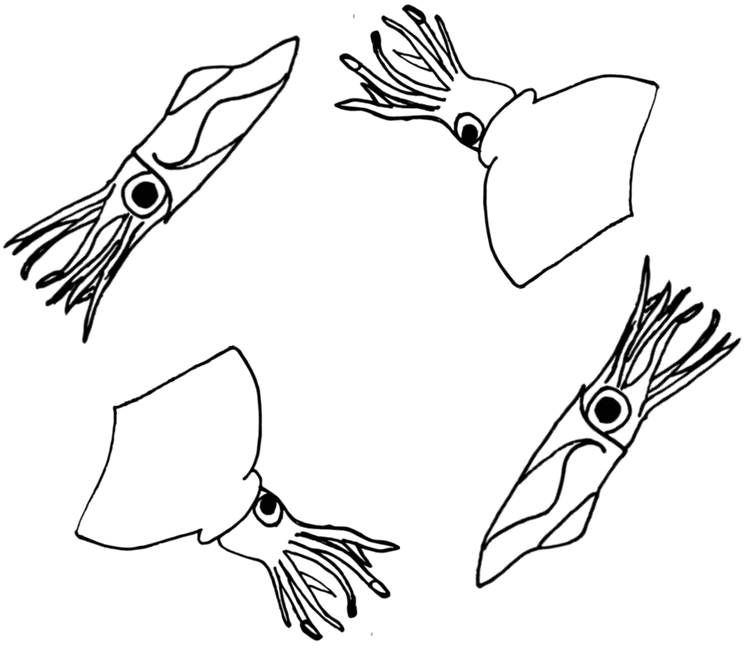
Kraken the Code: On Decoding Deep-sea Squids Via Host-associated Microbiota (Cephalopoda: Oegopsida)

Marina Maranzana

A thesis submitted to
Auckland University of Technology
In fulfilment of the requirements for the degree of
Doctor of Philosophy
2025



Faculty of Health and Environmental Science
School of Science
AUT Lab for Cephalopod Ecology and Systematics

Abstract

Microbial symbiosis with marine hosts facilitates the adaptation and survival of the host, expanding the range of habitable niches available to them in the deep ocean. Research on the microbiomes of deep-sea organisms is increasing due to their recognised importance for providing insight into the physiology, ecology, and behaviour of host organisms; however, much remains to be studied. The microbiota of deep-sea oegopsid squids is an emerging field, with the majority of oegopsid squids' microbiota still unknown. Deep-sea squids are of considerable economic, scientific, and ecological importance, yet there is a lack of data on their life histories and ecologies. This is due to the challenges of studying them *in situ* and the inaccessibility of deep-sea samples, which limits research availability. Therefore, this PhD thesis aimed to increase our limited understanding of Southern Ocean deep-sea oegopsid squids by investigating their bacterial communities for the first time using 16S rRNA gene amplicon sequencing. Nine different squid species from five distinct families belonging to the order Oegopsida were sequenced. To start, a single species was used to assess the intraspecific variability between sexes by analysing the digestive tract microbiota of five females and five males of Aotearoa's endemic Southern arrow squid, *Nototodarus sloanii*. The core microbial taxa observed were *Mycoplasma* and *Brachybacterium,* and no significant differences were observed between the sexes. When no significant differences were observed among any individuals within a species, the bacterial community composition variability among species within the same genus and between genera within the same family was investigated. No significant difference in microbial richness was observed among species or between genera; however, a significant difference in microbial beta diversity was observed between squid genera. *Mycoplasma* and *Brachybacterium* (which has not been previously reported in cephalopods) are the two most common and abundant microbial taxa found in all researched deep-sea squids. *Mycoplasma* was seen to be mainly associated with the digestive tract and beak of all squid species, and the BD1-7 clade was the most abundant bacterium in the gills of all the ommastrephid squids. The opportunistic collection of a single species, *Todarodes filippovae*, at two distinct locations (the Chatham Rise and the sub-Antarctic) enabled a preliminary description of differences in body site microbiota between the two locations within a single species. Both the Chatham Rise and sub-Antarctic digestive tract samples mainly consisted of

