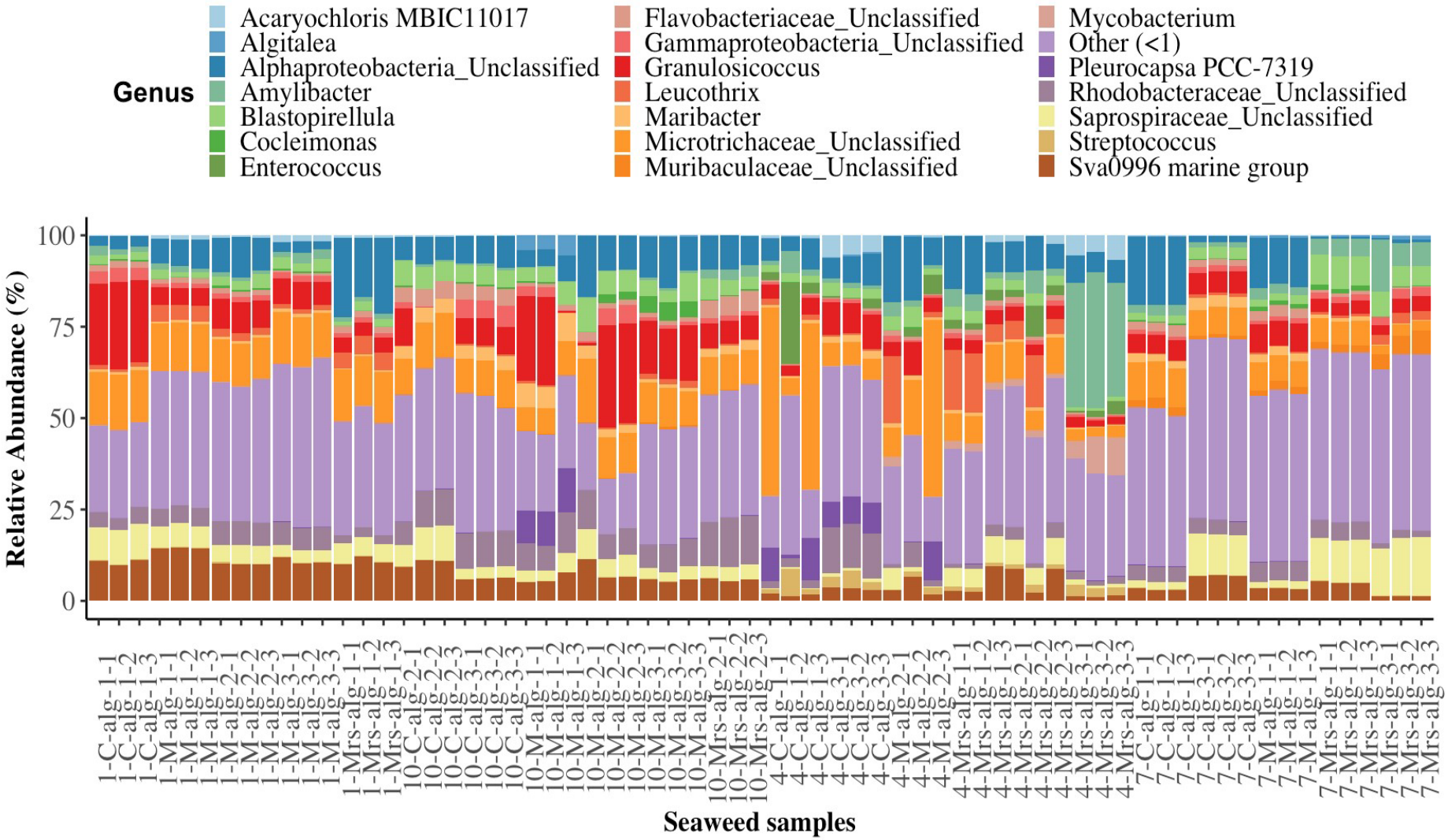


Figure 3. 3. The top 20 bacterial genera in seaweed samples collected from Cullercoats, Whitley Bay and Marsden Bay through different seasons. Sample codes are listed in Appendix B with additional information regarding sampling sites and months.

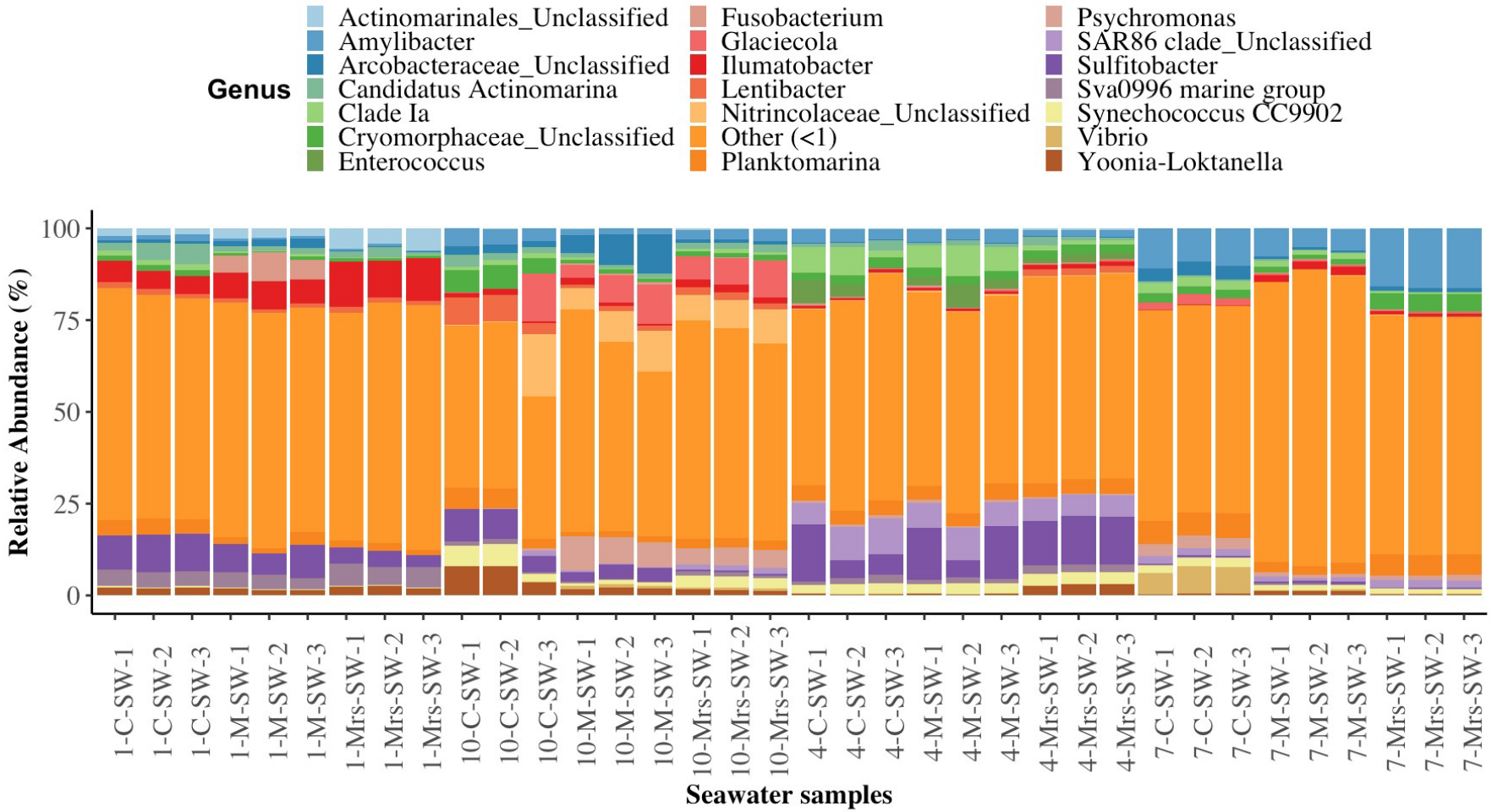


Figure 3. 4. The top 20 bacterial genera in seawater samples collected from Cullercoats, Whitley Bay and Marsden Bay during sponge sampling. Sample codes are listed in Appendix B with additional information regarding sampling sites and months.

3.4.2 Beta diversity analysis

For the beta diversity, Bray–Curtis distance using the pairwise PERMANOVA method between samples ASVs, highlights the significant differences in microbial communities between sponge, seaweed and seawater samples. The distinct clusters indicate differences among sample types, while some overlap suggests shared microbial taxa (Figure 3.5 A). There were, however, very few ASVs shared between sponge and seawater samples; in contrast, others were shared only between seaweed and seawater samples. Furthermore, the clustering in microbial taxa in both brown and red seaweed with sponge samples (Figure 3.5 B & C) indicates that both seaweeds have different microbial compositions, with minimal overlap between groups. The microbial diversity of the sponge was significantly different among the other sample types, seawater and seaweed ($P < 0.05$). Moreover, brown and red seaweed samples were significantly different from their attached sponge microbial community ($P = 0.001$). To clarify, Figure 3.5 explains the beta diversity test according to Bray-NMDS distance under a stress value of less than 0.1. The distance between points represents the level of difference. Stress values lower than 0.2 indicates that the NMDS analysis is reliable.

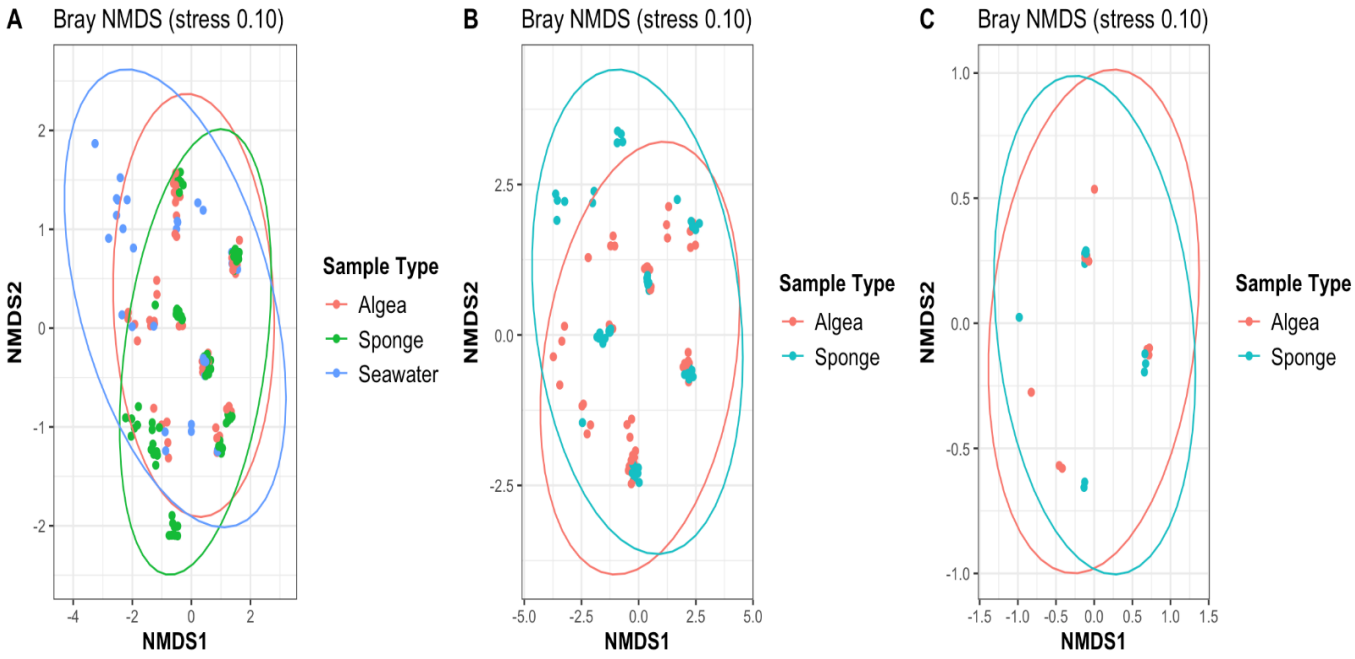
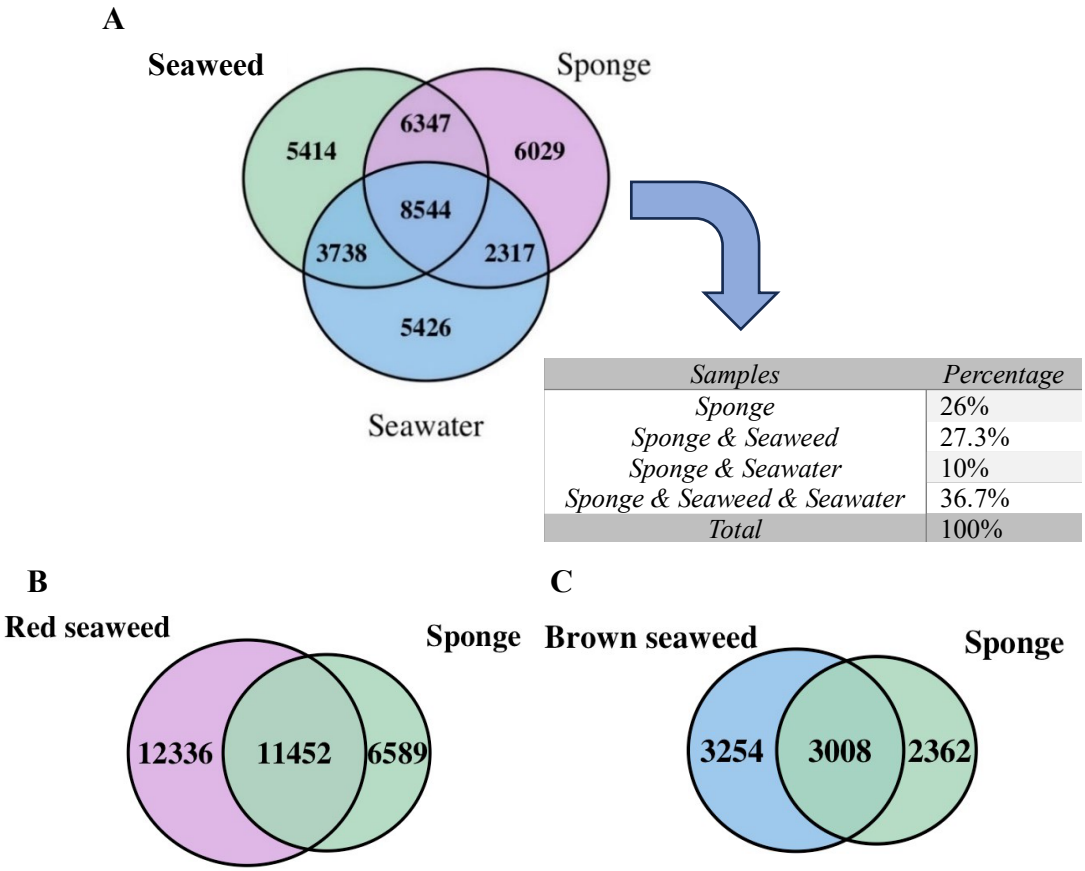


Figure 3. 5. Ordination plot of Beta-diversity indices (Bray-NMDS) for all samples under stress < 0.2, where each point in the graph represents one sample. (**A**) All samples (sponge, seawater and seaweed). (**B**) Sponge and red seaweed samples. (**C**) Sponge and brown seaweed samples.

3.4.3 Venn diagram for the shared ASVs

The evidence strongly suggests that the sponge microbial community is mostly acquired from the surrounding environment, in this case, seawater and seaweed. The Venn diagram illustrates the shared and unique microbial taxa among the three sample types (sponge, seaweed and seawater) (Figure 3.6 A). More than half (64 %= 14,891 ASVs) of the sponge ASVs were shared with the attached seaweed samples, whereas 47% (= 10,861 ASVs) of the sponge ASVs were shared with seawater samples. However, each sample type has unique ASVs reinforcing the distinct community structure. This supports the hypothesis of environmental acquisition (horizontal transfer) of the sponge microbiome. Seaweed Venn diagrams (Figure 3.6 B and C) clearly show that a large proportion of the *H. panicea* microbiome is associated with red seaweed (11500 ASVs = 63%) rather than brown seaweed (3008 ASVs = 56%). This may reflect that relatively few of the sponge samples were attached to brown-coloured seaweed.



Samples	*Percentage*
Sponge	26%
Sponge & Seaweed	27.3%
Sponge & Seawater	10%
Sponge & Seaweed & Seawater	36.7%
Total	100%

Figure 3. 6. Shared ASVs between different samples. (**A**) Sponge, seaweed and seawater samples. (**B**) Red seaweed and attached sponge. (**C**) Brown seaweed and attached sponge.

3.4.4 Analysis of combined data (OTU Table): Abundance bar plots, Indicator species analysis, and Function prediction

After examining the sponge OTU table, OTU_1 (Appendix C) was selected for further investigation because it was the most dominant OTU present in all sponge samples, with the aim of determining its potential origin. OTU_1 has been taxonomically assigned as an unclassified species within the genus *Amylibacter*, belonging to the class Alphaproteobacteria, according to the SILVA 138 database. Furthermore, its closest match in the NCBI database is *Amylibacter marinus*, with a 96% sequence similarity (accession no. NZ-BSNN01000013.1, Query cover= 100%).

The relative abundance of OTU_1 varied between different environments and sample types. In the ex-situ samples (Figure 3.7-A), OTU_1 was nearly absent, while it represented a substantial proportion (approximately 80%) in the wild samples. This lower abundance of OTU_1 in ex-situ sponges suggests that OTU_1 is likely acquired from the surrounding environment, rather than being an intrinsic component of the sponge microbiome. In contrast, when examining different sample types (Figure 3.7-B), OTU_1 was most abundant in sponge samples, accounting for approximately 90% of the total OTUs. Interestingly, OTU_1 also exhibited relatively high abundance in seaweed samples, representing over 35% of the total OTUs, while it was nearly absent in seawater samples.

According to the indicator species analysis, 72 species were identified as indicators for sponge samples, whereas 27 were identified as indicators for the combined group of sponge and seaweed. The indicator value for OTU_1 is very high (0.992) in the combined group of sponge and seaweed (Table 3.2), indicating that OTU_1 is equally strongly associated with both environments. This highlights OTU_1 as a significant indicator species in the combined sponge and seaweed samples. Conversely, the indicator value for OTU_1 is 0 in the seawater, sponge, and seaweed groups when considered separately, suggesting that OTU_1 is not individually associated with any of these samples.

The predicted functions of OTU_1 indicate its significant involvement in several key metabolic pathways within the sponge (Figure 3.8). The most abundant function was "Glycolysis / Gluconeogenesis," which plays a central role in carbohydrate metabolism and energy production. Other notable functions include the "Citrate cycle (TCA cycle)" and "Pentose phosphate pathway," essential for cellular respiration and biosynthesis. Additionally, OTU_1 is involved in diverse sugar metabolism and fatty acid biosynthesis, elongation, and degradation, indicating its metabolic versatility. Moreover, the predicted functions of OTU_1 highlight its role in fatty acid metabolism, including biosynthesis,

elongation, and degradation, and its involvement in lipid production and energy regulation within the sponge.

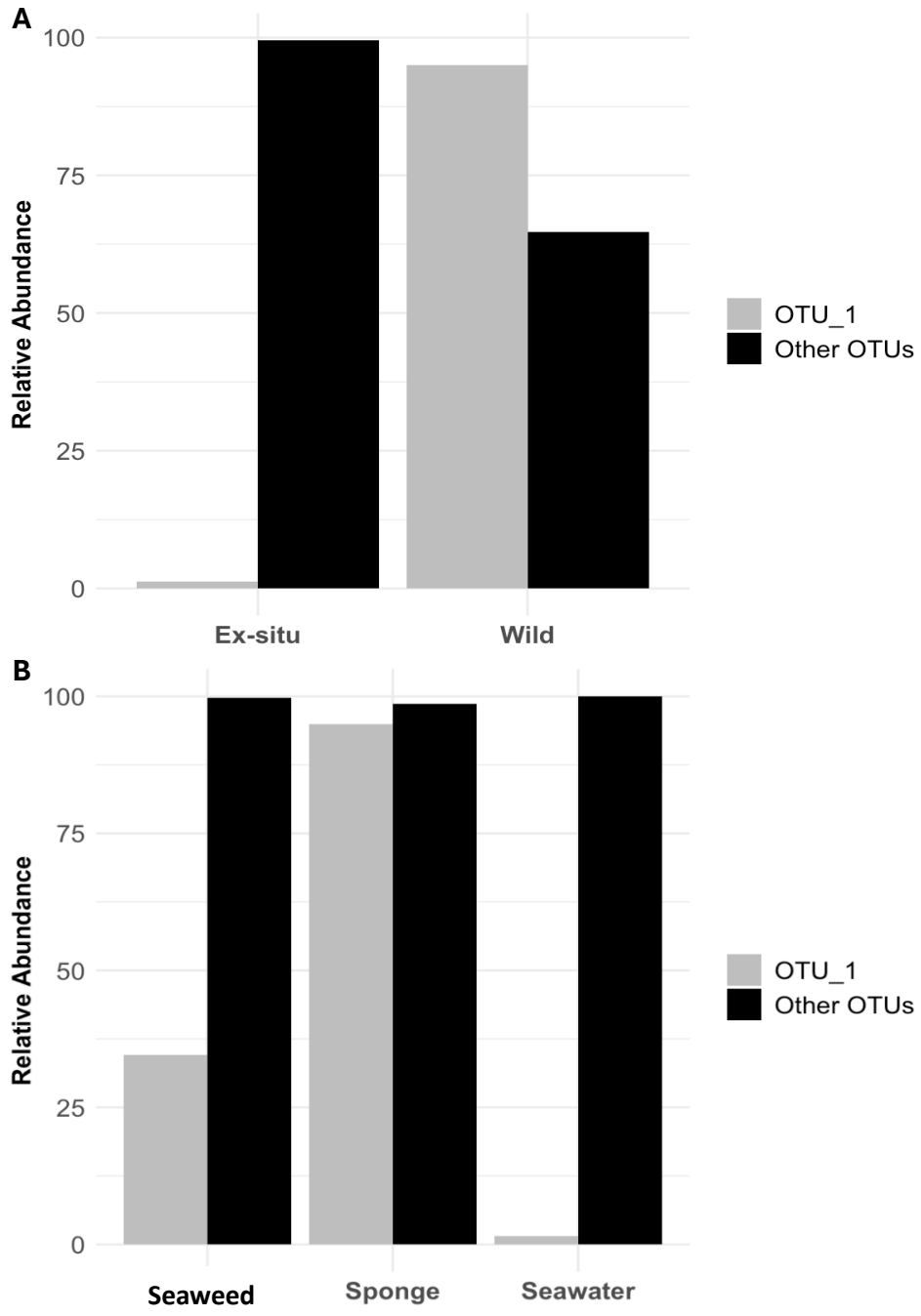


Figure 3. 7. Relative abundance of OTU_1 and other OTUs in *H. panicea* sponge across different environments and sample types. (**A**) between ex-situ and wild conditions. (**B**) among different sample types: seaweed, sponge, and seawater. The grey bars represent OTU_1, while the black bars represent all other OTUs.

Table 3. 2. Indicator species analysis results for OTU_1 in different sample types (sponge, seaweed and seawater) under significant level $p < 0.05$.

Group	Stat-value	P-value
Sponge	Not significant	-
Seaweed	Not significant	-
Seawater	Not significant	-
Sponge + Seawater	Not significant	-
Sponge + Seaweed	Significant (0.992)	0.001

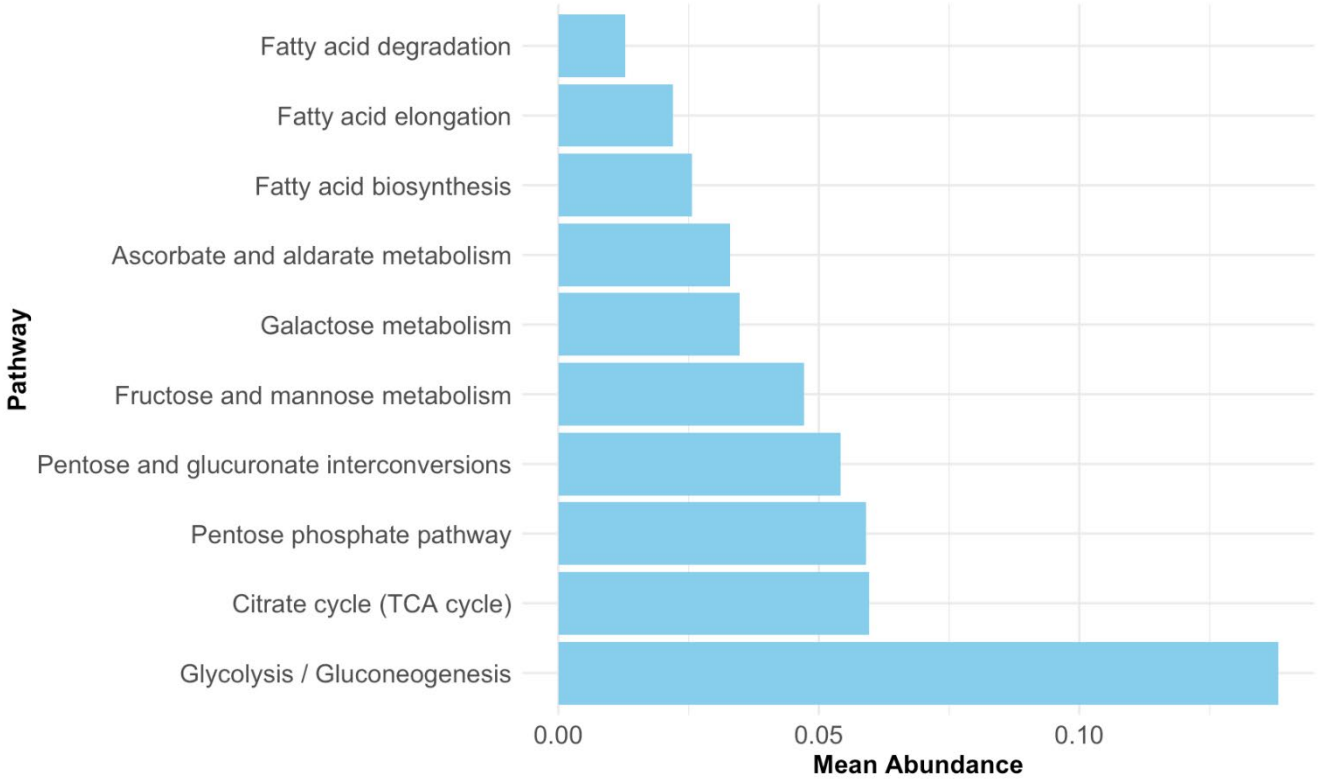


Figure 3. 8. Bar plot illustrates the top 10 predicted metabolic functions associated with OTU_1, based on mean abundance. The functions are ranked according to their predicted contribution to the host.