Brachybacterium. However, the sub-Antarctic beak samples were primarily composed of *Mycoplasma*, while the Chatham Rise beak samples were mainly made up of *Aurantivirga*. Once the intra- and inter-genus variability was established, the four ommastrephid deep-sea squids were compared with four different deep-sea oegopsid squids known for storing ammonia in their muscle tissues for buoyancy. This was done to assess variability in the microbiota among deep-sea squids with different body chemistries. A significant difference was observed between the ammoniacal and non-ammoniacal squids. Across all squids except *Onykia robsoni,* the beaks harboured similar bacterial communities but with different relative abundances. For all squid species, the same was true for the brain, inner eye fluid, and ommastrephid gills. In contrast, for all squid species except *Taningia danae*, the siphon and reproductive organs showed the greatest variation in both microbial abundance and diversity among squid taxa, although some general trends could be observed within the ammoniacal (which contained more *Mycoplasma)* and non-ammoniacal squid groupings (which had more BD1-7 clade). Lastly, due to the opportunistic collection of a rare deep-sea oegopsid squid in the Ross Sea, the colossal squid (*Mesonychoteuthis hamiltoni*), and the availability of a public dataset on NCBI on the microbiota of seawater from the Ross Sea a preliminary assessment of the transfer of microorganisms between the surrounding environment and host organism was conducted, as well as a first description of the microbiota of six body sites of the colossal squid. The bacterial genus *Pseudomonas* was the only observed taxon in both datasets; however, another bacterial taxon, *Pseudoalteromonas*, which was observed in all the Ross Sea seawater depth samples, was also observed in several deep-sea squids investigated in this thesis. *Mycoplasma*, which was seen to be the most abundant bacterium in the colossal squid, was not present in any of the seawater samples from the Ross Sea dataset. Investigating the microbiota of deep-sea squids is essential in furthering our understanding of their life history and ecology. This will also provide insights into how changes in oceanic conditions resulting from anthropogenic pressures could affect their health, as their microbial symbionts are thought to be linked to pathogen prevention and nutrient assimilation. This body of work represents the first microbiota study of cephalopods of the Southern Ocean and the first microbiota study worldwide of deep-sea squids belonging to the families: Octopoteuthidae, Onychoteuthidae, and Cranchiidae.

Contents

List of Figures

List of Tables

List of Appendices

Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor used artificial intelligence tools or generative artificial intelligence tools (unless it is clearly stated, and referenced, along with the purpose of use), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

4/04/2025

Signature
Date

Co-Authored Works

STUDENT AND SUPERVISOR APPROVALS

By signing you are confirming that the co-author contributions stated in the table(s) below a accurate.

Student Name	Marina Maranzana	**Signature**		**Date**	4/12/2025
Supervisor Name	Kat Bolstad	**Signature**		**Date**	4 Dec 2025

Chapter Number:	**Chapter 2**
Manuscript Title:	**The digestive tract microbiota of female and male *Nototodarus sloanii***
Publication Status:	**Unpublished/Ready for submission for Publication**
Reference if published:	

AUTHOR SURNAME: (order as per manuscript)	**CONTRIBUTION** (May copy from the guidelines above)
Maranzana	**Preparation and sequencing of samples, primary analysis and interpretation of results, preparation of figures, manuscript writing and editing**
Hermans	**Supervision of lab work, analysis and interpretation of results, and manuscript editing**
McClean	**Interpretation of results and manuscript editing**
Lee	**Experimental design, interpretation of results, and manuscript editing**
Bolstad	**Experimental design, collection of samples, supervision of lab work, analysis and interpretation of results, manuscript editing**

Chapter Number:	**Chapter 3**
Manuscript Title:	**Intraspecific and interspecific variability in deep-sea squid microbiota (family: Ommastrephidae)**
Publication Status:	**Unpublished/Ready for submission for Publication**
Reference if published:	
AUTHOR SURNAME: (order as per manuscript)	**CONTRIBUTION** (May copy from the guidelines above)
Maranzana	**Preparation and sequencing of samples, primary analysis and interpretation of results, preparation of figures, manuscript writing and editing**
Hermans	**Supervision of lab work, analysis and interpretation of results, and manuscript editing**
McClean	**Interpretation of results and manuscript editing**
Braid	**Manuscript editing**
Lee	**Experimental design, interpretation of results, and manuscript editing**
Bolstad	**Experimental design, collection of samples, supervision of lab work, analysis and interpretation of results, manuscript editing**

Chapter Number:	**Chapter 4**
Manuscript Title:	**The role ammoniacal vs non-ammoniacal body chemistry plays in determining the microbiota of deep-sea squids**
Publication Status:	**Unpublished/Ready for submission for Publication**
Reference if published:	
AUTHOR SURNAME: (order as per manuscript)	**CONTRIBUTION** (May copy from the guidelines above)
Maranzana	**Preparation and sequencing of samples, primary analysis and interpretation of results, preparation of figures, manuscript writing and editing**
Hermans	**Supervision of lab work, analysis and interpretation of results, and manuscript editing**
McClean	**Interpretation of results and manuscript editing**
Lee	**Experimental design, interpretation of results, and manuscript editing**
Bolstad	**Experimental design, collection of samples, supervision of lab work, analysis and interpretation of results, manuscript editing**

Chapter Number:	**Chapter 5**
Manuscript Title:	**The microbiota of the colossal squid *Mesonychoteuthis hamiltoni***
Publication Status:	**Unpublished/Ready for submission for Publication**
Reference if published:	
AUTHOR SURNAME: (order as per manuscript)	**CONTRIBUTION** (May copy from the guidelines above)
Maranzana	**Primary analysis and interpretation of results, preparation of figures, manuscript writing and editing**
Hermans	**Analysis and interpretation of results and manuscript editing**
McClean	**Interpretation of results and manuscript editing**
Lee	**Preparation and sequencing of samples, interpretation of results, and manuscript editing**
Bolstad	**Experimental design, collection of samples, supervision of lab work, analysis and interpretation of results, manuscript editing**

Acknowledgements

First and foremost, the squids for their sacrifice. This thesis would literally not have been possible without them. The squid collections were made possible with the help of all the incredible researchers and crew aboard the RV Tangaroa, as well as the indispensable help of the New Zealand Scientific Observer Programme. Thank you very much!

Much like a baby, it takes a village to complete a PhD, so thank you to my village for the love and support you have all shown me these past few years. A special shout-out goes to the following people:

A special and warm thank you to my supervisory team. Kat, thank you for answering my first email four years ago, which eventually led to me moving across the world and starting this PhD. Thank you for your advice throughout the years and your unwavering belief in me and in this project. Your enthusiasm for science and for my research has been contagious and fundamental, especially in the last few months.

Thank you, Kevin, for taking a chance on me and encouraging me to cross the world to live in Aotearoa. Thank you for picking me up at the airport and being the first friendly face, I met in Aotearoa. I appreciate you sticking with me until the end.

A special thank you to Syrie for coming in at the end to support me during arguably the toughest time during this journey. Your advice and jokes have been a lifesaver all the times I have wanted to quit. Also, a big thank you to Māika for being the best therapy dog ever and for letting me procrastinate by going on lots of walks.

Thank you, Luke, for taking the time to discuss, debate, troubleshoot and reassure me about the statistics at the end. Thank you for having the same conversation with me 100 times for my peace of mind. I could not have done it without you.

Heather, you are an incredible friend and a reassuring presence at university. I appreciate you always being available for me to rant and whine and being a solid sounding board for all my troubleshooting and last-minute doubts about life and squids. Thank you for taking the time and for your patience in helping me, and for jumping on board at the last minute for chapter three reading and editing. I could not have done it without you.

Donna, Colleen and Gardette, thank you so much for the constant advice and support throughout the past few years. Your knowledge of molecular and microbial biology has been indispensable. Thank you for giving up your precious time to explain and talk things through with me when needed.

A big and massive thank you to all the lab technicians for all of your help and sense of humour, especially Tim, Matt, and Adrian. This PhD would have literally been impossible

without you. A massive thank you to all the research groups that have made up my time at AUT: ALCES, Molecular, and Micro, for the support and feedback in all of our meetings. As well as a big special shout-out and thank you to Karen!

Thank you to all the friends I've made in the office during these three years, who allowed me to share my frustrations and double my wins. Thank you for always having the time to have tea with me both metaphorically and literally. A special thank you to Edward and Olivia for keeping me sane during the last month.

Thank you to the family of friends who have adopted me in Aotearoa and made me feel at home. Thank you for dragging me to the beach when I need it and allowing me to drag you all camping on a whim. I love you all! Thank you for putting up with me these last few years. Especially to Livvy, Levi, Julia, Marie, Ryan, and Kirsten thank you!