3.4 Discussion

The metabarcoding analysis of the 16S rRNA sequence of *H. panicea* and samples from its surrounding environments (seaweed and seawater) has provided a better understanding of the origin of the *H. panicea* sponge essential microbiome. The sponge gains most of its microbiome from its surrounding environment (Figure 3.6; Turon *et al*., 2018). However, some bacteria associated with sponges are only present in sponge samples. As members of the Desulfobacterota and Nitrospirota phyla were only abundant in sponge samples (Figure 3.1), it suggests that they play a major role in recycling nutrients as part of their symbiotic relationship. Based on transcriptomic studies of marine sponge microbial communities, Desulfobacterota can oxidise ammonia and/or methane through amoABC/pmoABC genes (Burgsdorf *et al*., 2022), while Nitrospirota play a significant role in the nitrogen cycle (Lücker *et al*., 2010) by oxidising nitrites through the second step of nitrification (Koch *et al*., 2015). Also, within the phylum Nitrospirota, magnetotactic bacteria (MTB) play an important role in biogeochemical cycles through their ability to biomineralise large amounts of magnetite magnetosomes and cycle key elements such as carbon, sulfur, nitrogen and iron (Zhao *et al*., 2023). Members of Campilobacterota were found in both sponge and seawater samples, and were not detected in seaweed samples. This phylum comprises free-living bacteria that colonise abiotic and biotic surfaces (Kalenitchenko *et al.,* 2016; Petersen *et al*., 2010; Pjevac *et al.,* 2018), so they could be transmitted horizontally from the surrounding seawater to the sponge.

In contrast, certain bacterial phyla were only associated with seaweed samples. For example, Acidobacteriota were detected in both red and brown seaweeds but was less abundant or even absent in other samples. Moreover, Deinococcota are found only in red seaweeds, (Gefen-Treves *et al*., 2021; Hollants *et al*., 2013), while Patescibacteria are only found in brown seaweeds. While the diversity of the bacterial community in the brown seaweed *Fucus vesiculosus* was similar to the same species studied in different geographical areas (Lachnit *et al*., 2011; Parrot *et al*., 2019; Stratil *et al*., 2013), the percentage of abundance in terms of the ASVs/OTUs differed among studies.

Phylum Proteobacteria (mostly class Alphaproteobacteria*)* was the main phylum in the sponge samples (Figure 3.1) with *Amylibacter* being the most dominant genera within this phylum. Bacterial genera belonging to class Alphaproteobacteria have been found in many marine sponges

(Bibi *et al*., 2020; Mohamed *et al*., 2008; Thomas *et al*., 2016). *H. panicea* is known to be dominated by a core taxon that belongs to class Alphaproteobacteria and is closely related to *Amylibacter* genera that have been detected in the sponge from different geographical areas and have recently been named "*Candidatus Halichondribacter symbioticus*" (Knobloch *et al*., 2019b; Rusanova *et al*., 2021; Schmittmann *et al*., 2022; Wichels *et al*., 2006). The result of this study supports the findings of *H. panicea* core taxon, where it was found that the most dominant bacterial OTU in the sponge from North-east of England waters, White Sea and Iceland waters belongs to *Amylibacter* genera (class Alphaproteobacteria). Studies have linked the abundance of this bacterial genera in the *H. panicea* sponge to horizontal transmission from the surrounding seawater (Knobloch, *et al*., 2019b; Rusanova *et al*., 2021). However, there were only trace amounts of this genera in the collected seawater samples (some were negative). Furthermore, OTU_1 (*Amylibacter* genus) was found in high abundance in the seaweed samples (Figure 3.7-A), ranking among the top 20 genera (Figure 3.3) and according to indicator species analysis (Table 3.2) it was found that this OTU is associated with both sponges and seaweed, indicating a potential ecological or functional link between these two habitats, while its near absence in seawater highlights that it is not a ubiquitous component of the surrounding water column. Hence, they may have been transmitted horizontally from seaweed rather than from seawater. The presence of these bacterial genera is highly related to their symbiotic relationship with the sponge. To further explore the symbiotic relationship between the sponge and its microbial community, the analysis of the predicted metabolic functions associated with OTU_1 revealed its involvement in several key metabolic pathways (Figure 3.8). The most abundant predicted function, Glycolysis/ Gluconeogenesis indicates that OTU_1 plays a central role in primary carbohydrate metabolism, crucial for energy production and carbon flow (Song *et al*., 2022) within the sponge. Additionally, significant functions such as the Citrate cycle (TCA cycle) and the Pentose phosphate pathway are essential for cellular respiration and nucleotide synthesis (Wang *et al*., 2021), while pathways related to Pentose and glucuronate interconversions, Fructose and mannose metabolism, and Galactose metabolism suggest the ability of OTU_1 to metabolise diverse sugars (Jeckelmann & Erni, 2020). Moreover, its involvement in Ascorbate and aldarate metabolism and Fatty acid biosynthesis, elongation, and degradation reflects a broader metabolic versatility, encompassing lipid and antioxidant metabolism (Elferink *et al*., 2020). These findings underscore the multifaceted role of OTU_1 in supporting the health and homeostasis of the host sponge, contributing to various metabolic processes that enhance its resilience in the marine environment.

3.5 Conclusion and Future study

This study has provided evidence consistent with the hypothesis that the core microbiome of the LMA sponge *H. panicea* is acquired through horizontal transmission from the environment. This accords with previous findings that the microbial communities of LMA sponges, which are dominated by Proteobacteria, are broadly similar to the surrounding seawater (Bayer *et al*., 2014; de Voogd *et al*., 2018; Rix *et al*., 2020), but seaweed appears to contribute more to the sponge microbial communities than seawater. Moreover, the most dominant OTU (taxonomically assigned to *Amylibacter*) was abundant in both *H. panicea* and attached seaweed samples but was only detected in trace amounts in seawater and it was a significant indicator species in the combined sponge and seaweed samples. The predicted functional profile of OTU_1 revealed its involvement in key metabolic pathways, including carbohydrate metabolism, fatty acid biosynthesis, and energy production, highlighting its potential role in supporting the host sponge's health and resilience. Further analysis is required to study the ecological role of this core bacterial taxa (OTU_1) within the sponge which would help to gain a better understanding of how they contribute to the host's health and survival.

Chapter 4. Does the Sponge Microbiome Play a Role in the Different Colour Morphs of *Halichondria panicea*?

4.1 Introduction

Sponges are associated with rich and diverse microbial communities (Bibi *et al*., 2021; Taylor, Radax, *et al*., 2007). Microbes can make up to 40-60% of sponge volume, with cyanobacteria often predominant (Grozdanov & Hentschel, 2007; Kallscheuer *et al*., 2020; Thacker & Freeman, 2012). There is a general pattern to the distribution of bacteria within sponges. Microorganisms capable of photosynthetic activity, such as cyanobacteria and eukaryotic algae, reside in the outer layers of tissues exposed to light (Devaprakash *et al*., 2024; Rützler, 1990; Wilkinson, 1992). In contrast, the inner core is populated by heterotrophic and possibly autotrophic bacteria (Hentschel *et al*., 2003). Photosynthetically active microorganisms in the sponge's outer layers play a crucial role in sponge colour variation (Thakur & Müller, 2004; van Opstal, 2023).

Cyanobacteria appear to be the most important group of sponge symbionts because of their photosynthetic activity and their ability to produce secondary metabolites (Mutalipassi *et al*., 2021; Sara *et al*., 1998; Taylor, 1973; Thomas, Kavlekar, *et al*., 2010). Generally, sponge and cyanobacteria interactions are mutualistic, in which the sponge host provides a sheltered habitat to cyanobacteria. In contrast, the cyanobacteria symbionts provide sponges with supplemental nutrition (through photosynthesis and nitrogen fixation) (Hudspith *et al*., 2022; Raven, 2002), chemical defense (through the production of bioactive compounds) (Konstantinou *et al*., 2020b), and UV protection (Curdt *et al*., 2022; Gao & Garcia-Pichel, 2011; Webster & Taylor, 2012). Sponges acquire cyanobacterial symbionts vertically, through parent-offspring transmission (through larvae), horizontally, from the surrounding environment, or a combination of the two (de Oliveira *et al*., 2020; Oren *et al*., 2005). Many sponge species have a common cyanobacteria clade, like coccoid cyanobacteria morphs (*Synechococcus* spp., *Prochlorococcus* spp., *Aphanocapsa* sp. and *Synechocystis* spp.), which are found in sponges more frequently than the filamentous morphs (*Oscillatoria spongeliae* and *Leptolyngbya* spp.) (Konstantinou *et al*., 2018). Studies on *Halichondria panicea* from different geographical areas showed that the bacterial diversity differed markedly across sampling sites and revealed that cyanobacteria are part of the sponge core microbiome (Knobloch *et al*., 2019b; Moitinho-Silva, Nielsen *et al*., 2017; Naim *et al*., 2014; Schmittmann *et al*., 2022; Steinert *et al*., 2017).

H. panicea is an abundant coastal marine sponge that can be found in two colour morphs, either green or cream-yellow. Its colour is primarily influenced by sunlight exposure, endobionts, and parasitic green algae such as *Microspora ficulinae* (Christensen, 1985). Other microalgae of the genus *Desmodesmus* may also contribute to the green colour of *H. panicea* (Gorelova *et al*., 2012; Kravtsova *et al*., 2013). The sponge-associated *Desmodesmus* strains are phyletically distinct from other symbiotic *Desmodesmus* species and display a contrasting pattern of response to N-starvation when compared to free-living strains with prominent differences in ultrastructure and fatty acid composition (Baulina *et al.,* 2016).

Many studies have been conducted on the relationship between sponge colour and cyanobacterial abundance (Alex *et al*., 2012; Konstantinou *et al*., 2020b; Pagliara *et al*., 2020). In contrast, microbial eukaryotes associated with marine sponges have received relatively little attention, especially regarding their ecology and function (Nascimento-Silva *et al*., 2022; Thomas *et al*., 2016). The unicellular eukaryotic community has recently been examined using the 18S rRNA gene metabarcoding analysis revealing no-host specificity to the studied sponge species (Cleary, 2019; de Mares *et al.,* 2017). Moreover, the molecular approaches have shown that the eukaryotic communities found in seawater are similar to those of the sponge hosts (de Mares *et al*., 2017; Cleary, 2019; Hardoim *et al*., 2021), while symbiotic cyanobacteria differ remarkably from those found in seawater (de Mares *et al.,* 2017; Hayami *et al*., 2023). This indicates that, despite the important role the unicellular eukaryotic community plays in ecosystem functioning, stochastic processes dominate their assembly (Hardomin *et al*., 2021).

A common marker for detecting and classifying aquatic eukaryotic protists is the 18S rRNA gene, also known as the small ribosomal subunit gene (SSU). In many studies on the diversity of eukaryotes, the V1–V2, V3, V4, and V9 regions of 18S rDNA have been used to investigate microbial community diversity (Bradley *et al*., 2016; Choi & Park, 2020; Tanabe *et al*., 2016). V9 (de Vargas *et al*., 2015) and V4 (Minicante *et al*., 2019; Hardoim *et al*., 2021; Piredda *et al*., 2017) are, however, most commonly used and offer conserved primer binding sites for PCR to amplify broadly taxonomic groups, allowing some degree of taxonomic resolution. In silico analysis of the V9 region of 18S rRNA reveals the diversity of eukaryotes, while the V4 region is more commonly used to determine phylogenetic relationships among eukaryotes (Choi & Park, 2020; Hu *et al*., 2015).

The cyanobacterial and microalgal communities in *H. panicea* have yet to be studied extensively, and their relationship to sponge colour morphs is not fully understood. Therefore, the present study aimed to assess the diversity of the eukaryotic and cyanobacterial community in the most common intertidal LMA marine sponge, *H. panicea*, using metabarcoding analysis (16S and 18S rRNA) to determine the nature of the different colour morphs of this sponge species. A total of 108 sponge samples were collected from three locations along the Northumberland coast, north-east England, during different seasons. The sponge samples were categorised based on their colour morphs and examined for differences in cyanobacterial communities. Only ten isolates of extreme colour morphs (five yellow and five green) were selected from these samples to investigate the differences in chlorophyll fluorescence and concentration as well as the diversity and abundance of eukaryotic communities of both colour morphs.

4.2 Methodology

4.2.1 Sponge sampling and identification

H. panicea samples were collected in triplicate from the intertidal zone at three sites in north-east England: Cullercoats Bay (55.035539, -1.431161), Marsden Bay (54.983112, -1.378978) and Whitley Bay (55.064639, -1.450110) during four seasons (January, April, July and October). Ten sponge samples were chosen with extreme green or yellow colours photographed prior to collection (Figure 4.1). A sterile blunt knife was used to scrape a 3 cm^2 piece of sponge tissue off the rock, which was transferred to a plastic bag with seawater from the surrounding environment. Samples were stored in an icebox for transport to the laboratory. Upon arriving in the laboratory, the samples were washed three times with sterile artificial seawater (ASW; Tropic Marin classic salt, Germany) of salinity equal 35 ppt and kept in sterile 50 mL Falcon™ tubes for identification and DNA extraction.

The sponge sample's identity was confirmed via spicule structure and external morphological traits. The protocol followed for spicule preparation was as described by Hooper (2000). The sponge samples from the three locations (108 sponge samples) were categorised according to sponge colour and tested for cyanobacterial community composition. To study the eukaryotic communities, ten sponge samples were chosen with extreme green or yellow colours: five yellow and five green.

Figure 4. 1. *Halichondria panicea* different colour morphs. (**A**) Sample 7-C-sp-1-1, a green sponge. (**B**) Sample 10-Mrs-2-2, a yellow sponge.

4.2.2 Chlorophyll extraction

To study the differences between chlorophyll concentrations in *H. panicea's* colour morphs (green and yellow), six representative sponge samples (three green and three yellow) were extracted according to the protocol of Thacker *et al.* (2007). Approximately 0.25 g (wet mass) of each freshly collected sponge sample was cut into small pieces and placed in 10 mL of 90% acetone. The mixture was vortexed for 5-10 min and left overnight at 4 °C (in the dark) to extract all the pigments from the samples. The next day, samples were centrifuged for 1 min at 4000 rpm to remove the suspended solids and left at room temperature to warm before being transferred to a glass cuvette. Solvent extract absorbances were measured at different wavelengths: 480, 510, 630, 647 and 664 nm in a UV-visible spectrophotometer (CARY 100 Bio, VARIAN) with 90% acetone as a reference blank. Chlorophyll *a*, *b* and *c* concentrations were calculated based on the equations of Parsons *et al.* (1984) and standardised to sample wet weight.

Parson's chlorophyll estimating equations:

$$\text{Chlorophyll } a = 11.85\ E_{664} - 1.54\ E_{647} - 0.08\ E_{630}$$
$$\text{Chlorophyll } b = 21.03\ E_{647} - 5.43\ E_{664} - 2.66\ E_{630}$$
$$\text{Chlorophyll } c = 24.52\ E_{630} - 1.67\ E_{664} - 7.60\ E_{647}$$
$$\text{Plant carotenoids} = 7.6\ (E_{480} - 1.49\ E_{510})$$

4.2.3 Epi-fluorescence microscopy

Upon reaching the laboratory, sponge samples were directly analysed by inverted epifluorescence microscopy (Leica DMi8, Germany) to determine the type and density of

photosynthetic symbionts. The sponge samples were washed with sterile ASW, cut into a thin cross-section using a sterile razor blade and examined under the microscope. Sponge symbionts were also extracted by squeezing the recently washed samples with sterile ASW several times and examining a few drops of the extracted solution under the microscope to check the different groups of symbionts within the sponge. Sponge samples (green and yellow) were exposed to green light using the Texas Red fluorescence filter set (540–580 nm excitation and 592-668 nm emission filters) to detect the distribution and concentration of chlorophyll auto-fluorescence. Then to investigate the number of fluorescent cells, cell counts were measured for each scanned image using QuPath software (v. 0.051; Bankhead, *et al.*, 2017).

4.2.4 DNA extraction

To study the eukaryotic and microbial communities of the sponge samples, the 18S and 16S rRNA amplicon sequencing was analysed, respectively. The DNA was extracted using a DNA extraction kit (DNeasy Power Soil Pro, Qiagen), where the manufacturer guidelines were slightly modified for each sample type to obtain maximum DNA yield.

A small piece of the sponge, approximately 0.125-0.2 g, was added to the PowerBead Pro tube with 800 µL of solution CD1. The sample was mixed briefly, then vortexed horizontally using a 1.5-2 mL tube size vortex adapter (cat. no. 13000-V1-24) for 10-15 min (until the sponge tissue disintegrated) at maximum speed; this was followed by centrifugation at 15,000 x *g* for 1 min. Next, the supernatant was transferred to a clean microcentrifuge tube (2 mL), and 200 µL of solution CD2 was added, vortexed for 5 s, and centrifuged at 15,000 x *g* for 1 min. The supernatant (up to 700 µL) was transferred to a clean microcentrifuge tube (2 mL), 600 µL of solution CD3 (saline solution) was added, and vortexed (5 s). Only 650 µL of the mixed solution (lysate) was transferred to a MB spin column, centrifuged at 15,000 x *g* for 1 min, and the flow-through discarded. This step was repeated 2-3 times until the whole lysate was processed. The MB spin column was transferred to a new clean collection tube (2 mL), 500 µL of EA solution (wash buffer) was added, centrifuged at 15,000 x *g* for 1 min, and the flow-through was discarded. Next, 500 µL of cold C5 solution (ethanol-based wash solution stored at -20 °C) was added to the column, closed tightly and inverted gently to wash off any residual buffer that may have adhered to the edges of the column. The column was then incubated for 5 min at 4 °C before being centrifuged at 15,000 x *g* for 1 min. After discarding the flow-through, an extra washing step with cold molecular grade

80% ethanol (stored at –20 ℃) was added to the column, which was closed tightly and inverted gently again to wash off any residual buffer that may be adhered to the edges of the column. The solution was again incubated in the column for 5 min at 4 ℃ before being centrifuged at 15,000 x *g* for 1 min. After discarding the flow-through, a dry centrifugation step at 16,000 x *g* for 2-3 min was done to ensure the removal of any ethanol traces from the DNA sample. Finally, the MB spin column was transferred to a new 1.5 mL Eppendorf tube to which 70 µL of C6 solution (elution buffer) was added to the centre of the filter membrane, centrifuged at 15,000 x *g* for 1 min, the MB column was discarded, and the obtained DNA was stored at –20 ℃.

4.2.5 DNA sequencing and taxonomic assignment

Bacteria (16S rRNA)

The primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify bacteria 16S rRNA gene V3/V4 hypervariable regions. PCRs were conducted in a 25 µL mixture containing 12.5 µL of Phusion® Hot start flex 2X Master Mix (New England Biolabs), 2.5 µL of each primer (10 µM), 50 ng of template DNA, and made up to 25 µL with double-distilled water. Thermal cycling was performed at 98 °C for 30 s , followed by 35 cycles at 98 °C for 10 s, 54 °C for 30 s, 72 °C for 45 s, and finally at 72 °C for 10 min. PCR products were isolated by 2% agarose gel, followed by purification with AMPure XT beads (Beckman Coulter Genomics, USA). The products were finally quantified by Qubit assay (Invitrogen, USA). After PCR, the products were analysed by Agilent 2100 Bioanalyzer (Agilent, USA), and KAPA Library Quantification Kits (Kapa Biosciences, Woburn, MA, USA) were used to generate the sequencing library. Qualified libraries had concentrations > 2nM. Sequencing was carried out on NovaSeq 6000 platform, 2x250 bp (NovaSeq 6000 SP Reagent Kit, 500 cycles) at LC Sciences (Houston, USA). Raw reads of 400–500 bp were generated with a depth of ~50,000 reads per sample.

Eukaryotic microorganisms (18S rRNA)

To study the whole eukaryotic microorganisms in sponge samples, the V4 hypervariable region of the 18S rRNA gene was chosen for sequencing. The best primer set was selected by comparing primers used in different studies (Choi & Park, 2020; Fadeev *et al*., 2018; Ferreira & Cleary, 2022; Konstantinou *et al*., 2020a; Kumar *et al*., 2021) using the PR2 primer database (https://app.pr2-primers.org/pr2-primers/) and choosing the set with the higher percentage of

amplified sequences of most of the chlorophyll-containing eukaryotes. Therefore, the general eukaryotic primers TAReuk454FWD1 (5′-CCAGCASCYGCGGTAATTCC-3′) and TAReukREV3 (5′-ACTTTCGTTCTTGATYRA-3′) (Stoeck *et al*., 2010) were chosen for this study. The purity of the extracted DNA was checked on a 1% agarose gel, and the DNA concentration was diluted with sterile water to 1 ng/µL by Novogene (UK) before amplifying the 18S rRNA gene using the specified primers (TAReuk454FWD1 and TAReukREV3). The PCR reactions used a 25 µL mix of 2.5 µL microbial DNA (5 ng), 5 µL of forward and reverse primers (1 mM), and 12.5 µL of Phusion® High -Fidelity PCR Master Mix (New England Biolabs). Thermal cycling was performed at 95 °C for 3 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and finally at 72 °C for 5 min. PCR products were separated on a 2% agarose gel, followed by purification with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated according to the manufacturer's recommendations using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA). The quality of the library was evaluated using a Qubit@ 2.0 Fluorometer (Thermo Scientific) and an Agilent Bioanalyzer 2100. Sequencing was conducted on the Illumina NovaSeq platform, 2x250 bp (Novogene, UK). Raw reads of 400–500 bp were generated with a depth of ~30,000 reads per sample.

4.2.6 Bioinformatic pipeline

The 16S rRNA and the 18S rRNA raw sequences were processed with the same bioinformatics pipeline described by the dada2 tutorial (https://benjjneb.github.io/dada2/tutorial.html), except that different reference databases were assigned at the “assign taxonomy” step according to gene type. The 16S rRNA inferred taxa were assigned to SILVA v.138 prokaryotic SSU database (http://www.arb-silva.de/) and the 18S rRNA gene taxa were assigned to the PR2 database (PR2 version 5.0.0, May 2023), which contains only eukaryotic taxa (Guillou *et al*., 2013).

4.2.7 Phyloseq analysis and community structure

The next step of the analysis was carried out using the R package Phyloseq (version 1.42.0), which is dedicated to the representation and analysis of microbiome data (McMurdie & Holmes, 2013). This package was used to combine the following data into one dataset: ASV table, taxa table, sampling data including any metadata collected, and phylogenetic tree. Next, data in the phyloseq object were cleaned from empty columns, Eukaryota, Chloroplast and Archaea, by using

“subset_samples” function. Then "prune_taxa" was used to filter taxa to a total of above 5 for the sum of shared ASVs in sponge samples. The taxa number was reduced from 55,346 to 22,417. To study the cyanobacterial community within sponge samples, “subset_taxa” function was used to subset the cyanobacteria in sponge samples. For further analysis, “subset_samples” function was used to create a phyloseq object for each sponge colour from the created Cyanobacteria phyloseq object. Then, "prune_taxa" was used again to filter taxa for taxa_sums >0 in each sponge colour phyloseq object. The community structure profiles for the cyanobacteria in each sponge colour were created by transforming the taxa counts to relative abundance using “transform_sample_counts” function from the phyloseq package. Taxa were agglomerated using “tax_glom”, and the taxa table was melted using “psmelt” functions. The produced taxa data frames were visualised using the ggplot2 package (Wickham, 2016) as pie charts for top cyanobacterial families and bar plots for the top 20 bacterial genera.

For the 18S rRNA analysis of the eukaryotic organisms within sponge samples, the ASV table, taxa table and metadata were combined into a phyloseq object using the Phyloseq package. The data were cleaned from empty columns, Porifera and Chloroplast, by using the “subset_samples” function. The taxa number was reduced from 788 to 726. To compare the eukaryotic community of the green and yellow samples, “subset_samples” function was used to create two phyloseq objects for each sponge colour. The green sponge had 422 taxa, while the yellow sponge had 464 taxa. The community structure profiles were created as described previously for the bacterial communities, but they were visualised as bar plots using the ggplot2 package.

4.2.8 Predicted functional genes using PICRUST

PICRUST (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was used in this study to predict the functional genes of the common symbiotic bacterial community within the green and yellow sponge samples . Bacterial functions were predicted using picrust2 (Douglas *et al*., 2020) in Galaxy/Hutlab (https://huttenhower.sph.harvard.edu/galaxy/). The picrust2 output files were generated by uploading ASV’s fasta (ASV’s sequences) and biom (OTU abundance table) files created in R, . Fasta file was created using “write” function from the R “Base” Package, while biom file was created using “write_biom” function from “biomformat” R package. Picrust2 output files were analysed and interpreted using the R package ggpicrust2 (Yang *et al*., 2023). A pathway error bar

was created to check the differences between symbiotic functions between green and yellow sponge samples. The first step to obtaining the required error bar was translating the KEGG orthology (KO) data into Kyoto encyclopedia of genes and genomes (KEGG) using the "ko2kegg" function, followed by "pathway_daa", which added the metadata table and specified the comparison group; in this study, sponge colour was used, along with differential abundance analysis based on the "LinDA" method. The resulting data were visualised using "pathway_errorbar" function with p-value threshold equal to 0.05. Pathways related to the human genome were eliminated, and only the pathways that showed significant differences between different colour morphs were presented in the plot.

4.3 Results

4.3.1 Chlorophyll concentration

The green sponge samples contained higher concentrations of pigments than yellow sponges (Figure 4.2), including chlorophylls *a*, *b* and *c*, and carotenoids. The concentrations of chlorophyll *a* and plant carotenoids were higher in green than yellow sponges.

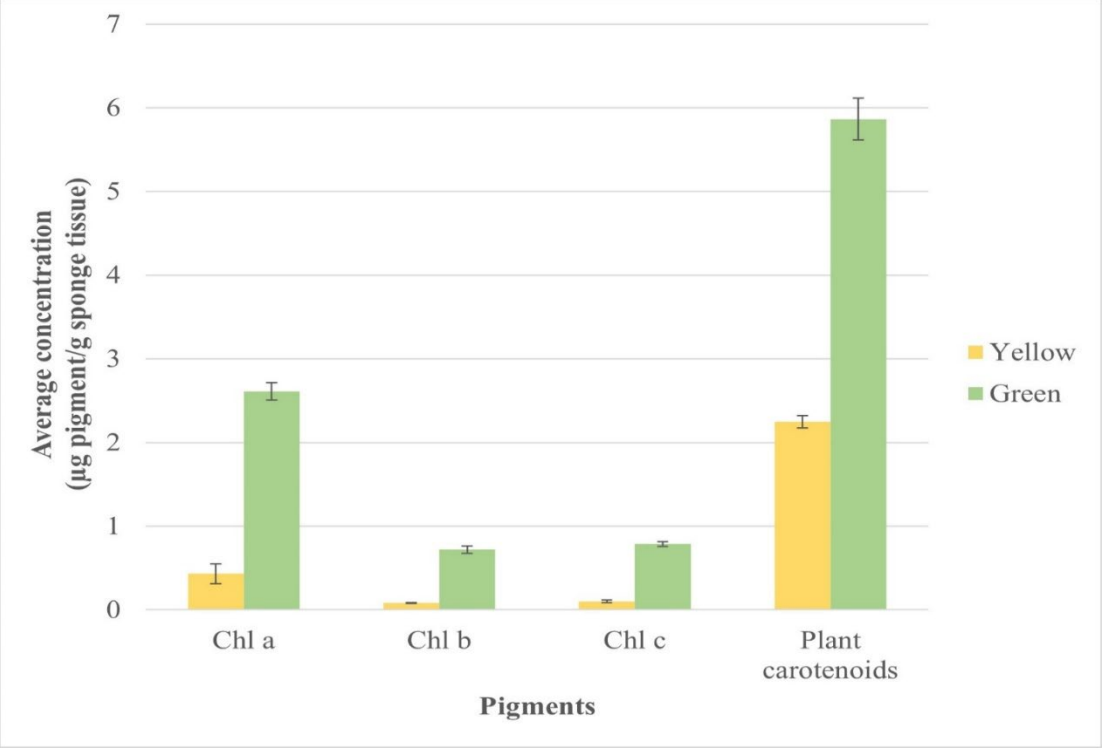


Figure 4. 2. Average concentration of pigments in three yellow and three green *H. panicea* samples. Error bars refer to standard error (n = 3).

Microscopic examination revealed that green sponge tissue showed higher chlorophyll (*a* and *b*) fluorescence than yellow sponge tissue (Figure 4.3). Using QuPath, the fluorescent cell count for both slides were measured, showing that green sponges had higher chlorophyll containing cells (647 cells), than yellow sponge tissue (161 cells). The size of counted cells ranges from 8 - 95 µm. Moreover, no photosynthetic eukaryotes were detected in either sample; thus, chlorophyll concentrations are directly correlated with the abundance of cyanobacteria.

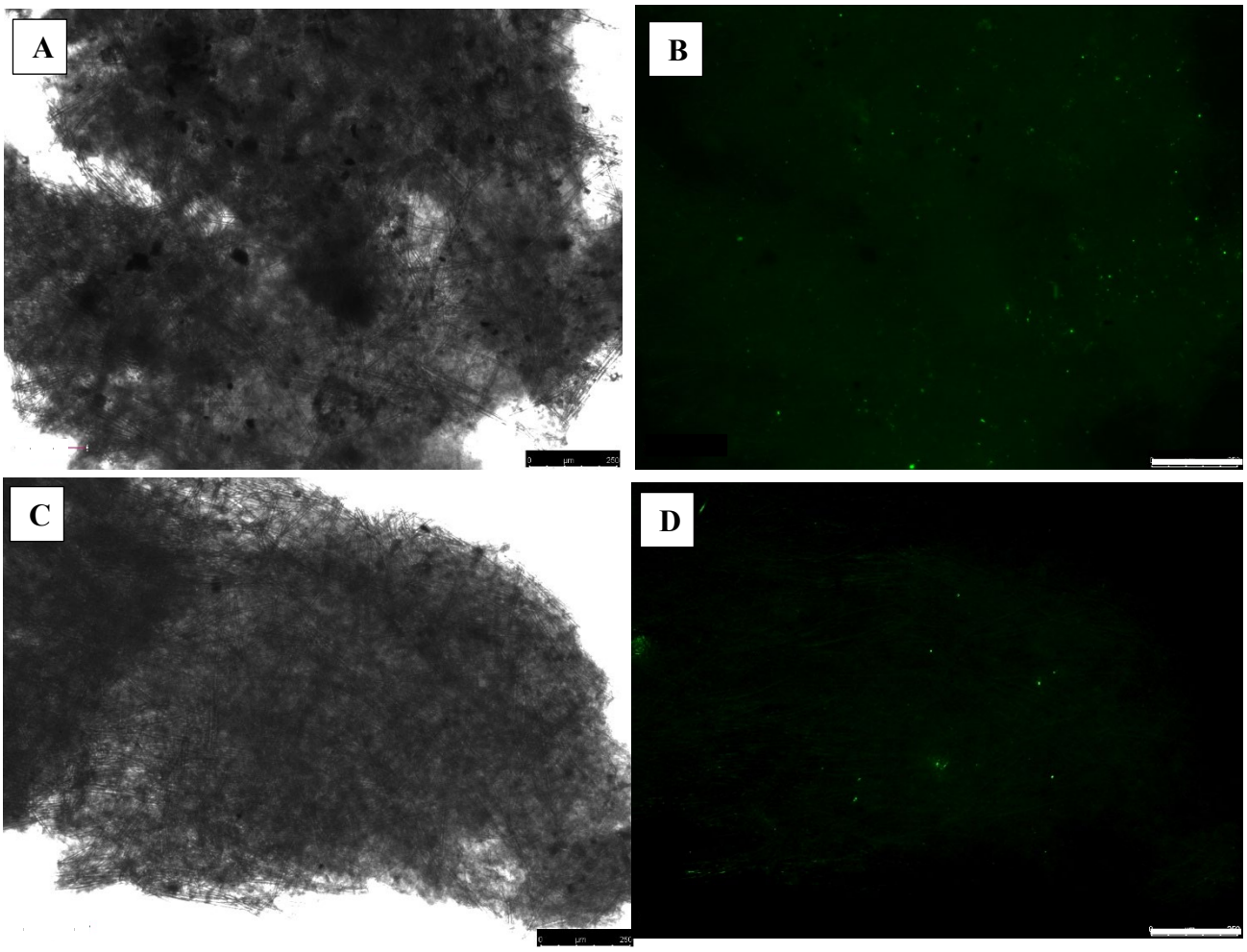


Figure 4. 3. Microscopic images of sponge tissues of the two colour morphs of *H. panicea*. (**A**) and (**B**) green sponge viewed without and with the Texas red filter set, respectively. (**C**) and (**D**) yellow sponge viewed without and with the Texas red filter set, respectively. Scale bars are 250 µm.

4.3.2 Microbiome community structure of the two sponge colour morphs

The community structure of 108 sponges was examined; 45 samples were categorised as green sponges, and 63 were yellow sponges. The top five bacterial phyla were consistently found

across *H. panicea* sponge samples, regardless of different colour morphs. These phyla vary slightly in relative abundance between green and yellow sponges (Table 4.1). Yellow sponges are richer in Proteobacteria and Firmicutes bacterial members, while green sponges in contrast have a higher relative abundance of Actinobacteriodota, Bacteroidota, and Cyanobacteria. The numbers of identified taxa in green and yellow sponges were 17845 and 17996, respectively. The relative abundance of bacterial community structure based on 16S rRNA is presented in Figure 4.4. The dominant genera across both color morphs (yellow and green) are *Amylibacter* (green= 75% and yellow= 78%), Alphaproteobacteria_Unclassified (green= 2.11% and yellow= 1.72%) and *Synechococcus CC9902* (green= 1.10% and yellow= 1.10%). Most bacterial communities (top 20) were shared between green and yellow sponges, however, there were some differences in genera: *Acaryochloris* MBIC11017 (0.54%), *Blastopirellula* (0.19%), *Ilumatobacter* (0.37%), *Nitrospira* (0.25%), PS1 clade_Unclassified (0.30%), and *Woeseia* (0.37%) were only present in green sponges, and *Aurantivirga* (0.21%), *Bacteroides* (0.14%), *Faecalibacterium* (0.88%), *Kistiminas* (0.25%) and *Lactobacillus* (0.36%) were unique to yellow sponges.

Table 4. 1. Top five bacterial phyla by relative abundance in green and yellow colour morphs of *H. panicea* sponge samples.

Top Phyla by rank	Green	Yellow
First	Proteobacteria (87.20%)	Proteobacteria (88%)
Second	Acidobacteriota (2.89%)	Firmicutes (3.98%)
Third	Bacteroidota (2.70%)	Bacteroidota (2.67%)
Fourth	Firmicutes (2.62%)	Acidobacteriota (2.22%)
Fifth	Cyanobacteria (1.87%)	Cyanobacteria (1.42%)

Green sponges contained 255 cyanobacterial taxa, while yellow sponges had 206 taxa. The top 10 cyanobacterial families that dominated the communities are presented in Figure 4.5. The two colour morphs shared most of their cyanobacterial taxa (see also Appendix D - Figure D1). Cyanobiaceae was the most dominant family (relative abundance equals 67.6% and 76.1% in green

and yellow sponges, respectively) followed by Acaryochloridaceae (green= 23.9%and yellow= 17.9%) and Xenococcaceae (green= 3.6% and yellow= 2.7%) in both colour morphs. The green colour morph had higher cyanobacterial diversity than the yellow colour morph (Appendix D - Figure D2). Three cyanobacterial families – Desertifilaceae (0.07%), Obscuribacteraceae (0.05%) and Oscillatoriaceae (0.16%) - were confined to green sponges, while Coleofasciculacea (0.33%) and Sericytochromatia_Unclassified (0.01%) cyanobacterial families were only found in yellow sponges.

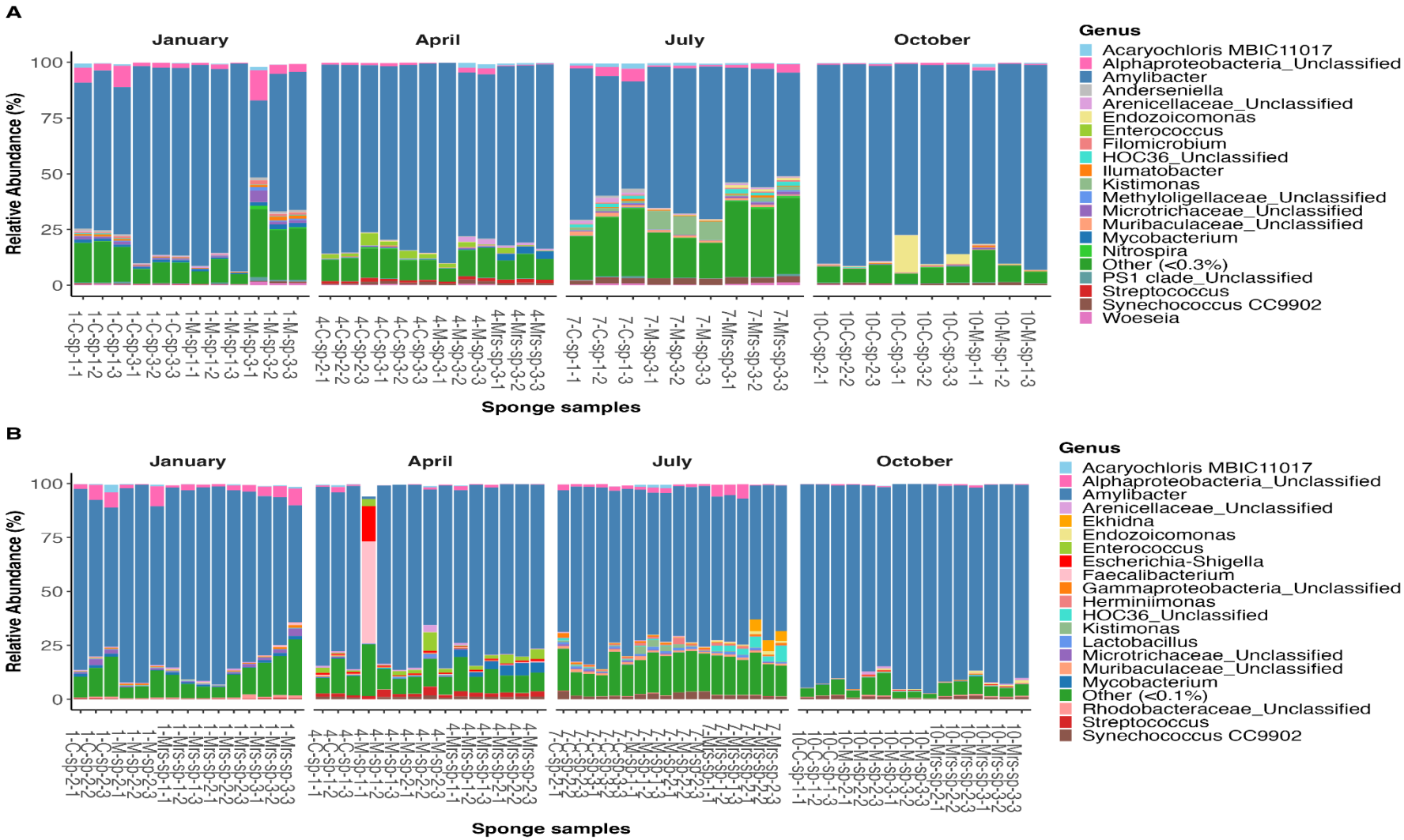


Figure 4. 4. Seasonal variation in the relative abundance of bacterial genera in *H. panicea* different colour morphs across different sampling months. The bar plots depict the relative abundance (%) of the top bacterial genera identified in (A) green and (B) yellow colour morphs of H. panicea collected in January, April, July, and October. Sample codes in the x-axis indicate the sampling month (1= January, 4= April, 7= July, 10= October)- location (C= Cullercoats, M= Whitley Bay, Mrs= Marsden Bay)- sample type (sp = sponge)- sample number (biological replicate) and sample replicate (technical replicate), with relative abundances displayed on the y-axis.

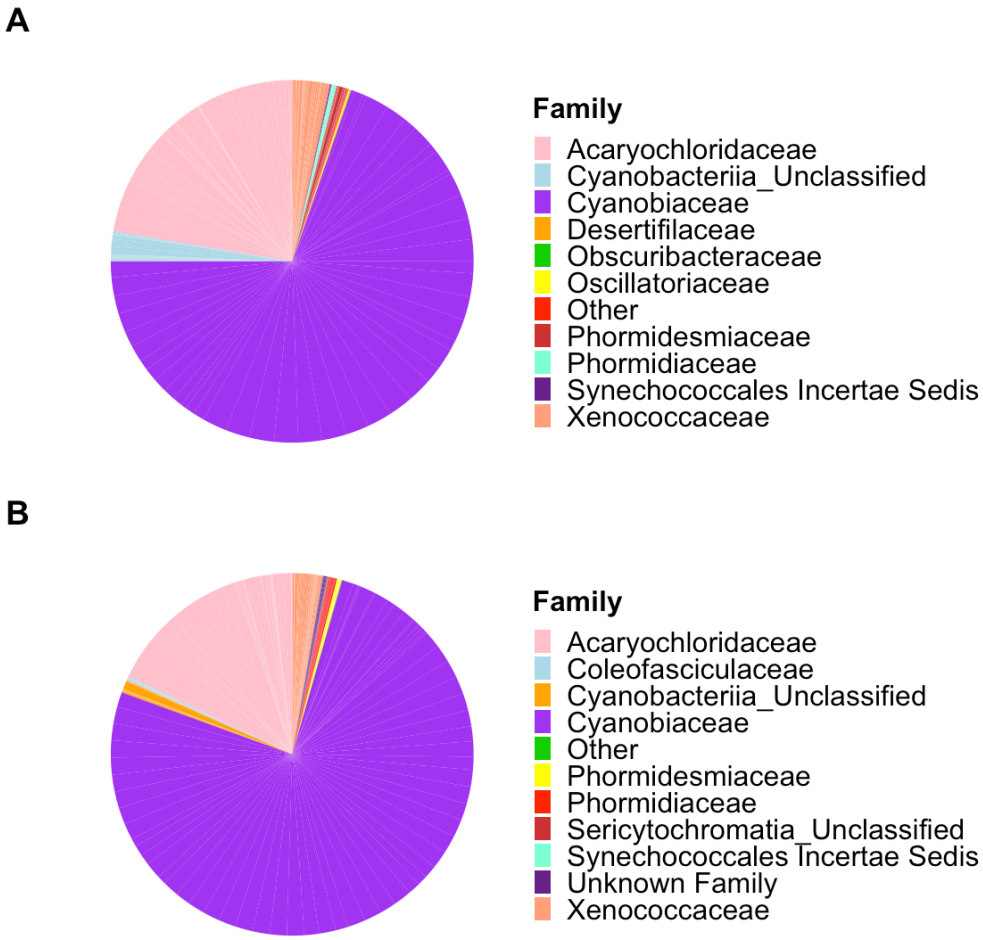


Figure 4. 5. Relative abundance of the top 10 cyanobacterial families in (**A**) green and (**B**) yellow colour morphs of *H. panicea* sponge. Different colours in the legend represent different cyanobacterial families.

Each colour morph had a predominant eukaryotic genus (Figure 4.6). A high relative abundance of Dino-Group-1-Clade-1 was found in yellow sponges that belong to the class Florideophyceae. Conversely, green sponges had a greater abundance of *Phymatolithon* that belongs to the class Syndiniales. Other presented genera show distinct differences in their relative abundances between the two sponge colours, reflecting the differing eukaryotic communities associated with these sponge samples.

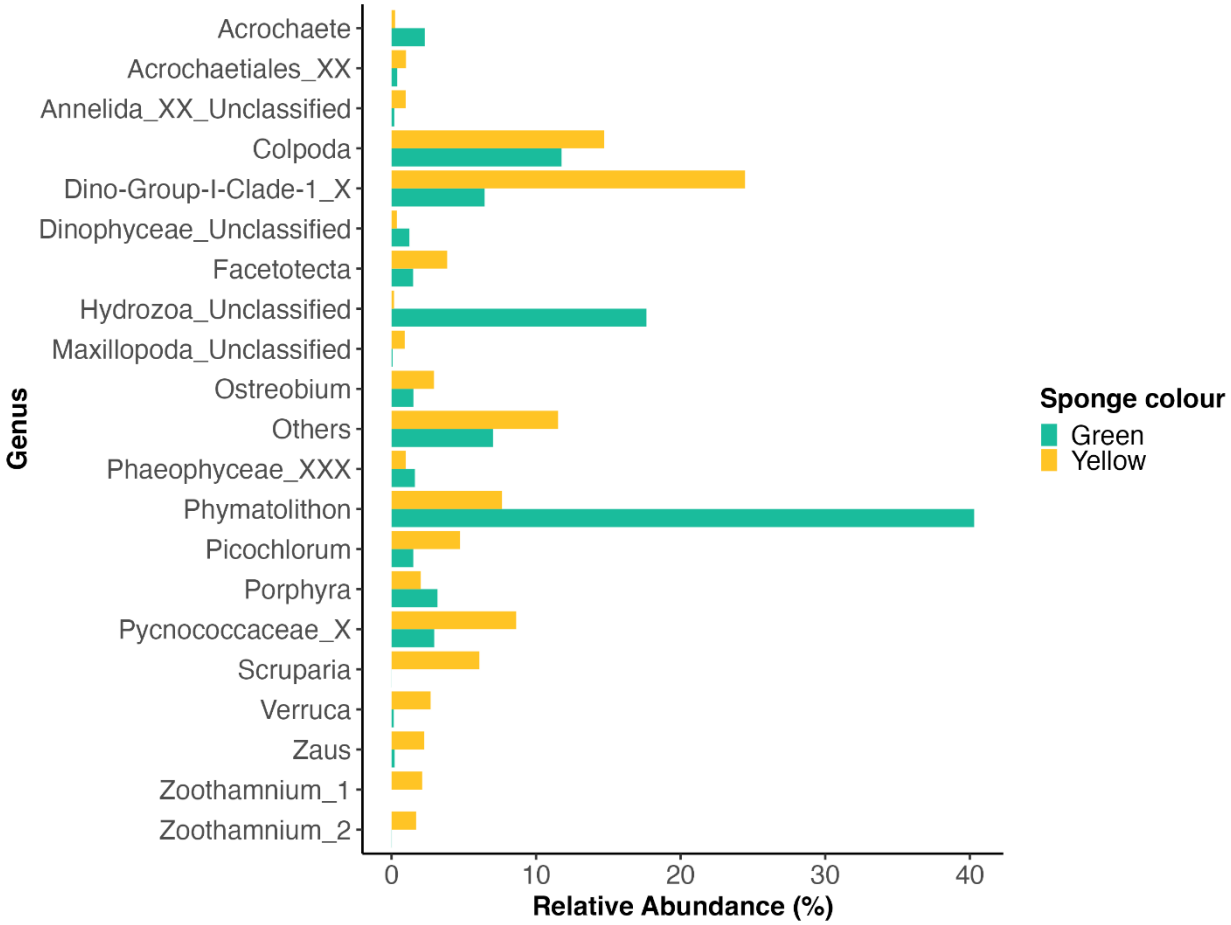


Figure 4. 6. Relative abundance of different eukaryotic genera detected in green and yellow colour morphs of *H. panicea* sponge samples. Genera are listed on the y-axis, and their relative abundance is represented by the length of the bars on the x-axis.

4.3.3 Differences in the predicted functions for the sponge microbiomes of green and yellow colour morphs

There was considerable overlap in the microbiome communities of green and yellow *H. panicea* (Figure 4.4) but they differed in diversity and abundance. Only 17 KEGG pathways were significantly ($p < 0.05$) different between green and yellow sponges (Figure 4.7). The most abundant pathways were for genetic information processing (nucleocytoplasmic transport and proteasome) and metabolism (flavonoid, n-glycan, steroid, steroid hormone and alpha-linolenic acid biosynthesis). The green sponges had a higher relative abundance of all the specified pathways.

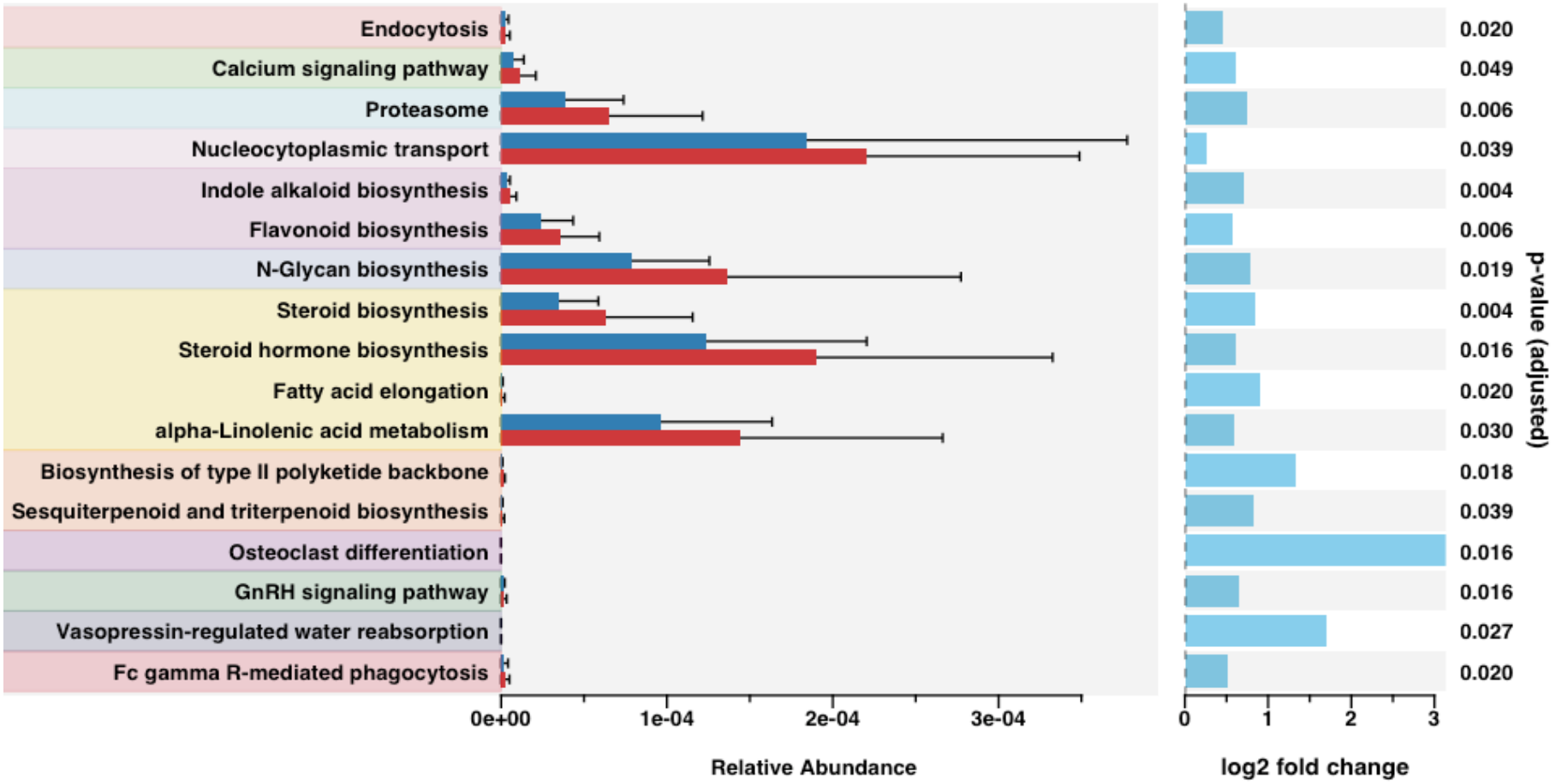


Figure 4. 7. Error bar plot of the KEGG pathways that differed significantly ($p < 0.05$) between the two *H. panicea* colour morphs. The y-axis shows the pathway term, and the x-axis shows the relative abundance and log fold change of each KEGG pathway. Bar plots on the left side display the log fold change relative abundance of each KEGG pathway. Bar plots on the right show the differences (adjusted p-value) in log fold change between the two indicated groups. Red bars = green sponges and blue bars = yellow sponges.

4.4 Discussion

The sponge *Halichondria panicea* has two colour morphs that vary in relative abundance between seasons and sometimes locations (Hiscock, 2008). The sponge microbial community has a great influence on its colour morph, which has been clearly observed through chlorophyll concentration and metabarcoding analysis of the V4 region of 16S and 18S rRNA. Green *H. panicea* had higher pigment content, especially chlorophyll *a* and plant carotenoids, compared to the yellow sponges (Figure 4.2) and this was supported by epifluorescence microscopy of chlorophyll (Figure 4.3). To determine if variability in microbial communities could account for these pigment differences, 16S and 18S metabarcoding analysis was performed. The 16S rRNA analysis (Figure 4.4) revealed a more abundant and diverse microbial community in the green colour morph. including *Acaryochloris* MBIC11017, *Blastopirellula, Ilumatobacter, Nitrospira,* Pirellulaceae_Unclassified, and *Woeseia*. In contrast, *Aurantivirga, Bacteroides, Faecalibacterium, Kistiminas, Lactobacillus*, and SVa0096 marine group were abundant in yellow sponges. Members of the Pirellulaceae family, associated with various corals and sponges, are able to convert ammonia into nitrate (Mohamed *et al*., 2010; Kellogg, 2019), while *Nitrospira* members oxidises nitrite to nitrate in various sponges (Daims *et al*., 2015; Off *et al*., 2010; Palomo *et al*., 2016; Sánchez-Suárez *et al*., 2022; van Kessel *et al*., 2015). The abundance of these two bacterial genera and their associated functions may contribute to the sponge's biological fitness. *Acaryochloris* MBIC11017 was more abundant in green sponges (the top 20 genera) than in yellow sponges and may contribute to the green colouration since it contains chlorophylls *a* and *d* (Miyake *et al*., 2020; Nagao et al., 2023). Likewise, the higher abundance of cyanobacterial taxa in green sponges (Appendix D – Figure A) and their associated chlorophyll pigments, primarily chlorophyll *a* (Curdt *et al*., 2022; Gan & Bryant, 2015), will likely be an important contributor to the sponge's green colour. This suggestion is supported by the high community abundance (75-89%) of cyanobacteria in healthy green specimens of the sponge *Lubomirskia baicalensis*, while community abundance was very low (10-20%) in diseased yellow sponges (Chernogor *et al*., 2020). Other eukaryotic organisms, such as Pycnococcaceae_Unclassified, *Ostreobium, Acrochaete,* and *Picochlorum* that are present in both colour morphs but more dominant in green sponges, and are also suggested to contribute to the green colouration.