To my friends back home and spread across the world. Thank you for being a phone call away at any and all times. Your friendship has meant the world to me and your efforts to stay in touch and support me from a distance have truly made this journey so much easier. Special shout out to Leo, Zaven, Elly, Nicole, Meel, Jamesy, and Molly, I am forever grateful for your constant presence in my life. I love you!

Elianna and Laura, I love you, and without you, I would never have stayed and finished. You have pushed me, held me, and seen me through the worst of it. Words cannot describe how much you mean to me and how much you have helped me during this journey. I would not have wished to share it with anyone else. My fimps, I am forever grateful and in your debt.

Hannah, thank you for your never-ending encouragement, enthusiasm for my work, and your patience while I slowly lost my mind with this PhD. You have been such a safe harbour during these crazy last months. Thank you for baking with me, cooking with me, and especially for always making me laugh. Ti amo.

Alla mia famiglia: grazie di cuore per il vostro entusiasmo nei miei studi fin da quando ero piccolo. Grazie per le risate, per l'amore infinito, per il cibo e per tutte le chiacchiere. Vi voglio tantissimo bene!

Parents, grazie mille per tutto il vostro amore infinito e per aver creduto in me fin da quando, a sei anni, ho detto che volevo diventare una biologa marina… ce l'abbiamo fatta! Questa tesi è per voi e grazie a voi: al coraggio costante che mi date ogni giorno e alla vostra pazienza senza fine mentre impazzivo in questi ultimi tre anni. Il vostro sostegno infinito mi ha dato la forza di andare dall'altra parte del mondo inseguendo i miei sogni; ve ne sarò sempre riconoscente e grata. Vi voglio più che un mare di bene, e sono stra orgogliosa di essere vostra figlia. Grazie mille!

Ethics Approval

Collection of all material used in this thesis was carried out under existing permits and ethical approvals held by the Earth Sciences New Zealand, formerly known as the National Institute for Water and Atmospheric Research, Ltd (NIWA).

Chapter 1 Literature Review and Research Objectives

1.1 Introduction

Deep-sea squids are integral components of the marine food web, functioning as both predators and prey across numerous trophic levels. As with many deep-sea organisms, their study is hindered by the difficulty of obtaining samples. Their microbiota, which have been shown to increase host organisms' adaptations to challenging deep-sea environments, remain poorly understood (Osman & Weinnig, 2022). Learning about their unique microbial communities can offer exciting insights into their physiology and how they thrive in their habitats. ‘True’ squids, which this review will focus on, are classified within the order Teuthoidea and are further divided into the suborders Myopsida and Oegopsida (Clarke, 1988). Myopsid squids, typically inhabiting pelagic and demersal coastal waters, are characterised by a corneal membrane covering their eyes and the presence of accessory nidamental glands in their reproductive system (Jereb et al., 2010). This suborder comprises two families: Australiteuthidae and Loliginidae, with this review concentrating on Loliginidae, as no microbiota studies on Australiteuthidae have been conducted to date. Conversely, oegopsid squids, which inhabit the open ocean, lack a corneal membrane, and do not possess accessory nidamental glands, with some species having hooks instead of sucker rings. Oegopsids are considered the most morphologically diverse cephalopods, comprising at least 24 distinct families (Fernández-Álvarez et al., 2022).

While much of the deep sea encompasses habitats with relatively constant physical conditions (cold, stable temperatures; salinity ~35ppt), it also contains a range of localised habitats with variable and sometimes extreme conditions, such as hydrothermal vents, brine pools, and cold seeps, leading to an evolutionary need for extreme environmental microorganisms to perform specialised functions (Hongxiang et al., 2008; Osman & Weinnig, 2022). Microbe–host interactions like these are believed to confer an evolutionary advantage to the invertebrate hosts. The term “microbiota” refers to a community of microorganisms, such as fungi and bacteria, that coexist within a specific environment or body region (Lederberg & McCray, 2001). Improving our understanding of squids’ microbiota can provide valuable insights into the ecological and anatomical differences between these two suborders, allowing for a more comprehensive understanding of the order Teuthoidea. Kang et al. (2022) demonstrated that host phylogeny is a critical determinant of gut microbiome composition in marine molluscs, particularly cephalopods, with dietary and environmental factors also contributing significantly. Most deep-sea organisms harbour distinct microbial communities whose functions increase the host's ability to survive in extreme environments (Kennedy et al., 2014).