Among the eukaryotic community of green *H. panicea*, the genus *Phymatolithon,* which belongs to the class of multicellular red algae, the Florideophyceae, was abundantly represented.

The coralline *Phymatolithon* has a non-geniculate habit and is highly abundant in UK coastal areas (Chamberlain, 1990; Jeong *et al*., 2021). Sponges interact most frequently with algae, corals and crustose coralline algae, where coralline algae represent approximately 14 percent of the substratum cover (González-Murcia *et al*., 2023). As noted previously (Figure 4.1), both colour morphs were found on rocks covered in coralline algae, as sponges prefer to attach to non-living substrates (crustose coralline algae, algae, ascidians and zoanthids) and to interact with non-coral organisms (González-Murcia *et al*., 2023). Thus, coralline algae may have been introduced into sponge samples as a result of cross-contamination while collecting them from coralline algae-covered rock surfaces. In contrast, Dino-Group-I-Clade-I of the class Syndiniales was abundant in yellow sponges. This genus belongs to group I of Syndiniales (Dinophyceae, Alveolata), known for its parasitic behaviour (Anderson & Harvey, 2020; Nagarkar & Palenik, 2023), and is found in many marine organisms (Guillou *et al*., 2008; Jung *et al*., 2016; Skovgaard *et al*., 2005; Stentiford & Shields, 2005), including sponges (Cleary, 2019; Rodríguez-Marconi *et al*., 2015). This genus has not been studied yet in relation to marine sponges including how it infects them. However, some sponges can acquire specific dinoflagellate species when they digest coral substratum (Achlatis *et al*., 2021; Hill *et al*., 2011) or from the surrounding seawater (Ferreira & Cleary, 2022), and they can be either symbiotic to their sponge-host or parasitic. It is conceivable, therefore, that the Dino-Group-1-Clade-1 genus is parasitic on the sponge, and their abundance in yellow *H. panicea* resulted in a decrease in the abundance and diversity of the bacterial community, including cyanobacteria (Appendix D - Figure A & B). Moreover, the golden-brown pigmentation of the Dino-Group-1-Clade-1 genus contributed to the yellow colouration of the sponge. Indeed, some dinoflagellate species (mixotrophic or heterotrophic) are capable of feeding on bacteria, including cyanobacteria (Jeong *et al*., 2010; Jeong *et al*., 2005).

Higher microbial diversity and functional redundancy are linked to greater community stability, which is vital in obligate symbiotic systems like sponge holobionts (Allison & Martiny, 2008; Girvan *et al*., 2005; Robinson *et al*., 2010). This specialization results in limited functional redundancy, meaning that the lower relative abundance of key microbial symbionts—such as those observed in the yellow sponges—cannot be easily compensated for by other species (Allison & Martiny, 2008; Mao-Jones *et al.*, 2010). Consequently, the yellow morph's reduced microbial diversity and functionality could make these sponges more vulnerable to environmental stress, reflecting a compromised or unstable state. Therefore, the shift observed in the yellow sponges

aligns with the understanding that obligate symbiotic systems are particularly sensitive to environmental perturbations, further supporting the conclusion that the yellow color morph is indicative of a sponge under stress. This conclusion is supported by Posadas *et al*. (2022) study on *Leucetta chagosensis* sponge, where they found that the sponges under acidification and warming stress will have a less diverse microbiome and consequently lower predicted functional redundancy.

4.5 Conclusion and Future study

This study investigated the diversity and abundance of bacterial and eukaryotic communities in *H. panicea* of different colour morphs using 16S and 18S rRNA metabarcoding techniques to examine whether the microbiome plays a role in the colour morphs. The difference in colour was found to be due to certain eukaryotic microalgae and cyanobacteria. The presence of cyanobacteria along with microalgal genera such as Pycnococcaceae_Unclassified, *Ostreobium, Acrochaete*, and *Picochlorum* in green sponges contributed to their colouration. In contrast, yellow sponges contained fewer cyanobacteria and showed a high abundance of the Dino-Group-I-Clade-I genus. The golden-brown pigments of this genus strongly influenced the sponge's colour. The yellow colour may also be the result of parasitic behaviour associated with the Dino-Group-I-Clade-I genus, which is known to cause diseases in marine organisms. This is supported by the lower dominance of bacteria, cyanobacteria, and eukaryotic communities in yellow sponges as compared to green sponges, as well as lower abundances of predicted functions of the microbial communities. A more complete understanding of differences between the colour morphs would require a consideration of other organisms, such as fungi. Additionally, the use of different 18S rRNA primers and different regions (like V9) will help in providing a better understanding of the diversity of eukaryotes in both colour morphs.

Chapter 5. The Effect of Antifouling Paints on the Microbial Community of the Sponge *Halichondria panicea*

5.1 Introduction

Marine biofouling is the accumulation of microbes, plants, and aquatic animals on artificial surfaces submerged in seawater (Abitbol *et al*., 2020; Selim *et al*., 2017; Yebra *et al*., 2004). Biofouling causes several problems on ships, including increased fuel consumption due to water resistance and weight gain; in aquaculture, it decreases water exchange through the net mesh (Lindholdt *et al*., 2015; Papadopoulos *et al*., 2023). Therefore, antifouling paints have been developed to prevent fouling organisms from attaching to underwater surfaces (Koutsaftis & Aoyama, 2007; Sadan *et al*., 2022). Antifouling paints usually contain biocides to minimise the settlement and development of fouling organisms. Consequently, biocides are released into aquatic environments, and may adversely affect non-target organisms and compromise ecosystems (Dafforn *et al*., 2011; Paz-Villarraga *et al*., 2022). The main biocide in antifouling paints is copper oxide (Paz-Villarraga *et al*., 2022).

Copper is an essential trace element, important to many enzymatic reactions, but in excess is toxic (Kong, 2022). The ability to regulate the bioavailability of copper, through for example, metallothioneins and inorganic granules, is important to its detoxification (Calvo *et al*., 2017). Evidence of this essential process has been obtained for many organisms and may lead to the bioaccumulation of high tissue concentrations (de Almeida Rodrigues *et al*., 2022; Cai & Wang, 2019; Chen *et al*., 2017; Li, Tsim, *et al*., 2021). The bioaccumulation of copper can also pose a health risk at higher trophic levels.

Several studies have been conducted on marine sponges to study the effect of copper pollution on sponge health and the associated microbiome. An apparent decrease in the symbiotic autotrophic bacterial community was observed in *Haliclona cymaeformis,* where the heterotrophs and bacteriophage communities increased significantly, causing damage to sponge cells (Tian *et al.,* 2014). The sponge *Rhopaloeides odorabile* showed a decline in diversity and density of its total microbial community after exposure to copper ion treatment (Cu^{2+}), particularly for highly tolerant bacteria (Webster *et al*., 2001). Copper oxide and copper sulphate inhibited sponge phagocytosis and antioxidative defence responses when tested on *Eunapius carteri* for 14 days. Exposure to

copper oxide nanoparticles increased the generation of reactive oxygen species. In both cases, lysozyme and phosphatase activity were decreased, and membrane stability was reduced (Mukherjee *et al*., 2021).

To reduce coating erosion, most companies add zinc oxide (ZnO) to antifouling paints (Perelshtein *et al*., 2013; Watermann *et al*., 2005). The release of Zn from antifouling paints can have toxic effects on organisms (Karlsson *et al*., 2010; Muller-Karanassos *et al*., 2021; Ytreberg *et al*., 2010), even though it is not classified as an active substance in the EU Biocidal Products Regulation (BPR, Regulation (EU) 528/2012). There have been several compounds utilised to formulate antifouling paints (Almeida *et al*., 2007; Castro *et al*., 2011; Kyei *et al*., 2020), including organic and organometallic biocides (Miller *et al*., 2020; Okamura & Mieno, 2006). These booster biocides work together with the main biocide, usually copper oxide, to increase efficacy against a broader range of marine organisms, including copper-tolerant species and a broader range of targeted marine organisms (Almeida *et al*., 2007; Evans *et al*., 2000; Voulvoulis *et al*., 1999; Ytreberg *et al*., 2021).

The effect of biocidal antifouling paints on marine organisms has been studied extensively (e.g., Al Ghais *et al*., 2020; Cima & Varello, 2022; Molino *et al*., 2019; Muller-Karanassos *et al*., 2021; Soroldoni *et al*., 2020). In contrast, only a few studies have evaluated the toxicity of biocide-free antifouling paint leachates (Feng *et al*., 2012; Muller-Karanassos *et al*., 2021; Okamura *et al*., 2012; Piazza *et al*., 2018; Watermann *et al*., 2005), highlighting the inconsistent toxic effects on selected model organisms. However, no study has yet to be conducted on the impact of biocide and biocide-free antifouling paints on marine sponge microbiome. Therefore, this study aimed to determine the toxicity of biocidal and non-biocidal antifouling paints on the model marine sponge *Halichondria panicea.* Toxicity was evaluated on the sponge microbiome community using 16S rRNA metabarcoding analysis and correlated with released Cu and Zn.

5.2 Methodology

5.2.1 Sponge ex-situ cultivation

H. panicea was sampled in June 2023 from the intertidal area of Whitley Bay in the Northeast of England (55.064639, -1.450110). Whitley Bay was selected for sponge sampling based on the findings in Chapter 2, where sponge samples showed a greater diversity and bacterial

composition than those collected at other sampling locations (Cullercoats and Marsden Bay). According to the bathing water quality of Whitley Bay, which was rated as excellent, less stress resulted in healthier sponges. Sponge samples were scraped off rock using a sterile blunt knife and stored in a plastic bag with seawater from the surrounding environment. The samples were kept in an icebox for transport to the laboratory. Upon arrival, the sponge samples were cleaned of any attached marine organisms and soil particles using sterile 35 ppt artificial seawater (Tropic Marin Classic Salt, Germany). Sponge samples were identified according to spicule structure and external morphological traits (as described in Chapter 2- section 2.2.5. Sponge identification). Several attempts were made to achieve the best conditions for ex-situ cultivation for this sponge species (Table 5.1, Appendix E).

Table 5. 1. The sponge ex-situ cultivation trials.

	pH	Temperature	Salinity (ppt)	Light exposure	Oxygen	Substrate	Water exchange	Survival
Trial 1	8.2± 0.2	Room temperature (15-17℃)	30-32	12h/12h light/dark (7.88 PAR)	+ve	24-well plate	2-3 days	Failed
Trial 2	8.2± 0.2	10-11℃	30-32	Direct warm light 12h/12h light/dark (189.8 PAR)	+ve	Mesh with nylon ties	24 h	Failed
Trial 3	8.2± 0.2	12-14℃	30-32	Room light 24h light exposure (2-4 PAR)	+ve	Mesh with nylon ties	24 h	Success

After several trials, the selected method for *H. panicea* sponge ex-situ cultivation was as follows: the samples were cut into small pieces (approximately 1 cm^3) with at least 2-3 oscula in each sample. The pieces were fixed on garden mesh (20 x 20 mm) using thin nylon cable ties. The fixed sponge samples were cultivated in a 4L bucket with continuous aeration (aquarium air pump) (Figure 5.1). The physical parameters settings for the sponge cultivation were pH = 8.2 ± 0.2, salinity= 32 ± 2 ppt, light intensity= 2-4 PAR/ 24 h and temperature= 15 ± 1 ℃. The sponge

cultivation for this experiment was carried out at a slightly higher temperature to be more similar to the natural environment, based on the seawater temperature at the time of sampling (15 ℃). A multi-parameter pocket tester kit (Apera PC60-Z Smart, Shanghai San-Xin Instrumentation, China) was used to measure pH, salinity, and temperature. The light intensity was measured using a PAR meter (Quantum PAR Meter, USA). An aquarium cooler was used to keep the temperature at the desired level. For the first two days, the sponge samples were cultivated in the lab with 2 L of seawater from the sampling site, which was subsequently filtered (0.22 μm). On the third day, sponge samples were cultivated in a 1:1 mix of filtered seawater and artificial seawater (Tropic Marin Classic Salt, Germany). From the fourth day onwards, sponge samples were cultivated in 2L artificial seawater (32 ± 2 ppt) that was replaced daily (every 24 h). After 14 days of sponge cultivation, healthy samples were chosen for the antifouling paint experiment.

5.2.2 Antifouling paints testing

Two antifouling paints – biocidal and non-biocidal - were studied for their effect on *H. panicea* (Table 5.2).

Table 5. 2. Overview of the two antifouling paints and primer investigated. Information about the active substance content was collected from the safety data sheets.

Paint	Product name	Manufacturer	Colour	Active substances (% by weight)
T1 (Biocidal)	Trilux 33	International	Red	Zinc oxide (10-25) Copper thiocyanate (10-25) Pyrithione Zinc (≤ 5)
T2 (Non-biocidal)	Ecopower Cruise	HEMPEL	Red	-
-	Primocon	International	Grey	-

A trial was done to estimate the biocidal effect of the antifouling paint on sponge samples. The healthy sponge samples were divided into three buckets, each with 10-12 pieces. The first bucket served as a control, the second for the biocidal antifouling paint and the third for the biocide-free antifouling paint (Figure 5.1-A). The paints were applied to both surfaces of 10 x 10 cm^2 glass plates (Figure 5.1-C). Two layers of grey primer (Primocon- -International) were applied and

allowed to dry followed by two layers of antifouling paint (Figure 5.1-B). Primocon was used as a primer for both antifouling paints (biocidal and non-biocidal) to facilitate comparison between the effects of both paints. The biocidal antifouling paint used was Trilux 33 from International (Australia), and the biocide-free paint was Ecopower Cruise from HEMPEL (Germany). To avoid exposing the sponge to the expected initial high release of biocide, the painted glass surfaces were kept in artificial seawater for five days before being introduced to the sponge tanks. On day 15 of sponge cultivation, the painted glass surfaces with antifouling paints were added to the tanks. The experiment ended after five days of exposure to the painted plates.

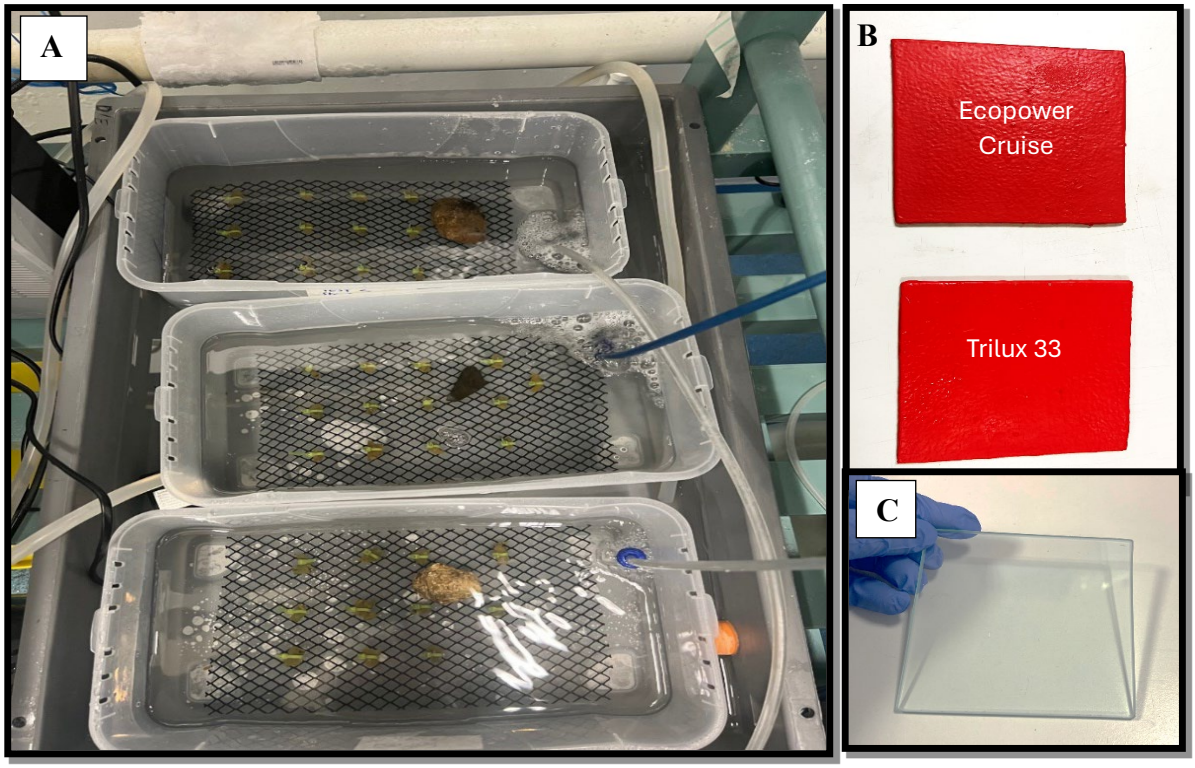


Figure 5. 1. *Halichondria panicea* sponge samples in the exposure tanks during the trial. **A**: Sponge samples in the seawater control, Test 1 and Test 2 tanks. **B**: Painted glass, Top= biocide-free antifouling paint (Ecopower Cruise), Bottom= biocidal antifouling paint (Trilux 33). **C**: 10 x10 cm glass before painting.

The conditions used for the main experiment were based on the trial results. Sponge samples were collected again in July 2023 from the exact location as for the trial (Whitley Bay). A similar cultivation method was used, except that the antifouling paint concentration introduced was changed (Figure 5.2). As part of the main experiment, the amount of paint was calculated based on

the number of days it took the sponges to die when exposed to the biocidal paint in the trial experiment. Consequently, this reduced the area of paint to be applied from 200 cm^2 to about 10 cm^2. Therefore, coatings were applied to one side of 3 x 3 cm^2 glass pieces. Two layers of the same primer were used, followed by two layers of antifouling paints. The painted surfaces were soaked in artificial seawater for five days prior to the experiment. On day 14 of sponge ex-situ cultivation (in the lab), the painted glass surfaces were introduced to the cultivation tanks and sponge samples were exposed for 21 days. The samples were then removed and stored at -20 °C for DNA extraction.

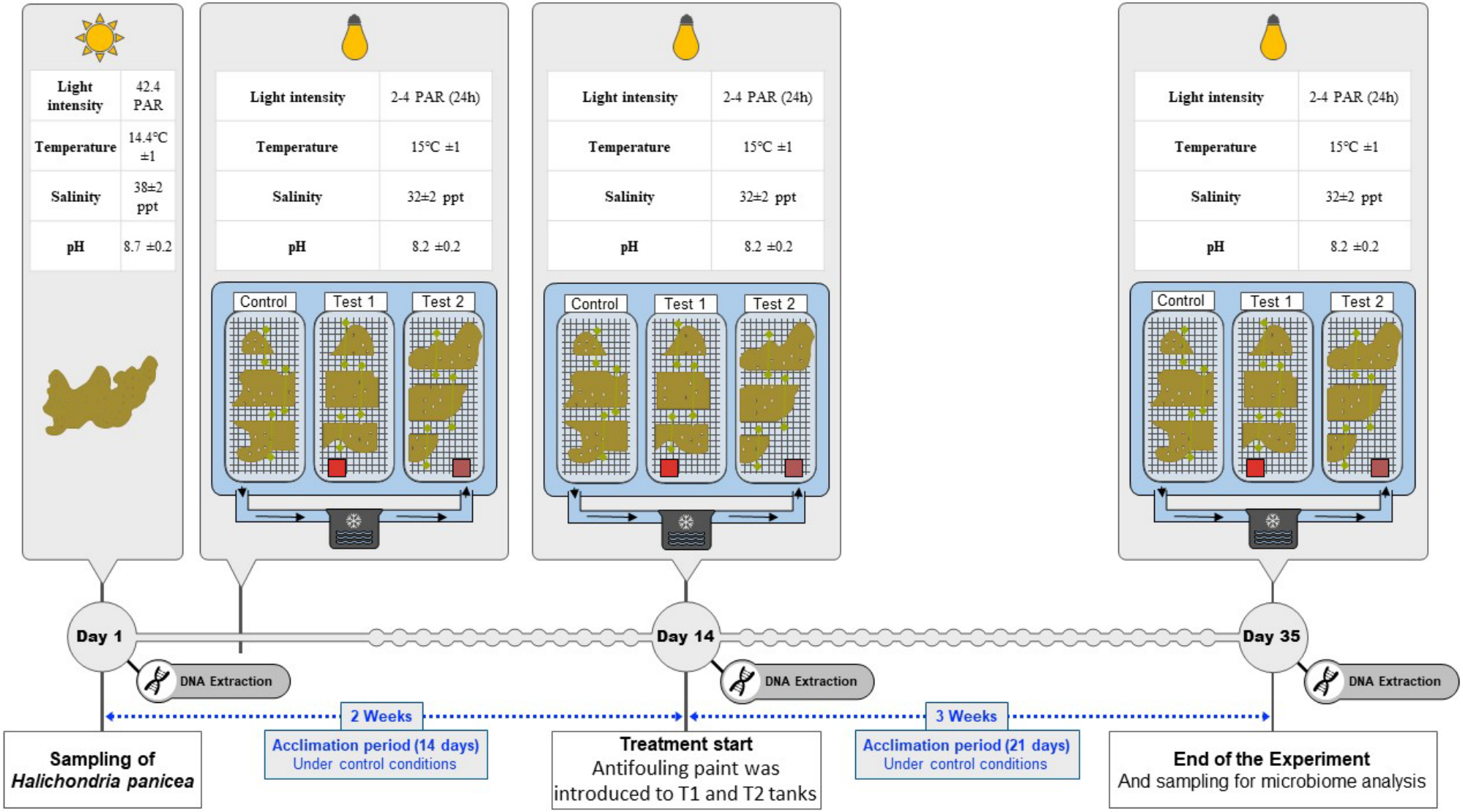


Figure 5. 2. Diagram summarising the main antifouling paint experiment on *Halichondria panicea* sponge.

5.2.3 Chemical analysis

Triplicate water samples were collected from the three tanks on days 1, 5, 10, 15 and 20. The water samples (25 mL) were filtered through sterile 0.22 µm cellulose nitrate filters (Manta Diagnostics™), acidified using 0.5 mL trace grade nitric acid per 25 mL sample, and sent for analysis to Newcastle University SAgE technical service department. The concentration of Zn and Cu in the seawater samples was analysed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). The average values of Cu and Zn concentration in T1 (biocidal antifouling paint) and T2 (non-biocidal antifouling paint) water samples were subtracted from the seawater control.

5.2.4 DNA extraction

Sponge samples were collected for DNA extraction on the day of collection (Day 0), and on day 14 and day 20 of sponge exposure to the coatings. A DNA extraction kit (DNeasy power soil Pro, Qiagen) was used as described in Chapter 2, and the samples were extracted in triplicate.

5.2.5 DNA sequencing

DNA samples were shipped to LC Sciences (Houston, Texas, USA) for sequencing. To study the V3–V4 hypervariable regions of bacterial 16S rRNA gene, primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used. PCR amplification, purification and quantification methods and kits were followed as described in Chapter 2. Sequencing was performed on NovaSeq 6000 platform, 2x250 bp (NovaSeq 6000 SP Reagent Kit, 500 cycles) at LC Sciences. Raw reads of 400–500 bp were generated with a depth of ~50,000 reads per sample.

5.2.6 Bioinformatics

The bioinformatics pipeline used for this study was accomplished using the dada2 R package version 1.26 for R version 4.2.3 (Callahan *et al*., 2016), and the method was followed as described in Chapter 2 except that the quality filtering parameters were changed according to the nature of the obtained sequences. Therefore, the used filterAndTrim command was as follows: truncLen = c(250,250), maxN = 0, minLen = 100, maxEE = 5, trimLeft = 20, truncQ = 2, and the ASVs were inferred using dada function with pooling command equals ”FALSE”.

5.2.7 Bacterial community analysis

As a next step, microbiome data was represented and analysed using the R package Phyloseq (McMurdie & Holmes, 2013). This package combined the following data into a single dataset: ASV table, taxa table and metadata table. Next, data in the phyloseq object was cleaned from empty columns, Eukaryota, Chloroplast and Archaea, using the "subset_taxa" function. Then, "prune_taxa" was used to filter taxa to a total above 0; the taxa number in sponge samples was reduced from 6249 to 6176.

The community structure profile for bacterial communities in sponge samples from different cultivation days was created using the full phyloseq data. The relative abundance of the bacterial genera was agglomerated using "tax_glom", and the tax table was melted using "psmelt" functions. A "unique" function was used to select the top 20 genera with abundances over 0.5, while genera with abundances below 0.5 were classified as "Others". The taxa data frames produced were visualised using the ggplot2 package (Wickham, 2016) as bar plots.

To prepare sponge samples for the subsequent analysis (alpha diversity analysis), they were rarefied using the "rarefy_even_depth" function to the minimum sequence depth of 49,073 bp.

5.2.8 Alpha diversity analysis

Following rarefaction, Observed richness, Chao, Shannon, and Inverse Simpson values were used for the alpha diversity analysis. The function "plot_richness" was used to estimate the difference in microbiome richness using Shannon and Simpson indices between sponges from the three experimental tanks. According to Shapiro test, the diversity metrics (Observed, Chao, Shannon, and Inverse Simpson) were not normally distributed. Consequently, differences between sample groups within these metrics were investigated through non-parametric test (Kruskal-Wallis) using the "kruskal.test" function from the stats package in R. The results were then visualised using ggplot2.

5.2.9 Venn diagram for the shared bacterial community among sponges from the antifouling paint experimental tanks.

A Venn diagram illustrates the similarities and differences between groups in a graphical form. Therefore, VennDiagram and UpSetR packages were used to compare the microbial

communities (bacterial genera) shared by the sponges from the three tanks (Control, T1 and T2). This was done using the functions "get_vennlist", "get_upset", and "venn.diagram".

5.2.10 Differential abundance for the top bacterial phyla

To detect differences between bacterial phyla among treatment and control samples, differential abundance analysis was chosen, which was conducted using the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) package in R (Lin & Peddada, 2020). Therefore, the “ancombc2” function was used, and the parameters were adjusted as follows: pairwise=TRUE, p_adj_method=”holm”, alpha= 0.05, global=FALSE, group= “tank.no”, pseudo=0, pseudo_sens=TRUE, dunnet=FALSE, struc_zero=TRUE, tax_level=”Phylum”. The produced table was prepared for the sensitivity test using the following packages: “DT” (Xie *et al*., 2023), “dplyr” (Wickham *et al*., 2023) and “Tidyverse” (Wickham *et al*., 2019). The sensitivity score and the pairwise comparison of the log fold change for the taxa abundance in the three tanks were estimated from the pseudo-count data and visualised using the ggplot2 package.

5.3 Results

The trial was terminated after five days because the sponge samples in the biocidal paint (Trilux 33) tank looked unhealthy (Figure 5.3-B). The sponge pieces were changing in colour from yellowish-green to pale yellow with black spots. Also, they were surrounded by a slime layer and their texture became soft and friable. In contrast, sponge samples in the seawater control and the non-biocidal antifouling paint (Ecopower cruise) appeared healthy.

In the main antifouling paint experiment, the sponges survived for 21 days without any obvious (or overt) disease symptoms. However, sponge shrinkage was noted in the biocidal antifouling paint tank (Figure 5.3-E) compared to other tanks (control and non-biocidal paint) (Figure 5.3-D & F respectively).

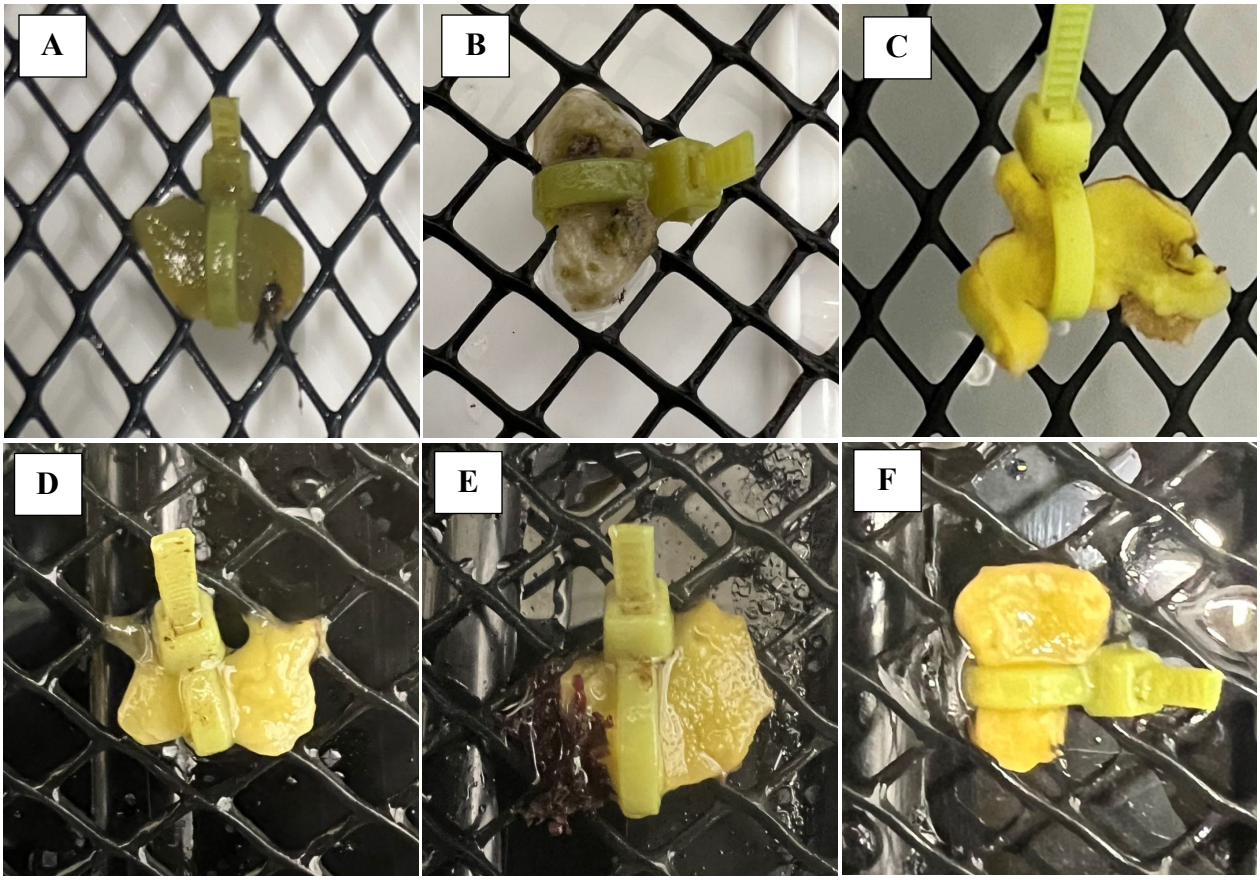


Figure 5. 3. Sponge samples at the end of the trial and the main experiment of the antifouling paint experiment. Each picture represents a sponge sample from a different tank; (**A**) Control (trial), (**B**) Biocidal antifouling paint (trial), (**C**) Non-biocidal antifouling paint (trial), (**D**) Control (main experiment), (**E**) Biocidal antifouling paint (main experiment), (**F**) Non-biocidal antifouling paint (main experiment).

5.3.1 Chemical analysis:

The concentrations of Cu and Zn in water samples collected from tank T1 (biocidal antifouling paint- Trilux 33) and tank T2 (nonbiocidal antifouling paint- Ecopower Cruise) are presented in Figures 5.4. The concentration of Cu released in tank T1 started at 0.5 ppb on day 1 and had decreased slightly by day 5 (0.46 ppb) and day 10 (0.35 ppb). On day 15, the Cu concentration showed a sudden increase to 3.94 ppb but declined sharply by day 20 (0.95 ppb). In contrast, Cu concentrations in tank T2 varied remarkably; there was an initial high release of Cu (3.46 ppb) on day 1, which decreased mid experiment - day 5 (0.04 ppb), day 10 (0.08 ppb) and day 15 (0.21 ppb), but increased again by day 20 (2.41 ppb).

The Zn concentration was the highest on day 1 in tank T2, measuring approximately 50 ppb, while in tank T1, it was highest on day 15, measuring around 14.09 ppb. From day 5 to 15, zinc concentrations for both T1 and T2 were low, the Zn concentration varied from 2.70 to 14.09 ppb in tank T1 and between 0.04 to 0.97 ppb in tank T2. A noticeable increase in Zn concentration was only observed in tank T1 on day 15 (=14.09 ppb). In summary, the concentration of the released Zn in the T1 tank was much higher than in tank T2. The highest Cu and Zn release was observed in the T1 tank on day 15, at 3.94 and 14.09 ppb, respectively. In contrast, the highest Cu and Zn release was observed in the T2 tank on day 1, at 3.46 and 50.10 ppb, respectively.

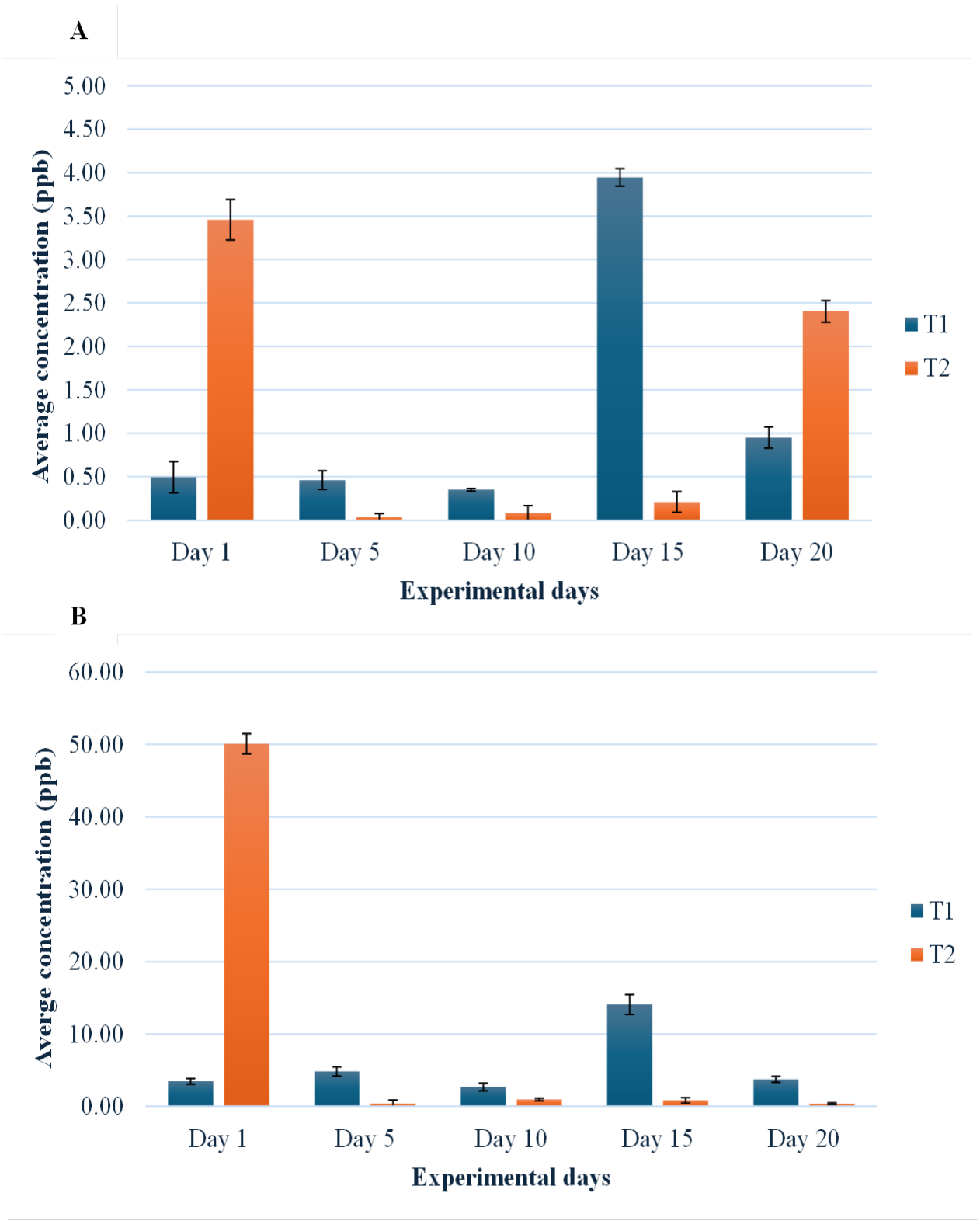


Figure 5. 4. The average concentration of copper and zinc released in tank T1 and tank T2 on Days 1, 5, 10, 15, and 20. (**A**) Copper. (**B**) Zinc. Error bars refer to standard error (n = 3).

5.3.2 Microbial analysis

The relative abundance of the top twenty bacterial genera in the sponge samples is presented in Figure 5.5. *Amylibacter* genus was highly abundant on the first sampling day (S), and the bacterial diversity was extremely low compared to the other sponge samples (B, C, T1, and T2). After 14 days of sponge ex-situ cultivation in the lab and before starting the antifouling paint experiment (B), the sponge samples showed a slight decline in bacterial community abundance accompanied by higher bacterial diversity than the sampling day (S). *Sulfitobacter, Kistimonas, Dasania* and *Aliiglaciecola* appear abundant at this stage (B). After 21 days of the start of the antifouling paints experiment (C, T1 & T2), the control sponge samples (C) showed an elevated abundance of new genera such as *Marinifilum, Shewanella, Colwellia* and *Cyclobacteriaceae_Unclassified* genera. Sponge samples in tank T1 (biocidal antifouling paint) contained 43.3% *Endozoicomonas* genus and had an abundance value of >50,000 ASVs. In contrast, *Alphaproteobacteria_Unclassified, Kistimona* and *Dasania* were among the top three genera in sponge samples from the T2 tank (non-biocidal antifouling paint).

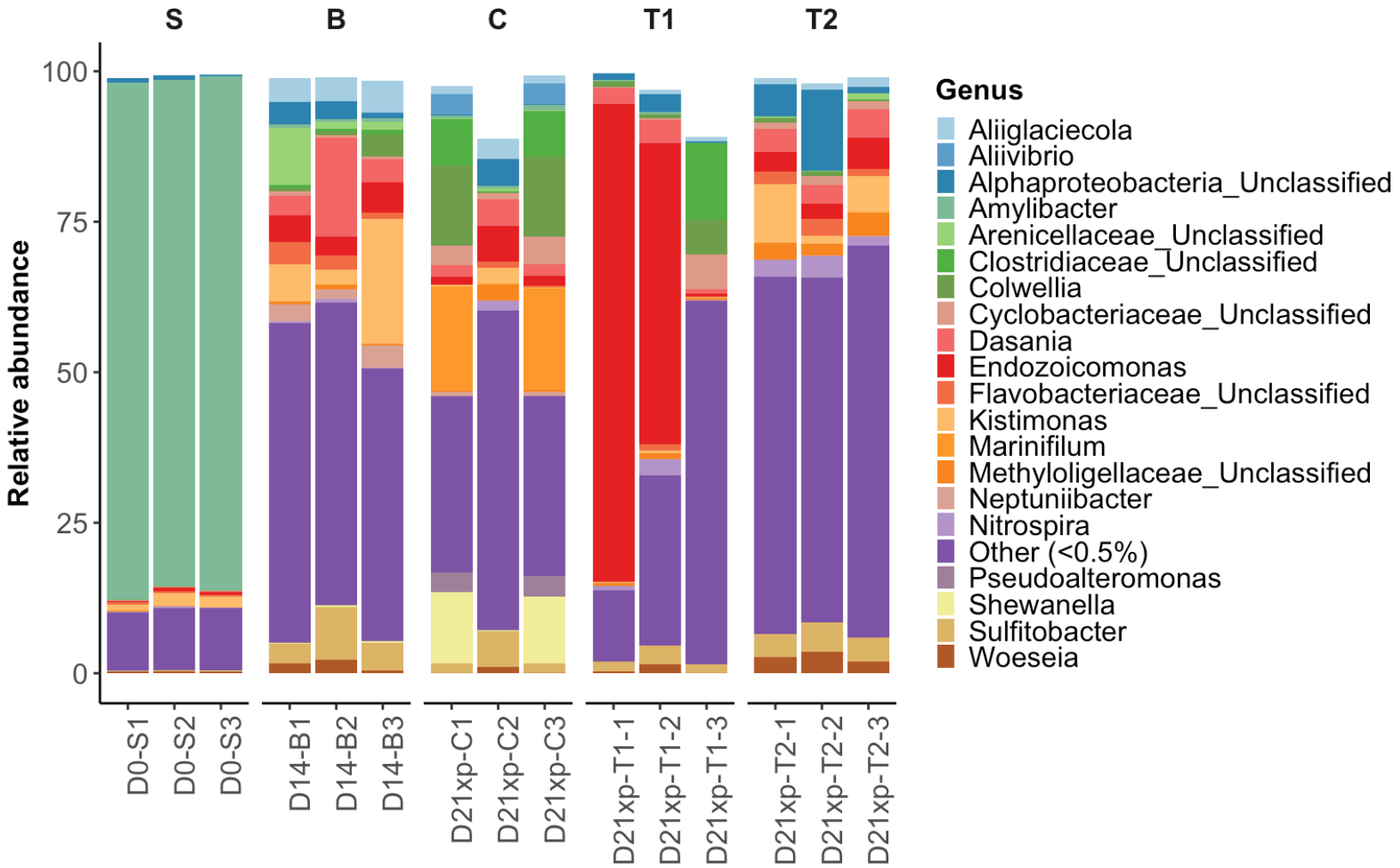


Figure 5. 5. The relative abundance of the top 20 genera in sponge samples at the start of the experiment (**S**), after 14 days of ex-situ cultivation and before starting the antifouling paints experiment (**B**), and the end of the antifouling paints experiment (21 days); Control tank (**C**), Biocidal antifouling paint tank (**T1**), nonbiocidal antifouling paint tank (**T2**).

The top five bacterial phyla of sponge samples varied throughout the experiment (Table 5.3). Proteobacteria dominated, however, its relative abundance declined over time, from 92.6% on the first day of sampling (S) to 60.6% in the control tank (C) at the end of the experiment. The relative abundance of Cyanobacteria declined after 14 days (B) of ex-situ sponge cultivation, which was evident in a change in sponge colour from dark green to yellow. Three bacterial phyla were detected in sponge samples from the three tanks (C, T1 & T2) of the antifouling paint experiment: Proteobacteria, Bacteriodota, and Firmicutes; however, their abundance varied.

Table 5. 3. The top five phyla in sponge samples throughout the experiment and from experimental tanks.

Top phyla by rank			**Tank**		
	S	**B**	**C**	**T1**	**T2**
First	Proteobacteria (92.6%)	Proteobacteria (76.9%)	Proteobacteria (60.6%)	Proteobacteria (75.4%)	Proteobacteria (68.3%)
Second	Firmicutes (3.04%)	Bacteroidota (9.26%)	Bacteroidota (19.1%)	Firmicutes (9.02%)	Bacteroidota (7.97%)
Third	Bacteroidota (1.80%)	Firmicutes (4.24%)	Firmicutes (10.3%)	Bacteroidota (5.69%)	Acidobacteriota (3.77%)
Fourth	Cyanobacteria (0.60%)	Planctomycetota (2.15%)	Campilobacterota (2.23%)	Campilobacterota (4.66%)	Planctomycetota (3.73%)
Fifth	Acidobacteriota (0.48%)	Verrucomicrobiota (1.77%)	Acidobacteriota (1.33%)	Nitrospirota (1.18%)	Firmicutes (3.46%)

A Venn diagram of the shared bacterial community (genera) between sponge samples from control, T1 and T2 tanks is presented in Figure 5.6. Sponge samples in the T1 tank had a relatively lower abundance of bacteria (435) compared to the T2 tank (512). The sponge samples from the three experiment tanks shared most of the bacterial community (288 genera). It is also worth mentioning that the control samples had a higher bacterial community abundance (505) than both T1 (435) and T2 (512) tanks. In summary, the bacterial abundance in sponge samples from antifouling paints and control tanks was ordered from highest to lowest: C>T2>T1.

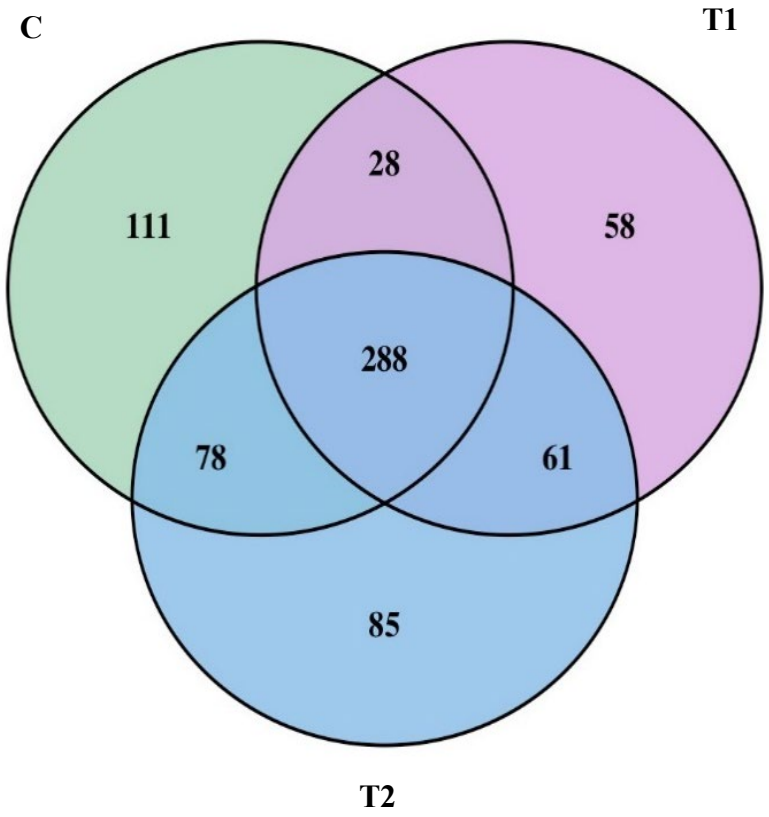


Figure 5. 6. Venn diagram of the shared bacterial genera between sponge samples in control tank (C), tank T1 (biocidal antifouling paint) and tank T2 (non-biocidal antifouling paint).

The alpha diversity analysis of sponge samples from the three experimental tanks is presented in Figure 5.7. The alpha microbial diversity in sponge samples from tank T2 was much higher than the ones from tank T1. The alpha microbial diversity in sponge samples from tank T2 was much higher than the ones from tank T1. Moreover, the microbial diversity (Shannon and Simpson) and richness (Observed and Chao1) did not differ significantly ($P > 0.05$) among sponge samples from the three tanks (C, T1 and T2). In general, the microbiome richness (Observed and

Chao1) and diversity (Shannon and Simpson) among tanks were ordered from highest to lowest as follows: T2> C> T1.

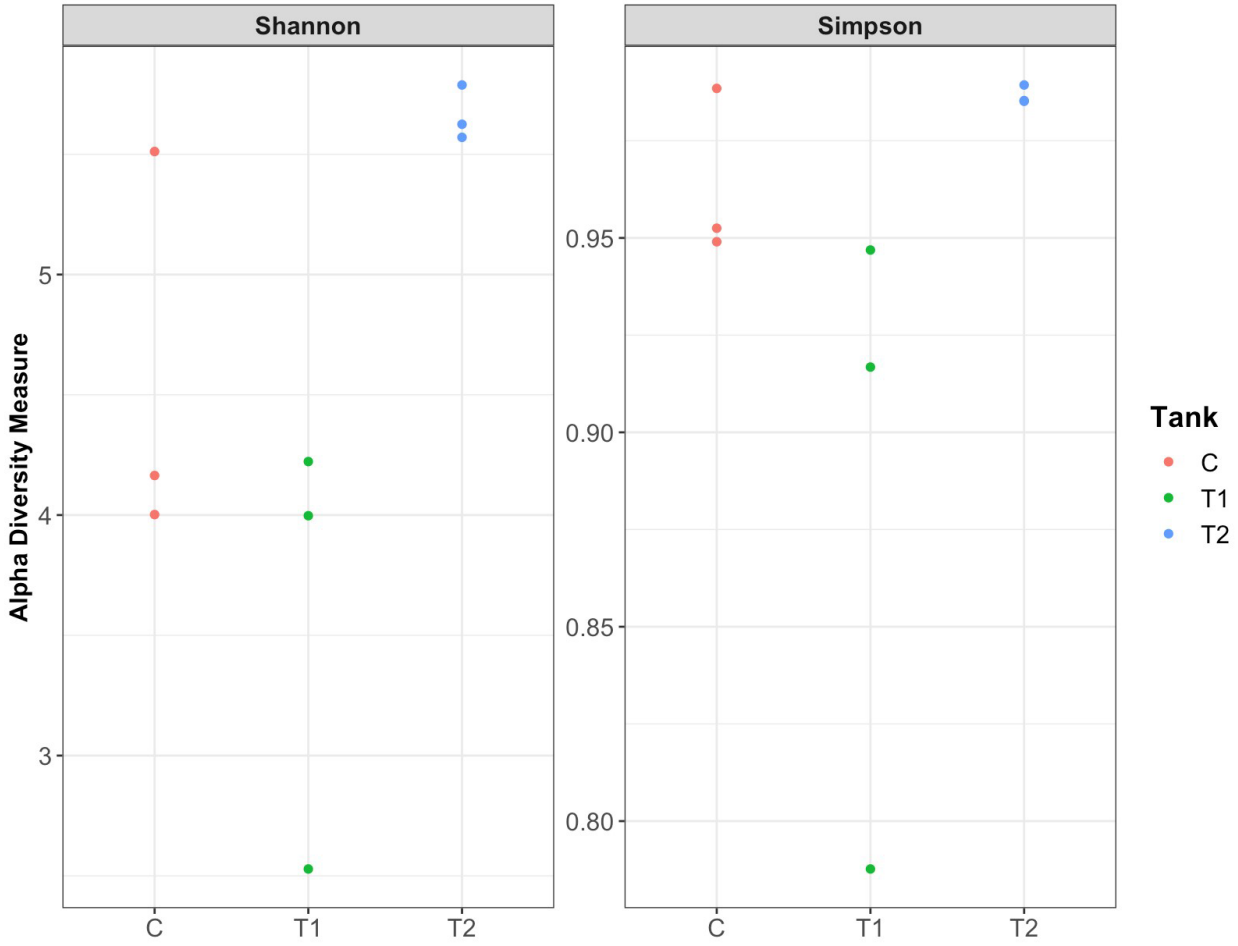


Figure 5. 7. The alpha diversity analysis of sponge microbial community from the three tanks (C, T1 and T2) using Shannon and Simpson indices.

The pairwise analysis test at the phylum level was done using the ANCOM-BC, and the outcomes are presented in Figure 5.8. There was no significant difference ($P > 0.05$) between microbial communities in sponge samples from the three tanks (C, T1, and T2). However, there was a slight change in the log fold abundance of specific bacterial phyla between the control tank versus the T1 and T2 tanks (Figure 5.9). In the C vs. T1 result, most bacterial phyla were more abundant in control tanks than in T1. Some bacteria phyla, such as Spirochaetota, Patescibacteria, Desulfobacterota, Dependentiae, Campilobacterota, and Bacteroidota, were in higher abundance

in the T1 tank. In contrast, only Spirochaetota, Firmicutes, Desulfobacterota, Campilobacterota, and Bacteroidota showed a higher abundance in the T2 tank compared to the control.