1.1.1 Importance of the invertebrate microbiota

While there is limited research on the microbiota of ‘true’ squid, some insights into the importance of microorganisms in host development can be gleaned from our knowledge of the microbiota of other, more well-characterised, invertebrates. Petersen and Osvatic (2018) demonstrated that the microbiota of invertebrates are generally less complex than those of vertebrates, thereby facilitating an equally less complex exchange between the host organism and its symbiotic microbes. Such symbiotic relationships have been observed throughout the host's lifespan, across multiple generations, and even over extended evolutionary timescales.

Invertebrates can host microbial symbionts both externally and internally, within host cells, and notably, within symbiont-specific cells and structures (Kerney et al., 2011; Petersen & Osvatic, 2018). They can acquire microbes via either vertical or horizontal transmission (Bright & Bulgheresi, 2010); however, mixed transmission modes have also been observed (Szafranski et al., 2014). Microbial symbionts can influence the morphogenesis of host organs, the development of the host immune system (Taschuk & Griebel, 2012), reproduction (Montgomery & McFall-Ngai, 1994), behaviour (Shropshire & Bordenstein, 2016), and susceptibility to certain diseases (Honda & Littman, 2012; Burgos et al., 2020). Conversely, the host immune system not only protects against microbial infections but also plays a vital role in selecting beneficial microbes and facilitating their colonisation within the host, fostering a mutualistic host–microbe relationship (Cerf-Bensussan & Gaboriau-Routhiau, 2010; Tseng et al., 2023). Microbial symbionts have been observed to evolve, speciate, and reproduce in conjunction with their hosts. These mutually beneficial interactions between hosts and their microbiota are thought to provide a distinct benefit to the host organism. For instance, deep-sea mussels (*Bathymodiolus*) have adapted to thrive under extreme conditions at hydrothermal vents and cold seeps in the deep ocean thanks to their symbiosis with horizontally acquired chemosynthetic methanotrophic Gammaproteobacteria endosymbionts in their gills (Fujiwara et al., 2000; Kellermann et al., 2012; Szafranski et al., 2014; Lasa & Romalde, 2021). Similarly, the giant mud-boring bivalve (*Kuphus polythalamia*) utilises its sulphur-oxidising chemoautotrophic symbiotic bacteria to consume sunken wood as a food source in the deep sea (Distel et al., 2017). Despite the significance of host–symbiont interactions, very little is known about the complex symbiotic relationships between most invertebrates and their microbiota (Petersen & Osvatic, 2018). However, the presence of healthy microbiota has been shown to confer important benefits to animal and plant hosts (McAnulty et al., 2023). Experimental removal and reduction of symbiotic bacteria, and sterile *in vitro* growth of host animals, can lead to developmental defects, decreased organ function, and a general decline in host health (McAnulty et al., 2023). Microbial symbionts appear crucial in shaping the behaviours of marine animals by altering the host's chemistry and, consequently, its physiology (Ezenwa et al., 2012; Kennedy et al., 2014). They can improve the organism's ability to survive in ‘extreme’ marine environments, such as low oxygen, low light, low temperature, and high-pressure zones, as well as other harsh conditions, where specific adaptations are vital for survival. These symbiotic, host-associated adaptations can also be seen through secondary, indirect physiological changes in the host. For instance, symbiotic bacterial biofilms are fundamental in the settlement behaviour of marine invertebrates such as cnidarians, echinoderms, and polychaetes (like the tubeworm *Hydroides elegans*; Ezenwa et al., 2012; Huang et al., 2012).

Most microbiota studies conducted on marine organisms have focused on the gut microbiota (Meziti et al., 2010; Star et al., 2013; Viver et al., 2017; Heys et al., 2020; Cheaib et al., 2021b; Rasmussen et al., 2021; Kang et al., 2022), including those conducted on squids (Figure 1.1a). The gut microbiota of three hadal amphipod species, as in numerous other invertebrates, has been reported to play a crucial role in their nutrient status, immune system development, and environmental adaptation (Dugas et al., 2016; Zhang et al., 2016; Chan et al., 2022). Variations in environmental conditions and genetic differences in the host are thought to affect the composition of the organism’s microbiota (Suzuki et al., 2017; Chan et al., 2021; 2022).