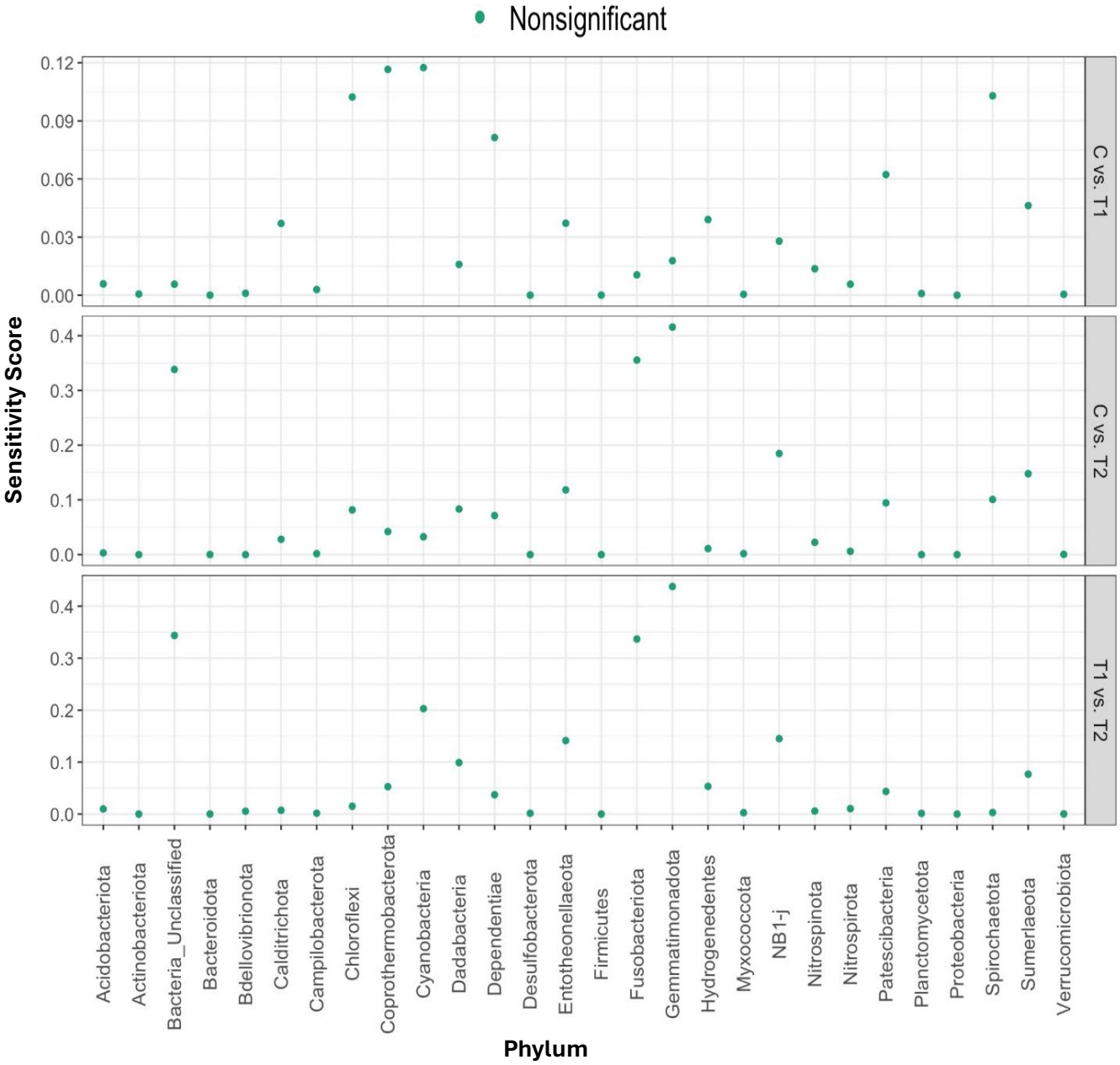


Figure 5. 8. ANCOM-BC sensitivity score test for the top thirty bacterial phyla among the three tanks (C, T1 & T2). The differential abundant test was adjusted to a P value < 0.05.

Figure 5. 9. ANCOM-BC log fold change with 95% confidence interval bars (two-sided; Holm adjusted) for the top 30 bacterial phyla compared between the control tank with T1 and T2 tanks.

5.4 Discussion

This study has provided a better understanding of the detrimental effects of released chemicals from antifouling paints on the health of the model sponge, *H. panicea*. The evidence suggests that these effects are mediated by changes in the host's microbial community.

A trial run prior to the main experiment established that the amount of the biocidal (T1- Trilux 33) antifouling paint applied to both sides of 10 x 10 cm^2 glass plates (approximately accounting for 200 cm^2 of surface area) was too high and quickly killed the sponge samples (after five days) with disease tissue appearing after only three days. This was highly related to the amount of released metals from the biocidal antifouling paint. Accordingly, a relatively small amount (approximately 10 cm^2 of surface area) of the antifouling paints was applied to only one side of 3 x 3 cm^2 glass plates for the experiment (Figure 5.2) with the aim of determining how the diversity and abundance of the sponge microbial community is affected by released Cu and Zn. The study was designed in the knowledge that the abundance of specific bacterial phyla has been reported to increase in diseased sponges (Luter *et al*., 2017).

As expected, Cu and Zn were detected in high amounts in the biocidal paint (T1) tank. The Cu concentration decreased gradually in T1 tank over the first ten days, with a marked increase by day 15; this is consistent with Lagerström *et al*. (2018), who noted a maximum release rate of Cu from the majority of the studied antifouling paints to be sometime between day 14 and 56. In contrast, the non-biocidal antifouling paint (T2) showed a maximum release of Cu and Zn on day 1. The detected Cu in the non-biocidal paint (T2) tank (Figure 5.4) is likely due to the coated primer layers, which leach Cu more readily in high salinity waters (salinity =27 ppt) (Lagerström *et al*., 2020). According to another study, Primocon primer releases other chemicals that may negatively affect native microbial diversity and abundance (von Ammon *et al*., 2018). The primer (Primocon) used for the antifouling paints was not recommended for the non-biocidal paint. However, it was used for both paints to facilitate comparison of their effects on the sponge microbiome.

Since the amount of introduced antifouling paints to the sponge samples in the main experiment was comparatively low, their effect on the sponge microbiome was not significant (Figure 5.8). The sponge microbial community was slightly affected by the released Cu and Zn from the antifouling paints, which was clearly observed in the biocidal antifouling paint tank (Figure 5.5). The effect of Cu stress has been investigated for several sponge species (Bauvais *et*

al., 2015; Cebrian *et al.,* 2006; Mukherjee *et al*., 2021; Webster *et al.,* 2001). For example, a high Cu concentration (1 mg/L) led to a decrease in the abundance of phototrophic bacteria, like cyanobacteria, and an increase in the heterotrophic species in the sponge *Haliclona cymaeformis* (Tian *et al*., 2014). The present study also observed a reduction in cyanobacterial abundance in sponge samples from the T1 treatment (Figure 5.9), which were exposed to elevated copper concentration released from antifouling paint.

In contrast to Cu, some marine sponges accumulate Zn in their tissue, and their tolerance to the Zn stress varies among species. *Crambe crambe* showed no significant difference in microbiome diversity between polluted and non-polluted environments (Gantt *et al*., 2017). Two common Mediterranean sponges (*Petrosia (Petrosia) ficiformis* and *Ircinia oros*) accumulated comparable amounts of metals in their tissues, including Cu and Zn. The microbiome community diversity and the metabolic functions were highly affected by the accumulated metals creating stress for both sponges; both sponges lost dominant ASVs and acquired a novel set of ASVs from their surrounding environment that mostly belonged to the phyla Proteobacteria, Bacteroidetes and Planctomycetes (Bulleri *et al*., 2022). Likewise, in the present study, microbiome relative abundance (Figure 5.5) and diversity (Figure 5.7) of *H. panicea* were lower after exposure to the biocidal antifouling paint (T1) compared to values for the non-biocidal antifouling paint (T2) exposure.

Some changes were noticed in the log fold abundance of the microbiome community among tanks at the phyla level (Figure 5.9). The abundance of Spirochaetota, Firmicutes, Desulfobacterota, Campilobacterota, and Bacteroidota microbial phyla was higher in both T1 and T2 tanks compared to the control. Their abundance is highly correlated with early signs of diseased sponges (Castro-Fernández *et al*., 2023; Gavriilidou *et al*., 2023; Gutleben *et al*., 2019; Luter *et al*., 2017). Spirochaetota is abundant in low microbial abundance (LMA) sponges as a critical member of their core microbiome (Alex *et al*., 2013; Matcher *et al*., 2017). As symbionts, Spirochaetota contributes to the production of secondary metabolites in their sponge hosts (Matcher *et al*., 2017). Therefore, their increased abundance in T1 and T2 tanks indicates chemical/ metal stress on these sponge samples; as microbes are known to produce secondary metabolites under stressful conditions for a variety of reasons, either to defend themselves (Yoon & Nodwell, 2014), to adapt to their surrounding environment, or to communicate with each other (Bhatnagar & Kim, 2010; Hutchinson, 2003). A slight increase in *Endozoicomonas* abundance was observed in T1 tank

sponge samples (43.3% of the top first bacterial genera). In addition to appearing as symbionts in sponges, corals, fish, molluscs, and corals (Alex & Antunes, 2019; Jensen *et al*., 2010; Neave *et al*., 2016), members of the *Endozoicomonas* have been observed to increase in abundance in sponges under heat stress (Castro-Fernández *et al*., 2023). Also, members of *Endozoicomonas* were found to cause mass mortality of king scallops (*Pecten maximus*) in south-west England in 2013 and 2014 (Cano *et al*., 2018) and sharp snout seabream fish (Katharios *et al*., 2015). Moreover, *Endozoicomonas* species were also observed in greater abundance in bleached corals from Ishikawabaru (Neave *et al*., 2016). Therefore, their abundance could reflect an early sign of a diseased state of *H. panicea* sponge under metal stress.

5.5 Conclusion and Future study

In conclusion, the studied antifouling paints were observed to negatively affect the bacterial community of *H. panicea* through the metals they released. The amount of metals released from the antifouling paints introduced in the experiment was very low in concentration (Cu_{median} = 0.5 and 0.21 ppb, Zn_{median}= 3.75 and 0.84 ppb for biocidal and non-biocidal paint, respectively), which slightly affected the sponge microbial community without showing any damage to the external structure of the sponge samples. Biocidal antifouling paint disturbed the sponge bacterial community, which appeared in lower diversity and abundance. In contrast, non-biocidal antifouling paints had a lesser impact on sponge's microbial community due to lower amounts of metals. The release of metals observed in the non-biocidal antifouling paint tank could be attributed to metal leaching from the Primocon primer that was incompatible with the paint. Despite this, most bacteria were shared between samples from the three tanks with no significant differences (Figure 5.9). In light of these results, it was found that *H. panicea* is tolerant to low concentrations of antifouling paint-released metals (Cu and Zn). Therefore, to better understand the tolerance level of *H. panicea* to these antifouling paints and their released chemicals, the incubation days should be increased to more than 21 days, or slightly increase the amount of introduced antifouling paints. Also, using a flow-through seawater system will create more realistic environmental conditions for evaluating the effect of antifouling paints on the sponges. In addition, further analysis should also consider checking the difference in the bacterial functional genes between control sponges and sponges under the stress of antifouling paints.

Chapter 6. Discussion: The Effect of the Surrounding Environment on the Breadcrumb Sponge *Halichondria panicea*

6.1 Project Synthesis

Marine sponges are important members of marine ecosystems, serving as crucial habitats and providing numerous ecological functions (Sheppard *et al*., 2017). The extent to which the microbial community associated with sponges – the microbiome - contributes to these functions, including nutrient recycling, biogeochemical activity, and host defense (Patel *et al*., 2023), is an active area of research. The sponge microbiome can be influenced by the surrounding environment (Lamb & Watts, 2023), with distinct microbial communities found in sponges from different sites (Meenatchi *et al*., 2020). Factors affecting the sponge microbiome in coastal areas include global stressors such as elevated sea surface temperature, ocean acidification, eutrophication, and poor water quality (Shore *et al.*, 2021). Local anthropogenic impacts, such as pollution from runoff, wastewater contamination, and heavy metal pollution, also play a role in shaping the sponge microbiome (Hardoim *et al*., 2023; Ramírez *et al*., 2023; Simister, Taylor, Tsai & Webster, 2012). The current study has shown that the surrounding environment plays a significant role in shaping the sponge microbiome, which affects the sponge's health and functioning, and that is brought together and discussed in this chapter.

Recent advances in metabarcoding have allowed scientists to study the effects of various coastal activities on marine sponges by studying their microbial communities (Busch *et al*., 2020; Hardoim *et al*., 2023; Shore *et al*., 2021). However, a comprehensive field study on the effect of anthropogenic activities and environmental factors in coastal areas influencing marine sponges has yet to be conducted. Hence, Chapter 2 examined the combined effect of different environmental and anthropogenic factors on the microbial community of *Halichondia panicea* over the course of a full year. The metabarcoding analysis of the V4 region of the 16S rRNA revealed significant differences in bacterial diversity and composition among sponge samples from the three locations. This indicates that pollution (measured by trace metals, nutrients, and faecal bacteria levels) and environmental factors (measured by seawater physicochemical properties) shape the sponge's microbial community. There was a noticeable seasonal shift in sponge microbiota, with some species flourishing in January and others emerging in April. In April and October, stormwater run-

off resulted in a high prevalence of faecal and coliform bacteria. Additionally, evidence of wastewater contamination was found in both sponge and seawater samples following rainy months. It was clearly shown that months with heavy rainfall and higher levels of stormwater run-off that carries high levels of contaminants to coastal marine ecosystems impacted sponges. Furthermore, bathing water quality had a significant impact on sponge microbiota. There was a greater range of intra-species dispersion in sponge microbiomes collected from sites with poor bathing water than those found in sites with excellent to good bathing water. In spite of the differences in bathing water quality between the three test locations, most of the sponge microbiomes were shared among the three sites (36%). There was a correlation between coliform and enteric bacteria in poor-quality bathing water and high levels of nutrients and trace metals, which negatively affected the diversity and composition of the sponge microbiome.

While Chapter 2 showed that the surrounding environment plays a major role in shaping the sponge microbiome, Chapter 3 investigated the origin of the sponge microbiome. In particular, where does the sponge acquire its core microbiome from? Most of the studies that have examined this question have concluded that sponges acquire their microbiomes from seawater (Busch *et al*., 2022; de Oliveira *et al*., 2020; Turon *et al*., 2018). However, organisms associated with sponges, such as seaweed, have not been investigated in this context. Therefore, in Chapter 3, the relationship between the microbial community of *H. panicea* and seawater and attached seaweeds was investigated. According to the study results, *H. panicea* acquired its microbiome horizontally from its environment. Seawater contributed 47% of the total ASVs to the microbial population of *H. panicea*, whereas 64% of the ASVs were shared between the sponge and attached seaweed. The differences in abundance and diversity of sponges and seaweed samples confirmed this as well. A distinct microbiome of *H. panicea*, OTU_1, has been traced to acquisition from the attached seaweed instead of the surrounding seawater. Indicator species analysis supported this conclusion, showing that this bacterial taxon (OTU_1, taxonomically assigned as *Amylibacter*) is an indicator species for sponge and seaweed combined. It was pointed out, however, that further analysis is required to study the ecological role of this core bacterial taxa (OTU_1) within the sponge which could provide valuable insights into how it supports the host's health and overall function.

Chapter 4 is based on observations made in different seasons. Although the two colour morphs, green and yellow, have been reported to occur in the summer and winter, respectively

(Hiscock, 2008), they were observed throughout the year in the present study. While some studies have provided evidence that the microalgal species *Microspora ficulinae* (Christensen, 1985) or *Desmodesmus* (Gorelova *et al*., 2012; Kravtsova *et al*., 2013) are responsible for the sponges' green colour, others have implicated cyanobacterial abundance (Alex *et al*., 2012; Konstantinou *et al*., 2020b; Pagliara *et al*., 2020). However, there has not been a comprehensive study using metabarcoding analysis of all chlorophyll-containing organisms in both colour morphs, which could provide the full picture of the reason for the different morphs. Consequently, this study investigated the chlorophyll concentration within the *H. panicea* sponge along with chlorophyll-containing organisms, such as cyanobacteria and eukaryotes, through 16S and 18S rRNA metabarcoding analysis. Green sponges had a higher chlorophyll concentration than yellow sponges. Additionally, the green morph exhibited higher diversity, abundance, and predicted biological functions of bacteria than the yellow morph. A higher eukaryotic abundance was observed in green sponges, along with a greater abundance of specific microalgal genera. On the other hand, yellow sponges had higher abundances of golden-brown dinoflagellates known as Dino-Group-I-Clade-I dinoflagellates. These dinoflagellates are known to parasitise marine organisms. In light of this, the yellow colour of *H. panicea* may indicate disease rather than seasonal changes.

There are a number of factors that contribute to the overflow of nutrients, metals and other contaminants into coastal environments, including wastewater from industrial, medical and agricultural activities (Quinete *et al*., 2023), severe storms, and terrestrial runoff, which, as a result, affect marine organisms (Shore *et al*., 2021). The effect of anthropogenic activities on marine organisms has been studied extensively, including antifouling paints (Al Ghais *et al*., 2020; Cima & Varello, 2022; Molino *et al*., 2019; Muller-Karanassos *et al*., 2021; Soroldoni *et al*., 2020). In contrast, a limited number of studies have examined the toxicity of biocide-free antifouling paint leachates (Feng *et al*., 2012; Muller-Karanassos *et al*., 2021; Okamura *et al.,* 2012; Piazza *et al.,* 2018; Watermann *et al.,* 2005), demonstrating inconsistent toxicity on selected model organisms. No comparable studies have been conducted on marine sponges. Thus, in Chapter 5, biocidal and non-biocidal antifouling paints were tested on *H. panicea* for their toxicity. Two levels of exposure to biocide-based (Trilux 33-International) and biocide-free (Ecopower Cruise-HEMPEL) antifouling paints were performed as a result of higher levels of antifouling paint exposure (200 cm^2 of surface area), the sponges displayed necrotic symptoms after three days; however, the non-

biocidal paints did not cause any discernible health problems (Figure 5.3). This was related to the higher release of Cu and Zn from the active substances of the biocidal antifouling paint, which were zinc oxide (ZnO: 10-25 wt%, ww) and copper thiocyanate (Cu: 10-25 wt%, ww). Conversely, sponges tolerated lower levels (approximately 10 cm^2 of surface area) of antifouling paints. In chemical analyses of water samples, copper and zinc metals were released from the biocidal paint in greater amounts, especially on the first day. In contrast, lower amounts of Zn and Cu were detected in the biocide-free antifouling paint tank that was released from the Primocon primer. The 16S rRNA metabarcoding analysis of the sponge samples was conducted after exposure to the lower levels of both antifouling paints for up to 21 days. In sponge samples exposed to biocidal antifouling paint, the bacterial community was the least abundant and diverse compared to seawater control and biocide-free samples. Moreover, at the end of the experiment, sponge samples from the three tanks (control, biocidal, and biocide-free paints), which had lower amounts of antifouling paint, did not exhibit any significant differences in bacterial communities at the top 10 phyla level. Nevertheless, the log fold abundance of bacteria in both biocidal and nonbiocidal antifouling paint tanks was higher than that of the control. These phyla included Spirochaetota, Firmicutes, Desulfobacterota, Campilobacterota, and Bacteroidota; other studies have shown a direct correlation between the abundance of these bacterial phyla and a diseased/stressed organism (Castro-Fernández *et al*., 2023; Gavriilidou *et al*., 2023; Gutleben *et al*., 2019; Luter *et al*., 2017).

6.2 Novelty of the study

There were a number of aspects covered in this project that had not been explored or were not presented in previous literature. Studies have begun to explore the effect of the surrounding environment and human activities on marine sponges and specifically on their microbiome, but a comprehensive study of a combined effect of seasonal and anthropogenic activities, including bathing water in coastal areas, has not been reported. Therefore, this study demonstrates how the surrounding environment affects the microbiome diversity and abundance of *H. panicea* sponges. According to the findings, sponge microbiomes (core and variable communities) vary depending on the quality of bathing waters. When water is clean (excellent), the sponge microbiome diversity and composition is high, and when water is polluted (poor), the reverse is true. Based on this finding, we may have an explanation for why the bacterial communities of the same sponge species are different across the globe. Accordingly, scientists should consider the season and pollution rate

in the area of sponge sampling before comparing its microbial community to that of the same species in other parts of the globe.

In this project, sponges and seaweeds in coastal areas were studied in order to understand the symbiotic relationship between them, providing evidence, for the first time, that seaweed may have a major impact on the sponge's symbiotic microbiome. Other potential contributors to the sponge microbiome should be investigated further as they will be useful for understanding how marine sponges relate to other marine organisms.

To fill in literature gaps, the differences between *H. panicea* colour morphs have been explored. A number of studies have examined *H. panicea* colour morphs and linked changes in colour to either cyanobacteria, eukaryotic algae or a variation in temperature (season). Therefore, this study investigated the three main factors - season, cyanobacteria, and microalgae - that could contribute to sponge colour morphs, along with the predicted functions of bacterial communities in both morphs. The evidence obtained here suggests that the yellow sponge colour indicates a stress state. This finding could open a new field of research to examine the differences in sponge colour morphs of the same species, which could help in predicting the early signs of sponge disease through its colour.

The effect of different concentrations of two types of antifouling paints (biocidal and biocide-free) was explored for *H. panicea* for the first time in this project. Antifouling paint toxicity was assessed by examining the diversity and abundance of the *H. panicea* sponge microbiome. The findings from this study could provide useful information for establishing regulations governing human-induced activities in marinas. Additionally, it will enable the development of new strategies for inhibiting the attachment and growth of biofouling organisms on submerged surfaces without the use of biocides or antibiotics. These strategies could prioritise surface modifications and design approaches that are effective in inhibiting biofilm formation without having any adverse effect on non-targeted organisms.

6.3 Limitations and Future Work

There are many anthropogenic activities occurring along coastlines worldwide, including urbanisation, shipping and fishing. The impact of these activities could be significant on marine ecosystems, including marine sponges, which are important habitats for a number of species. Future studies are required to assess the specific effects of anthropogenic activities on marine sponges and

coastal ecosystems in general. Further investigation in this field will enable the development of targeted conservation and management strategies to mitigate the negative impacts and promote the conservation of marine sponge communities. Coastal marine ecosystems are affected by many sources of anthropogenic activities, including oil spills and dispersants carried by winds and currents to coastal areas. The presence of this type of pollution would pose a threat to sponge communities in coastal areas by affecting their clearance rate and microbial community functions. Hence, it is necessary to assess how these contaminants affect sponge health and resilience. Moreover, plastic pollution is a serious anthropogenic issue that threatens marine organisms' survival and causes them to die. So, it is important to examine the impacts of plastics (micro and macro) on marine sponges in coastal areas in relation to the sponge microbiome and sponge functioning (pumping rate). Also, the presence of nutrient loads in coastal areas makes red tides more frequent. Thus, sponges' response to microalgae-causes red tides needs to be investigated from the perspective of their microbiome community and function. Pollution from other sources, such as medical waste, could also be seen near the coast, which is generated by illegal dumping, sewer overflow, and stormwater. Medical pollution in coastal environments has a significant impact on marine sponges, and this should be considered in light of the effects of pathogenic microorganisms, such as *Vibrio cholerae*, multidrug-resistant bacteria, and viruses, on sponge symbionts. Coastal waters are under greater stress due to urbanisation and the expansion of artificial structures. This impacts biodiversity and ecosystems, which may have physiological and behavioural consequences for the organisms that live there. In this regard, scientists should investigate the impact of urban development on the diversity and abundance of marine sponges and their microbiomes. Additionally, boating activities in marinas and ports can cause multi-source pollution, including the release of antifouling paint, bilge dumping, and ballast water. Ballast water could contain new microorganisms, such as microalgae, bacteria and viruses, from other parts of the world, which could become invasive and affect marine organisms. Consequently, it is necessary to assess the effects of these microbes on marine sponge health and functioning. Lastly, comparisons between sponges living in polluted and conserved environments should be made with regard to their metabolic genes, which can provide insight into the health and functions of sponges affected by pollution.

Marine sponges can serve as a habitat for many organisms in coastal environments. These organisms may play a role in sponge functioning, either in a symbiotic or parasitic relationship.

Consequently, there is a need for further investigation into the relationship between marine sponges and other organisms that can be found attached or living within the sponges, including polychaete worms, barnacles, crustaceans, nematodes and bivalve molluscs. Understanding the type of relationship between the sponge and other organisms requires studying their shared microbial community, including their metabolic genes and their evolutionary histories, to estimate species phylosymbiosis (the degree of similarity between the species' microbiomes with their host in a phylogenetic tree). The use of different primers to target different regions within 16S or 18S rRNA (other than V4) to study marine sponges is essential to the discovery of rare species that may play an important role in sponge functions. Further research is needed to determine which types of rocks are found along coastal areas and whether they are associated with certain sponge species. As coastal areas are made up of different types of rocks, each kind would have a different chemical composition that would support sponges with varying metal needs. The chemical composition of the rocks on which sponges can grow must be studied in order to determine whether these metals can contribute to shaping sponge microbes or fostering their symbiotic relationship.

The study provided an overview of the effect of the surrounding environment on the *H. panicea* microbiome. However, a few limitations were recorded throughout the study that were restricted to the thesis budget and the COVID-19 epidemic. The original aim was to repeat the study aspects over different years and to analyse and compare the outcomes to confirm seasonal effects on *H. panicea* sponge microbiomes; couldn't be done because of COVID. To understand the full effects of anthropogenic activities on the sponge microbiome, it was necessary to examine the full microbial community within the sponge by using eukaryotic 18S rRNA and fungi ITS primers along with the 16S rRNA primer used. By studying the sponge microbiome through transcriptomic analysis, we can better understand how symbiotic and transient bacteria contribute to its function. Focusing more on the functional analysis of sponge microbiota can help researchers better understand host-microbiome interactions and the influence of environmental factors on these interactions. As a result of this knowledge, strategies can be developed for mitigating the negative effects of environmental perturbations on sponge health and promoting the resilience of these important organisms and their associated ecosystems. Furthermore, future studies in this field can also explore the potential applications of sponge core microbiome functions in various industries, such as biotechnology and bioremediation. To understand the effect of antifouling paints on the sponge *H. panicea* in the same environment, in-situ cultivation (growing the sponge in its natural

habitat) would provide a better result; however, the lack of equipment and space to cultivate near the coast prevented us from doing so. The Newcastle University Dove marine lab aquarium (which is on the Cullercoats coast) was previously preserved for 6 months at the time I planned to start the experiment. As a limitation, there were issues with the experimental design of the antifouling paints experiment in so much as there was only a single replicate per treatment instead of three. Further, it was important to use a specific primer (Hempel Underwater Primer) that was suitable for biocide-free antifouling paint (Ecopower cruise - Hempel) rather than Primocon primer, which could provide better results when it comes to comparing the effects of biocidal antifouling paint on *H. panicea* sponge vs biocide-free antifouling paint. However, using the same primer for both paints showed both the negative effects of the biocidal antifouling paint and the possibility for the primer to release metals when coated with antifouling paint.

6.4 Conclusion

This thesis has clearly demonstrated that the surrounding environment strongly influences the microbiome of *H. panicea*, and it provides symbiotic bacteria essential to its survival and function that can be acquired from seawater or other marine organisms. Moreover, the microbiome plays a crucial role in sponge colour, with different colour morphs of *H. panicea* sponge reflecting their health state rather than their seasonal appearance. Seasonal transitions and human-induced activities influence the composition and diversity of bacterial communities within sponges. A notable difference in bacterial diversity and abundance was observed between sponges in polluted waters (poor quality bathing waters) and those in cleaner waters (excellent quality bathing waters), illustrating the adverse effects of pollution. Additionally, antifouling paint releases metals like copper and zinc that negatively impact the sponge microbiome, in turn affecting sponge health and survival. Taken together, the findings indicate a complex, interdependent relationship between the sponge and its surrounding environment within the marine ecosystem and confirm the horizontal transmission hypothesis of the sponge microbiome. Overall, understanding the factors that affect the sponge microbiome in coastal areas is important for predicting the status of coastal environments and mitigating the impacts of anthropogenic stressors.

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Appendix A

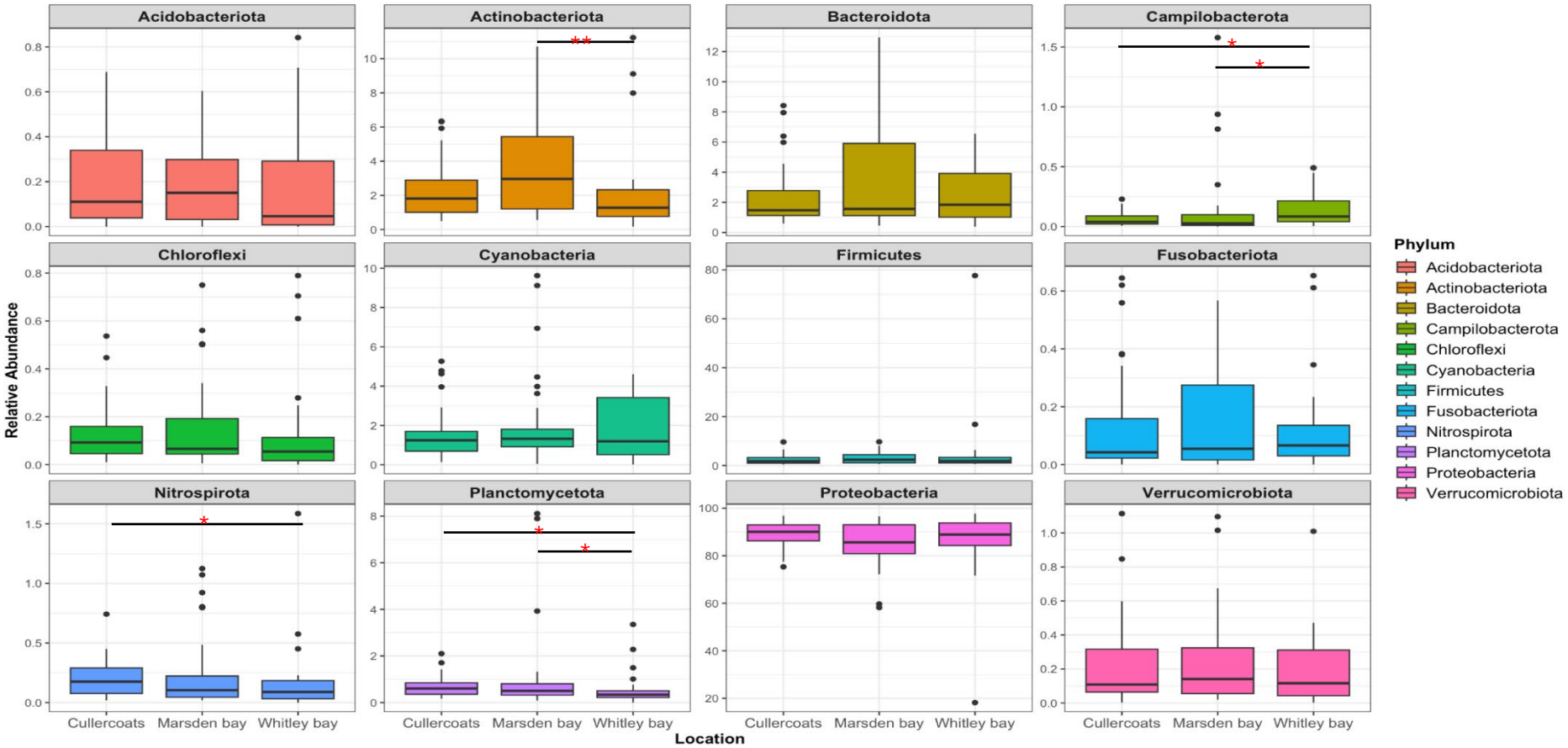


Figure A 1. Boxplot represents the relative abundance of the top bacterial phyla in sponge samples among sampling locations (Cullercoats, Marsden Bay and Whitley Bay). Red asterisks represent the significance of adjusted p-values from the Mann-Whitney test, *= $P < 0.05$, **= $P < 0.01$, *** =$P < 0.001$.

Table A 1. The percentage (relative abundance) of the top 10 bacterial phyla present in sponge samples collected during different months (January, April, July, October) from Cullercoats, Marsden Bay and Whitley Bay.

Cullercoats				
Bacterial phyla/sampling month	**January**	**April**	**July**	**October**
Proteobacteria	89.2	88.2	84.5	94.5
Bacteroidota	1.22	1.39	5.31	1.48
Cyanobacteria	1.15	1.22	2.78	1.03
Firmicutes	1.42	5.46	2.27	0.72
Actinobacteriota	4.75	2.23	1.52	0.85
Planctomycetota	0.96	0.35	0.71	0.77
Verrucomicrobiota	0.19	0.07	0.52	0.08
Acidobacteriota	0.17	0.08	0.53	0.03
Nitrospirota	0.11	0.25	0.37	0.10
Fusobacteriota	0.06	0.41	0.05	0.02
Marsden Bay				
Bacterial phyla/sampling month	**January**	**April**	**July**	**October**
Proteobacteria	90.6	84.1	80.9	84.4
Bacteroidota	1.42	1.45	8.78	2.85
Cyanobacteria	0.35	1.72	2.35	3.67
Firmicutes	1.99	6.13	2.39	1.30
Actinobacteriota	3.93	5.49	1.69	3.40

Planctomycetota	0.57	0.32	0.61	2.70
Verrucomicrobiota	0.10	0.07	0.49	0.26
Acidobacteriota	0.13	0.11	0.42	-
Nitrospirota	0.15	0.09	0.35	0.02
Fusobacteriota	0.17	0.35	0.05	0.01
Whitley Bay				
Bacterial phyla/sampling month	**January**	**April**	**July**	**October**
Proteobacteria	89.9	79.3	84.2	94.5
Bacteroidota	1.21	1.67	5.18	1.60
Cyanobacteria	0.67	1.69	3.83	1.07
Firmicutes	1.05	14.3	2.70	1.05
Actinobacteriota	4.31	1.94	1.06	0.65
Planctomycetota	0.95	0.29	0.38	0.44
Verrucomicrobiota	0.30	0.07	0.36	0.03
Acidobacteriota	0.23	0.06	0.41	0.01
Nitrospirota	0.35	0.07	0.15	0.06
Fusobacteriota	0.04	0.30	0.09	0.05

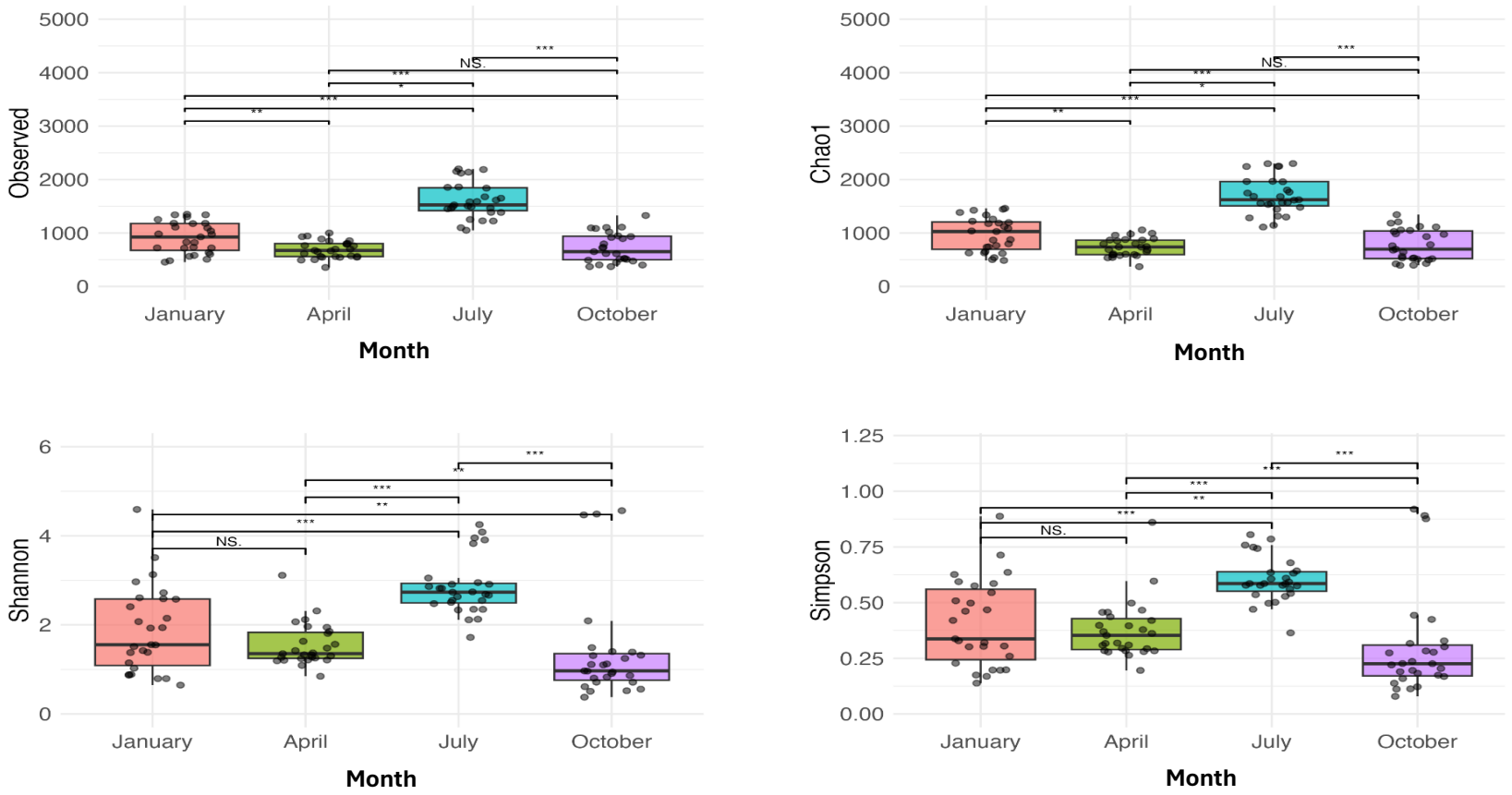


Figure A 2. Alpha diversity metrics (Observed, Chao1, Shannon and Simpson diversity index) for *H. panicea* sponge by sampling month (January, April, July, and October). Each boxplot shows the distribution of diversity metrics, with jittered points representing individual samples. Significance levels from Kruskal-Wallis tests are annotated, where *= P<0.05, **=P<0.01, ***=P<0.001, NS=not significant. The Observed and Chao1 metrics generally increase from April to July, indicating higher microbial richness during summer, while Shannon and Simpson indices show increased diversity in July, suggesting seasonal stability in microbial communities.

Table A 2. PERMANOVA values based on the Bray–Curtis distance under 999 permutations for the ASVs of sponge samples among locations on each season.

- **Global PERMANOVA (non-pairwise)**

January						
	Df	**Sums of Sqs**	**Mean Sqs**	**F. model**	**R2**	**Pr_F**
Location	2	3.5015	1.7508	10.5183	0.4671	0.001***
Residual	24	3.9948	0.1664	-	0.5329	-
Total	26	7.4963	-	-	1.000	-
April						
	Df	**Sums of Sqs**	**Mean Sqs**	**F. model**	**R2**	**Pr_F**
Location	2	0.1174	0.0587	0.2655	0.0226	0.735
Residual	23	5.0847	0.2211	-	0.9774	-
Total	25	5.2021	-	-	1.000	-
July						
	Df	**Sums of Sqs**	**Mean Sqs**	**F. model**	**R2**	**Pr_F**
Location	2	0.2140	0.1070	0.4981	0.0399	0.821
Residual	24	5.1554	0.2148	-	0.9601	-
Total	26	5.3694	-	-	1.000	-
October						
	Df	**Sums of Sqs**	**Mean Sqs**	**F. model**	**R2**	**Pr_F**
Location	2	0.3453	0.1726	0.4177	0.0383	0.875
Residual	21	8.6795	0.4133	-	0.9617	-
Total	23	9.0248	-	-	1.000	-

- **Pairwise PERMANOVA**

January			
Pairs	**t. values**	**p. values**	**p. adjusted**
Cullercoats vs Whitley Bay	14.183	0.001	0.003**
Cullercoats vs Marsden Bay	0.326	0.520	1.000
Whitley Bay vs Marsden Bay	14.008	0.002	0.006**
April			
Pairs	**t. values**	**p. values**	**p. adjusted**
Cullercoats vs Whitley Bay	0.157	0.558	1.000
Cullercoats vs Marsden Bay	0.420	0.310	0.93
Whitley Bay vs Marsden Bay	0.207	0.677	1.000
July			
Pairs	**t. values**	**p. values**	**p. adjusted**
Cullercoats vs Whitley Bay	0.423	0.576	1.000
Cullercoats vs Marsden Bay	0.491	0.598	1.000
Whitley Bay vs Marsden Bay	0.581	0.577	1.000
October			
Pairs	**t. values**	**p. values**	**p. adjusted**
Cullercoats vs Whitley Bay	0.028	0.900	1.000
Cullercoats vs Marsden Bay	0.662	0.513	1.000
Whitley Bay vs Marsden Bay	0.649	0.538	1.000

Table A 3. PERMANOVA values based on the Bray–Curtis distance under 999 permutations for the ASVs of sponge samples among seasons in each location.

- **Global PERMANOVA (non-pairwise)**

Cullercoats						
	Df	**Sums of Sqs**	**Mean Sqs**	**F. model**	**R2**	**Pr_F**
Season	3	7.002	2.334	9.906	0.489	0.001
Residual	31	7.304	0.236	-	0.511	-
Total	34	14.307	-	-	1.000	-
Marsden Bay						
	Df	**Sums of Sqs**	**Mean Sqs**	**F. model**	**R2**	**Pr_F**
Season	3	6.625	2.209	8.500	0.443	0.001
Residual	32	8.318	0.260	-	0.557	-
Total	35	14.943	-	-	1.000	-
Whitley Bay						
	Df	**Sums of Sqs**	**Mean Sqs**	**F. model**	**R2**	**Pr_F**
Season	3	6.531	2.177	7.674	0.418	0.001
Residual	32	9.078	0.284	-	0.582	-
Total	35	15.610	-	-	1.000	-

Table A 4. The percentage (relative abundance) of the top 10 bacterial phyla present in sponge samples collected during different months (January, April, July, October).

Bacterial phyla/sampling month	January	April	July	October
Proteobacteria	90	86.4	83.2	94
Bacteroidota	1.26	1.47	6.42	1.39
Cyanobacteria	0.72	1.60	2.99	1.09
Firmicutes	1.48	5.99	2.45	0.88
Actinobacteriota	4.32	3.29	1.42	0.79
Planctomycetota	0.82	0.33	0.57	0.64
Verrucomicrobiota	0.19	0.076	0.46	0.06
Acidobacteriota	0.17	0.08	0.45	0.02
Nitrospirota	0.20	0.14	0.29	0.074
Fusobacteriota	0.09	0.36	0.068	0.02

Table A 5. Global PERMANOVA values based on the Bray–Curtis distance under 999 permutations testing the influence of faecal indicator bacterial families (*Clostridiaceae, Enterobacteriaceae, Enterococcaceae* and *Pseudomonadaceae*) in sponges on the variance of sponge bacterial community composition.

	Df	Sums of Sqs	R2	F	Pr (>F)
Enterococcaceae	1	8.39	0.18	23.17	0.001***
Pseudomonadaceae	1	0.52	0.01	1.44	0.14
Enterobacteriaceae	1	0.26	0.01	0.71	0.68
Clostridiaceae	1	0.06	0.00	0.17	0.99
Residual	103	37.28	0.80	-	-
Total	107	46.51	1.00	-	-

Table A 6. Pairwise PERMANOVA values based on the Bray–Curtis distance under 999 permutations testing the influence of faecal indicator bacterial families (*Clostridiaceae, Enterobacteriaceae, Enterococcaceae* and *Pseudomonadaceae*) in sponges on the variance of sponge bacterial community composition among seasons.

January vs. April					
	Df	**Sums of Sqs**	**R2**	**F**	**Pr (>F)**
Enterococcaceae	1	7.28	0.36	29.5	0.001***
Pseudomonadaceae	1	0.56	0.03	2.25	0.04*
Enterobacteriaceae	1	0.27	0.01	1.08	0.36
Clostridiaceae	1	0.06	0.00	0.23	0.95
Residual	49	12.11	0.60	-	-
Total	53	20.26	1.00	-	-
January vs. July					
	Df	**Sums of Sqs**	**R2**	**F**	**Pr (>F)**
Pseudomonadaceae	1	0.99	0.05	2.69	0.03*

Residual	52	19.04	0.95	-	-
Total	53	20.03	1.00	-	-
January vs. October					
	Df	**Sums of Sqs**	**R2**	**F**	**Pr (>F)**
Model	0	0.000	0		
Residual	53	20.76	1.00	-	-
Total	53	20.76	1.00	-	-
October vs. April					
	Df	**Sums of Sqs**	**R2**	**F**	**Pr (>F)**
Enterococcaceae	1	6.47	0.30	21.76	0.001***
Pseudomonadaceae	1	0.48	0.02	1.62	0.08
Enterobacteriaceae	1	0.25	0.01	0.84	0.56
Clostridiaceae	1	0.06	0.00	0.19	0.99
Residual	49	14.56	0.67	-	-
Total	53	21.81	1.00	-	-
October vs. July					
	Df	**Sums of Sqs**	**R2**	**F**	**Pr (>F)**
Pseudomonadaceae	1	0.76	0.04	1.91	0.06
Residual	52	20.81	0.96	-	-
Total	53	21.58	1.00	-	-
April vs. July					
	Df	**Sums of Sqs**	**R2**	**F**	**Pr (>F)**
Enterococcaceae	1	8.19	0.44	43.09	0.001***
Pseudomonadaceae	1	0.58	0.03	3.04	0.02*
Enterobacteriaceae	1	0.30	0.02	1.56	0.20
Clostridiaceae	1	0.06	0.00	0.31	0.79

Residual	49	9.32	0.51	-	-
Total	53	18.45	1.00	-	-

Appendix B

Table B 1. Information about sponge (*H. panicea*), seawater and seaweed samples collected during the year 2022 from North-east England coastal waters (Newcastle Upon Tyne).

Sample code	Sampling month	Location	Sample type	Sample number	Sample replicate
1-C-alg-1-1	January	Cullercoats Bay	Algae	1	1
1-C-alg-1-2	January	Cullercoats Bay	Algae	1	2
1-C-alg-1-3	January	Cullercoats Bay	Algae	1	3
1-C-sp-1-1	January	Cullercoats Bay	Sponge	1	1
1-C-sp-1-2	January	Cullercoats Bay	Sponge	1	2
1-C-sp-1-3	January	Cullercoats Bay	Sponge	1	3
1-C-sp-2-1	January	Cullercoats Bay	Sponge	2	1
1-C-sp-2-2	January	Cullercoats Bay	Sponge	2	2
1-C-sp-2-3	January	Cullercoats Bay	Sponge	2	3
1-C-sp-3-1	January	Cullercoats Bay	Sponge	3	1
1-C-sp-3-2	January	Cullercoats Bay	Sponge	3	2
1-C-sp-3-3	January	Cullercoats Bay	Sponge	3	3
1-C-SW-1	January	Cullercoats Bay	Seawater	1	-
1-C-SW-2	January	Cullercoats Bay	Seawater	2	-
1-C-SW-3	January	Cullercoats Bay	Seawater	3	-
1-M-alg-1-1	January	Whitley Bay	Algae	1	1
1-M-alg-1-2	January	Whitley Bay	Algae	1	2
1-M-alg-1-3	January	Whitley Bay	Algae	1	3
1-M-alg-2-1	January	Whitley Bay	Algae	2	1
1-M-alg-2-2	January	Whitley Bay	Algae	2	2
1-M-alg-2-3	January	Whitley Bay	Algae	2	3
1-M-alg-3-1	January	Whitley Bay	Algae	3	1
1-M-alg-3-2	January	Whitley Bay	Algae	3	2
1-M-alg-3-3	January	Whitley Bay	Algae	3	3
1-M-sp-1-1	January	Whitley Bay	Sponge	1	1

Sample code	Sampling month	Location	Sample type	Sample number	Sample replicate
1-M-sp-1-2	January	Whitley Bay	Sponge	1	2
1-M-sp-1-3	January	Whitley Bay	Sponge	1	3
1-M-sp-2-1	January	Whitley Bay	Sponge	2	1
1-M-sp-2-2	January	Whitley Bay	Sponge	2	2
1-M-sp-2-3	January	Whitley Bay	Sponge	2	3
1-M-sp-3-1	January	Whitley Bay	Sponge	3	1
1-M-sp-3-2	January	Whitley Bay	Sponge	3	2
1-M-sp-3-3	January	Whitley Bay	Sponge	3	3
1-M-SW-1	January	Whitley Bay	Seawater	1	-
1-M-SW-2	January	Whitley Bay	Seawater	2	-
1-M-SW-3	January	Whitley Bay	Seawater	3	-
1-Mrs-alg-1-1	January	Marsden Bay	Algae	1	1
1-Mrs-alg-1-2	January	Marsden Bay	Algae	1	2
1-Mrs-alg-1-3	January	Marsden Bay	Algae	1	3
1-Mrs-sp-1-1	January	Marsden Bay	Sponge	1	1
1-Mrs-sp-1-2	January	Marsden Bay	Sponge	1	2
1-Mrs-sp-1-3	January	Marsden Bay	Sponge	1	3
1-Mrs-sp-2-1	January	Marsden Bay	Sponge	2	1
1-Mrs-sp-2-2	January	Marsden Bay	Sponge	2	2
1-Mrs-sp-2-3	January	Marsden Bay	Sponge	2	3
1-Mrs-sp-3-1	January	Marsden Bay	Sponge	3	1
1-Mrs-sp-3-2	January	Marsden Bay	Sponge	3	2
1-Mrs-sp-3-3	January	Marsden Bay	Sponge	3	3
1-Mrs-SW-1	January	Marsden Bay	Seawater	1	-
1-Mrs-SW-2	January	Marsden Bay	Seawater	2	-
1-Mrs-SW-3	January	Marsden Bay	Seawater	3	-
4-C-alg-1-1	April	Cullercoats Bay	Algae	1	1
4-C-alg-1-2	April	Cullercoats Bay	Algae	1	2

Sample code	Sampling month	Location	Sample type	Sample number	Sample replicate
4-C-alg-1-3	April	Cullercoats Bay	Algae	1	3
4-C-alg-3-1	April	Cullercoats Bay	Algae	3	1
4-C-alg-3-2	April	Cullercoats Bay	Algae	3	2
4-C-alg-3-3	April	Cullercoats Bay	Algae	3	3
4-C-sp-1-1	April	Cullercoats Bay	Sponge	1	1
4-C-sp-1-2	April	Cullercoats Bay	Sponge	1	2
4-C-sp-1-3	April	Cullercoats Bay	Sponge	1	3
4-C-sp-2-1	April	Cullercoats Bay	Sponge	2	1
4-C-sp-2-2	April	Cullercoats Bay	Sponge	2	2
4-C-sp-2-3	April	Cullercoats Bay	Sponge	2	3
4-C-sp-3-1	April	Cullercoats Bay	Sponge	3	1
4-C-sp-3-2	April	Cullercoats Bay	Sponge	3	2
4-C-sp-3-3	April	Cullercoats Bay	Sponge	3	3
4-C-SW-1	April	Cullercoats Bay	Seawater	1	-
4-C-SW-2	April	Cullercoats Bay	Seawater	2	-
4-C-SW-3	April	Cullercoats Bay	Seawater	3	-
4-M-alg-2-1	April	Whitley Bay	Algae	2	1
4-M-alg-2-2	April	Whitley Bay	Algae	2	2
4-M-alg-2-3	April	Whitley Bay	Algae	2	3
4-M-sp-1-1	April	Whitley Bay	Sponge	1	1
4-M-sp-1-2	April	Whitley Bay	Sponge	1	2
4-M-sp-1-3	April	Whitley Bay	Sponge	1	3
4-M-sp-2-1	April	Whitley Bay	Sponge	2	1
4-M-sp-2-2	April	Whitley Bay	Sponge	2	2
4-M-sp-2-3	April	Whitley Bay	Sponge	2	3
4-M-sp-3-1	April	Whitley Bay	Sponge	3	1
4-M-sp-3-2	April	Whitley Bay	Sponge	3	2
4-M-sp-3-3	April	Whitley Bay	Sponge	3	3

Sample code	Sampling month	Location	Sample type	Sample number	Sample replicate
4-M-SW-1	April	Whitley Bay	Seawater	1	-
4-M-SW-2	April	Whitley Bay	Seawater	2	-
4-M-SW-3	April	Whitley Bay	Seawater	3	-
4-Mrs-alg-1-1	April	Marsden Bay	Algae	1	1
4-Mrs-alg-1-2	April	Marsden Bay	Algae	1	2
4-Mrs-alg-1-3	April	Marsden Bay	Algae	1	3
4-Mrs-alg-2-1	April	Marsden Bay	Algae	2	1
4-Mrs-alg-2-2	April	Marsden Bay	Algae	2	2
4-Mrs-alg-2-3	April	Marsden Bay	Algae	2	3
4-Mrs-alg-3-1	April	Marsden Bay	Algae	3	1
4-Mrs-alg-3-2	April	Marsden Bay	Algae	3	2
4-Mrs-alg-3-3	April	Marsden Bay	Algae	3	3
4-Mrs-sp-1-1	April	Marsden Bay	Sponge	1	1
4-Mrs-sp-1-2	April	Marsden Bay	Sponge	1	2
4-Mrs-sp-1-3	April	Marsden Bay	Sponge	1	3
4-Mrs-sp-2-1	April	Marsden Bay	Sponge	2	1
4-Mrs-sp-2-2	April	Marsden Bay	Sponge	2	2
4-Mrs-sp-2-3	April	Marsden Bay	Sponge	2	3
4-Mrs-sp-3-1	April	Marsden Bay	Sponge	3	1
4-Mrs-sp-3-2	April	Marsden Bay	Sponge	3	2
4-Mrs-sp-3-3	April	Marsden Bay	Sponge	3	3
4-Mrs-SW-1	April	Marsden Bay	Seawater	1	-
4-Mrs-SW-2	April	Marsden Bay	Seawater	2	-
4-Mrs-SW-3	April	Marsden Bay	Seawater	3	-
7-C-alg-1-1	July	Cullercoats Bay	Algae	1	1
7-C-alg-1-2	July	Cullercoats Bay	Algae	1	2
7-C-alg-1-3	July	Cullercoats Bay	Algae	1	3
7-C-alg-3-1	July	Cullercoats Bay	Algae	3	1

Sample code	Sampling month	Location	Sample type	Sample number	Sample replicate
7-C-alg-3-2	July	Cullercoats Bay	Algae	3	2
7-C-alg-3-3	July	Cullercoats Bay	Algae	3	3
7-C-sp-1-1	July	Cullercoats Bay	Sponge	1	1
7-C-sp-1-2	July	Cullercoats Bay	Sponge	1	2
7-C-sp-1-3	July	Cullercoats Bay	Sponge	1	3
7-C-sp-2-1	July	Cullercoats Bay	Sponge	2	1
7-C-sp-2-2	July	Cullercoats Bay	Sponge	2	2
7-C-sp-2-3	July	Cullercoats Bay	Sponge	2	3
7-C-sp-3-1	July	Cullercoats Bay	Sponge	3	1
7-C-sp-3-2	July	Cullercoats Bay	Sponge	3	2
7-C-sp-3-3	July	Cullercoats Bay	Sponge	3	3
7-C-SW-1	July	Cullercoats Bay	Seawater	1	-
7-C-SW-2	July	Cullercoats Bay	Seawater	2	-
7-C-SW-3	July	Cullercoats Bay	Seawater	3	-
7-M-alg-1-1	July	Whitley Bay	Algae	1	1
7-M-alg-1-2	July	Whitley Bay	Algae	1	2
7-M-alg-1-3	July	Whitley Bay	Algae	1	3
7-M-sp-1-1	July	Whitley Bay	Sponge	1	1
7-M-sp-1-2	July	Whitley Bay	Sponge	1	2
7-M-sp-1-3	July	Whitley Bay	Sponge	1	3
7-M-sp-2-1	July	Whitley Bay	Sponge	2	1
7-M-sp-2-2	July	Whitley Bay	Sponge	2	2
7-M-sp-2-3	July	Whitley Bay	Sponge	2	3
7-M-sp-3-1	July	Whitley Bay	Sponge	3	1
7-M-sp-3-2	July	Whitley Bay	Sponge	3	2
7-M-sp-3-3	July	Whitley Bay	Sponge	3	3
7-M-SW-1	July	Whitley Bay	Seawater	1	-
7-M-SW-2	July	Whitley Bay	Seawater	2	-

Sample code	Sampling month	Location	Sample type	Sample number	Sample replicate
7-M-SW-3	July	Whitley Bay	Seawater	3	
7-Mrs-alg-1-1	July	Marsden Bay	Algae	1	1
7-Mrs-alg-1-2	July	Marsden Bay	Algae	1	2
7-Mrs-alg-1-3	July	Marsden Bay	Algae	1	3
7-Mrs-alg-3-1	July	Marsden Bay	Algae	3	1
7-Mrs-alg-3-2	July	Marsden Bay	Algae	3	2
7-Mrs-alg-3-3	July	Marsden Bay	Algae	3	3
7-Mrs-sp-1-1	July	Marsden Bay	Sponge	1	1
7-Mrs-sp-1-2	July	Marsden Bay	Sponge	1	2
7-Mrs-sp-1-3	July	Marsden Bay	Sponge	1	3
7-Mrs-sp-2-1	July	Marsden Bay	Sponge	2	1
7-Mrs-sp-2-2	July	Marsden Bay	Sponge	2	2
7-Mrs-sp-2-3	July	Marsden Bay	Sponge	2	3
7-Mrs-sp-3-1	July	Marsden Bay	Sponge	3	1
7-Mrs-sp-3-2	July	Marsden Bay	Sponge	3	2
7-Mrs-sp-3-3	July	Marsden Bay	Sponge	3	3
7-Mrs-SW-1	July	Marsden Bay	Seawater	1	-
7-Mrs-SW-2	July	Marsden Bay	Seawater	2	-
7-Mrs-SW-3	July	Marsden Bay	Seawater	3	-
10-C-alg-2-1	October	Cullercoats Bay	Algae	2	1
10-C-alg-2-2	October	Cullercoats Bay	Algae	2	2
10-C-alg-2-3	October	Cullercoats Bay	Algae	2	3
10-C-alg-3-1	October	Cullercoats Bay	Algae	3	1
10-C-alg-3-2	October	Cullercoats Bay	Algae	3	2
10-C-alg-3-3	October	Cullercoats Bay	Algae	3	3
10-C-sp-1-1	October	Cullercoats Bay	Sponge	1	1
10-C-sp-1-2	October	Cullercoats Bay	Sponge	1	2
10-C-sp-1-3	October	Cullercoats Bay	Sponge	1	3

Sample code	Sampling month	Location	Sample type	Sample number	Sample replicate
10-C-sp-2-1	October	Cullercoats Bay	Sponge	2	1
10-C-sp-2-2	October	Cullercoats Bay	Sponge	2	2
10-C-sp-2-3	October	Cullercoats Bay	Sponge	2	3
10-C-sp-3-1	October	Cullercoats Bay	Sponge	3	1
10-C-sp-3-2	October	Cullercoats Bay	Sponge	3	2
10-C-sp-3-3	October	Cullercoats Bay	Sponge	3	3
10-C-SW-1	October	Cullercoats Bay	Seawater	1	-
10-C-SW-2	October	Cullercoats Bay	Seawater	2	-
10-C-SW-3	October	Cullercoats Bay	Seawater	3	-
10-M-alg-1-1	October	Whitley Bay	Algae	1	1
10-M-alg-1-2	October	Whitley Bay	Algae	1	2
10-M-alg-1-3	October	Whitley Bay	Algae	1	3
10-M-alg-2-1	October	Whitley Bay	Algae	2	1
10-M-alg-2-2	October	Whitley Bay	Algae	2	2
10-M-alg-2-3	October	Whitley Bay	Algae	2	3
10-M-alg-3-1	October	Whitley Bay	Algae	3	1
10-M-alg-3-2	October	Whitley Bay	Algae	3	2
10-M-alg-3-3	October	Whitley Bay	Algae	3	3
10-M-sp-1-1	October	Whitley Bay	Sponge	1	1
10-M-sp-1-2	October	Whitley Bay	Sponge	1	2
10-M-sp-1-3	October	Whitley Bay	Sponge	1	3
10-M-sp-2-1	October	Whitley Bay	Sponge	2	1
10-M-sp-2-2	October	Whitley Bay	Sponge	2	2
10-M-sp-2-3	October	Whitley Bay	Sponge	2	3
10-M-sp-3-1	October	Whitley Bay	Sponge	3	1
10-M-sp-3-2	October	Whitley Bay	Sponge	3	2
10-M-sp-3-3	October	Whitley Bay	Sponge	3	3
10-M-SW-1	October	Whitley Bay	Seawater	1	-

Sample code	Sampling month	Location	Sample type	Sample number	Sample replicate
10-M-SW-2	October	Whitley Bay	Seawater	2	-
10-M-SW-3	October	Whitley Bay	Seawater	3	-
10-Mrs-alg-2-1	October	Marsden Bay	Algae	2	1
10-Mrs-alg-2-2	October	Marsden Bay	Algae	2	2
10-Mrs-alg-2-3	October	Marsden Bay	Algae	2	3
10-Mrs-sp-1-1	October	Marsden Bay	Sponge	1	1
10-Mrs-sp-1-2	October	Marsden Bay	Sponge	1	2
10-Mrs-sp-1-3	October	Marsden Bay	Sponge	1	3
10-Mrs-sp-2-1	October	Marsden Bay	Sponge	2	1
10-Mrs-sp-2-2	October	Marsden Bay	Sponge	2	2
10-Mrs-sp-2-3	October	Marsden Bay	Sponge	2	3
10-Mrs-sp-3-1	October	Marsden Bay	Sponge	3	1
10-Mrs-sp-3-2	October	Marsden Bay	Sponge	3	2
10-Mrs-sp-3-3	October	Marsden Bay	Sponge	3	3
10-Mrs-SW-1	October	Marsden Bay	Seawater	1	-
10-Mrs-SW-2	October	Marsden Bay	Seawater	2	-
10-Mrs-SW-3	October	Marsden Bay	Seawater	3	-

Appendix C

OTU_1 sequence:

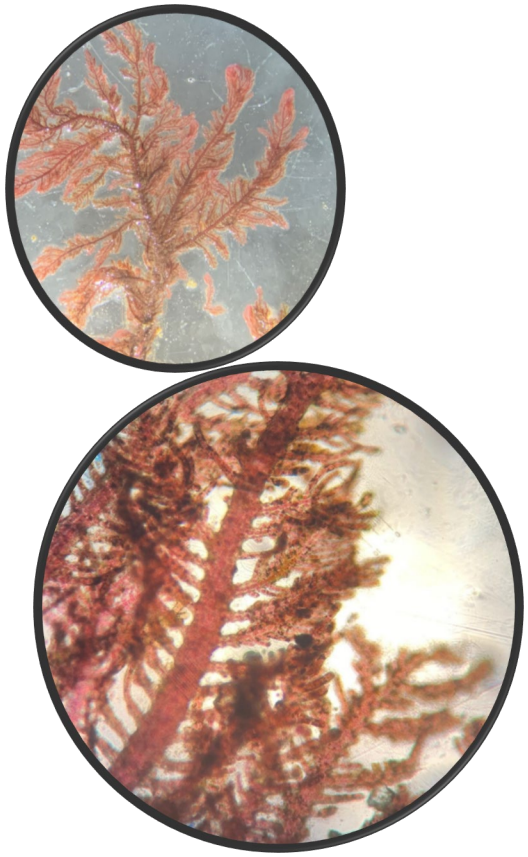
>OTU_1

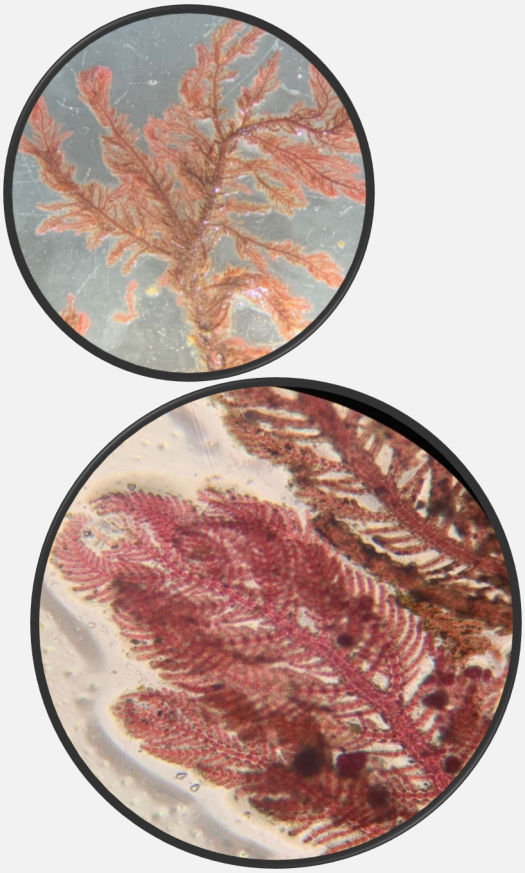
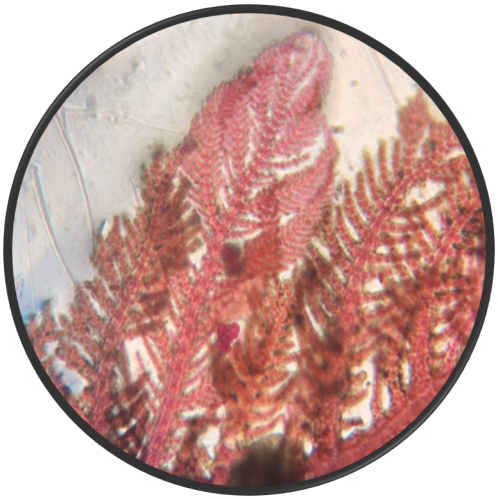
GGAATCTTGGACAATGGGGGAAACCCTGATCCAGCCATGCCGCGTGAGTGAAGAA
GGCCCTAGGGTCGTAAAACTCTTTCAACTGTGAAGATAATGACGGTAGCAGTAGAA
GAAGTCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGACTAGCGT
TGTTCGGAATTACTGGGCGTAAAGCGTACGTAGGCGGATTAGTAAGTTAGAGGTGA
AATCCCGGGGCTTAACCCCGGAACTGCCTTTAAAACTGCTAGTCTTGAGGTCGAGA
GAGGCGAGTGGAATTCCAAGTGTAGAGGTGAAATTCGTAGATATTTGGAGGAACA
CCAGCGGCGAAGGCGGCTCGCTGGCTCGATACTGACGCTGAGGTACGAAAGTGTG
GGGAGCAAACAG

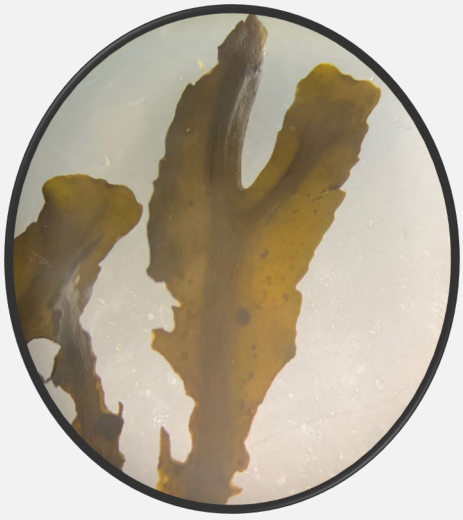
Table C 1. Information about the collected seaweed samples that were found attached to *H. panicea* samples.

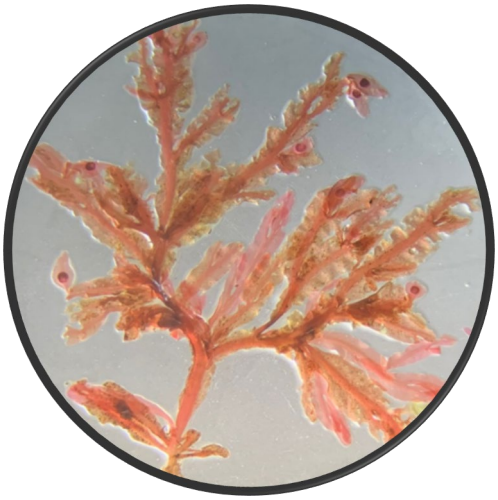
Sample code	Location	Month	Seaweed ID	Picture
1-C-alg-1	Cullercoats Bay	January	*Lomentaria articulata*	

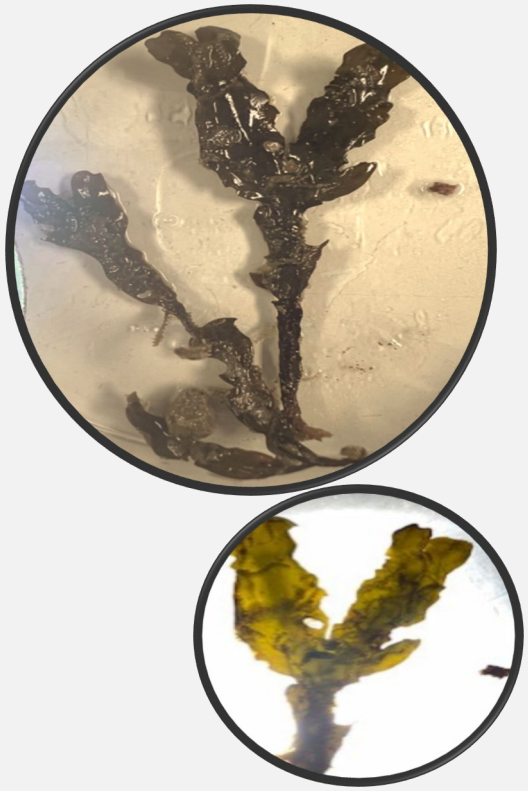
Sample code	Location	Month	Seaweed ID	Picture
1-C-alg-2	Cullercoats Bay	January	*Fucus vesiculosus*	 
1-M-alg-1	Whitley Bay	January	*Plumaria plumosa*	

Sample code	Location	Month	Seaweed ID	Picture
1-M-alg-2	Whitley Bay	January	*Plumaria plumosa*	

Sample code	Location	Month	Seaweed ID	Picture
1-M-alg-3	Whitley Bay	January	*Plumaria plumosa*	
1-Mrs-alg-1	Marsden Bay	January	*Plumaria plumosa*	

Sample code	Location	Month	Seaweed ID	Picture
4-C-alg-1	Cullercoats Bay	April	*Chondrus crispus*	
4-C-alg-3	Cullercoats Bay	April	*Chondrus crispus*	
4-M-alg-2	Whitley Bay	April	*Fucus vesiculosus*	

Sample code	Location	Month	Seaweed ID	Picture
4-Mrs-alg-1	Marsden Bay	April	*Membranoptera alata*	
4-Mrs-alg-2	Marsden Bay	April	*Plumaria plumosa*	
4-Mrs-alg-3	Marsden Bay	April	*Lomentaria articulata*	

Sample code	Location	Month	Seaweed ID	Picture
7-C-alg-1	Cullercoats Bay	July	*Fucus vesiculosus*	
7-C-alg-3	Cullercoats Bay	July	*Plumaria plumosa*	

Sample code	Location	Month	Seaweed ID	Picture
7-M-alg-1	Whitley Bay	July	*Fucus vesiculosus*	
7-Mrs-alg-1	Marsden Bay	July	*Palmaria palmata*	
10-C-alg-2	Cullercoats Bay	October	*Lomentaria articulata*	

Sample code	Location	Month	Seaweed ID	Picture
10-C-alg-3	Cullercoats Bay	October	*Lomentaria articulata*	
10-M-alg-1	Whitley Bay	October	*Chondrus crispus*	

Sample code	Location	Month	Seaweed ID	Picture
10-M-alg-2	Whitley Bay	October	*Chondrus crispus*	
10-M-alg-3	Whitley Bay	October	*Fucus vesiculosus*	

Sample code	Location	Month	Seaweed ID	Picture
10-Mrs-alg-2	Marsden Bay	October	*Lomentaria articulata*	

Appendix D

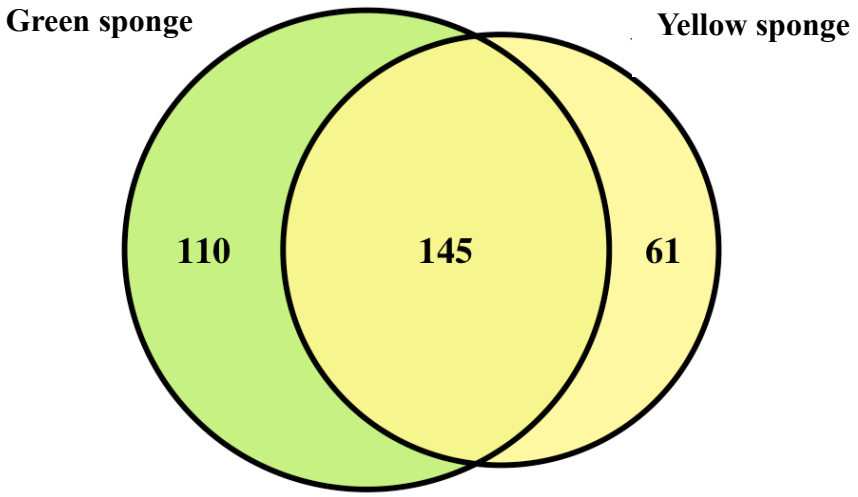


Figure D 1. Venn diagram showing the shared Cyanobacterial ASVs between green and yellow *H. panicea* sponges.

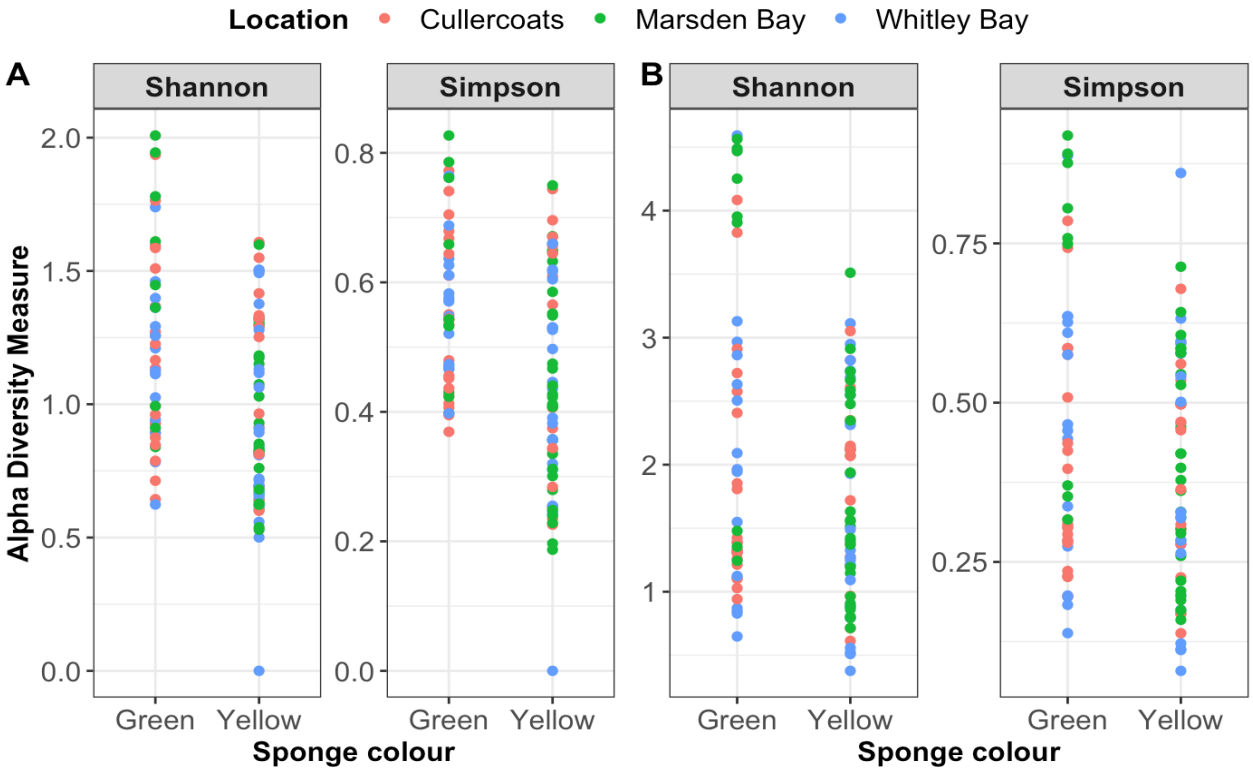
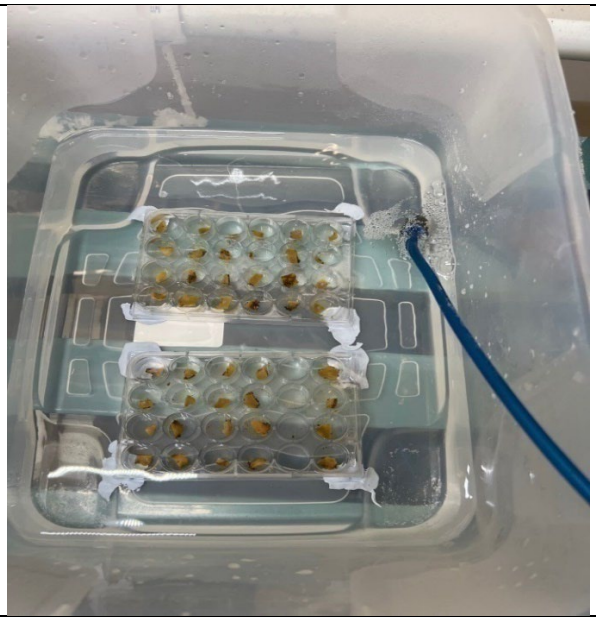


Figure D 2. The Alpha diversity analysis of sponges' microbial diversity from the three sampling locations using Shannon and Simpson diversity indexes. (A) Cyanobacterial diversity in green and yellow sponges. (B) Bacterial diversity in green and yellow sponges.

Appendix E

Table E 1. Sponge ex-situ cultivation trials of *H. panicea* sponge.

<table>
<tr><td colspan="6">Trial 1: 27th Feb- 3rd March 2023 - Failed</td></tr>
<tr><td>pH</td><td>Temperature</td><td>Salinity</td><td>Light exposure</td><td>oxygen</td><td>Water exchange</td></tr>
<tr><td>8.2±0.2</td><td>Room temperature (19-21℃)</td><td>32 ppt</td><td>Led light 12h/12h light/dark (7.88 PAR)</td><td>Oxygen prob used</td><td>Artificial seawater is exchanged every 2-3 days.</td></tr>
<tr><td colspan="6">Experiment picture:</td></tr>
<tr><td colspan="6">  </td></tr>
<tr><td colspan="6">Notes</td></tr>
<tr><td colspan="6">• Sponge pieces were kept in 24 well plates, each with 1-2 oscula.
• Sponge samples vary in colour from green to yellow.
• The sponges were first kept in seawater from the sampling location for two days. Then on the third day, ½ seawater was mixed with ½ artificial seawater. Sponges were kept in artificial seawater only on the fourth day and afterwards.
• The cultivation water (water in the aquarium) was exchanged every 2-3 days, and the pH and salinity were checked daily.</td></tr>
<tr><td colspan="6">Results</td></tr>
</table>

The experiment failed; the sponges started to turn black (diseased) after the 10th day of cultivation.

Trial 2: 8th March – 30 March 2023 – Failed

pH	Temperature	Salinity	Light exposure	oxygen	Water exchange
8.2±0.2	10-11°C (water cooler used)	30-32 ppt	Direct warm light 12h/12h light/dark (189.8 PAR)	Oxygen prob used	Artificial seawater is exchanged every 24h (daily).

Experiment picture:

Notes

- The sponge samples collected for this experiment were all green.
- Sponges were kept in natural seawater from the sampling location for the first day only.
- On the second day and afterwards, the sponges were kept in artificial seawater only.
- The aquarium water (artificial seawater) was exchanged daily (every 24 hours).
- Aquarium cooler was used to keep the water temperature at 10°C.
- The cultivated sponge samples have 2-3 oscula fixed in a plastic mesh using a plastic zipper.

Results

The experiment failed; Sponge tissue started to dissociate/ disintegrate after the 14th day of direct light exposure (189.8 PAR). And they turned pale yellow, starting from the edges and moving on to the center. It was observed that high light intensity exposure for 12 hours facilitates sponge decomposition (unfavorable condition for the sponge microbiome).

Trial 3: 8th March – 30 March 2023 (3 weeks) - Succeeded

pH	Temperature	Salinity	Light exposure	oxygen	Water exchange
8.2±0.2	12-14°C (the cold aquarium 2nd floor)	30-32 ppt	Room light 24h light exposure (2-4 PAR)	Oxygen prob used	Artificial seawater is exchanged every 24h (daily).

Experiment picture:

Notes

- The sponge samples collected for this experiment were all green. On the following day of ex-situ cultivation, the green colour faded and slowly turned yellow on the 3rd-4th day.
- Sponges were kept in natural seawater from the sampling location for the first day only.
- On the second day and afterwards, the sponges were kept in artificial seawater only.
- The aquarium water (artificial seawater) was exchanged daily (every 24 hours).
- The cultivated sponge samples have 2-3 oscula fixed in a plastic mesh using a plastic zipper.
- The aquarium water was always cold, ranging from 12-14°C according to the lab temperature.

Results

The experiment succeeded; Although sponge tissue started as green, it survived in the lab in a bright yellow colour. At the end of the experiment, sponge tissues were spongy and fresh as they were just sampled.