In cephalopods, microbial studies to date have mainly focused on commercially abundant or relatively easily reared species such as octopuses, cuttlefish, and bobtail squids (Lutz et al., 2019; Nyholm & McFall-Ngai, 2021; Costas-Imbernón et al., 2024). Mutualistic relationships have been observed between these species and various microbes, which seem to play important roles in cephalopod bioluminescence, reproductive development, and digestion. One notable example of such a symbiotic association is found in the 'Hawaiian bobtail' (*Euprymna scolopes*), where a well-documented relationship exists between the squid and the bacterium *Aliivibrio fischeri* (Nyholm & McFall-Ngai, 2021). The bobtail squid provides nourishment and protection to colonies of *A. fischeri*, while the bacterium emits light that helps reduce the squid's visibility to predators (Lasa & Romalde, 2021). Similarly, in other cephalopod species such as *Octopus mimus* and *Doryteuthis pealeii*, certain bacterial groups, such as Alphaproteobacteria and Gammaproteobacteria, play crucial roles in the reproductive system and egg defence (Barbieri et al., 2001; Iehata et al., 2016; Kerwin & Nyholm, 2017; Lutz et al., 2019). Investigations into cuttlefish microbiota have advanced our understanding of interactions between hosts and symbionts in cephalopod mariculture (Lutz et al., 2019). Additionally, investigations of the gastrointestinal microbiota of the North Atlantic common octopus (*Octopus vulgaris*) have provided valuable insights into the previously poorly understood larval migration from the coast to the open ocean (Roura et al., 2017).

A single teaspoon of seawater contains 5 million bacteria and 50 million viruses (Seymour, 2014). However, while marine invertebrates are exposed to a high number of microbes in the ocean, most studies to date have reported a limited range of bacterial species within their digestive tracts (Kang et al., 2022). Hosts are likely highly selective of the bacterial symbionts they tolerate and encourage (Petersen & Osvatic, 2018; Kang et al., 2022). Marine gastropods have a gut microbial composition more similar to that of marine bivalves and cephalopods than to their closer terrestrial relatives (Kang et al., 2022). This further emphasises the key importance of the host’s environment in microbial selection. When examining the gut microbial composition of cuttlefish and octopus, Kang et al. (2022) found an abundance of Mycoplasmatota and Pseudomonadota (formerly Tenericutes and Proteobacteria), with *Mycoplasma* and *Photobacterium* being the most abundant genera. In their study, they also concluded that the host’s diet influences the majority of the bacterial community observed in the digestive tract. Pseudomonadota are reported as one of the most abundant phyla in bacterial communities across most marine environments (Amaral-Zettler et al., 2010; Lawler et al., 2016). *Vibrio* and *Photobacterium* are common core microbial taxa in the gastrointestinal tracts of marine organisms, such as fishes (Tarnecki et al., 2017; Egerton et al., 2018; Lutz et al., 2019) and octopuses (Roura et al., 2017). This is likely because members of the Vibrionaceae family produce proteases, amylases, chitinases, and lipases, which are thought to aid in host digestion (Egerton et al., 2018; Lutz et al., 2019). Lutz et al. (2019) identified an abundance of *Vibrio* in the digestive tract and skin of European cuttlefish, *Sepia officinalis*, and a significant presence of Psicrickettsiaceae in its gills. The limited studies published on cephalopod microbiota to date suggest that microbes play a crucial role in their physiology; for instance, in the bioluminescence and egg survival strategies of Hawaiian bobtail squids (Kerwin & Nyholm, 2017; Douglas, 2019). Symbiotic bacteria associated with the bobtail squid's reproductive system are detectable in the environment, remain stable within the host and developing eggs, and are secreted into an egg jelly protective coat that helps prevent fouling and attacks by pathogenic micro-parasitic organisms (Vijayan et al., 2022).

The microbiota of most cephalopods remain largely unstudied. Most research on ‘true’ squids’ microbiota has been conducted on myopsid squids, with only four studies on oegopsid squids to date. There has also been considerably less sampling effort in the Southern Hemisphere (Figure 1.1b). Very little is known about the ecology, pre-mortem physiology, and microbial associations of oegopsid squids. This is mainly because of the significant challenges involved in studying the remote and extreme environments they inhabit. As a result, research on the ecology, pre-mortem physiology, and life history of deep-sea squids remains limited, highlighting the importance of establishing a database on their microbiota as a proxy for understanding their health and physiology.

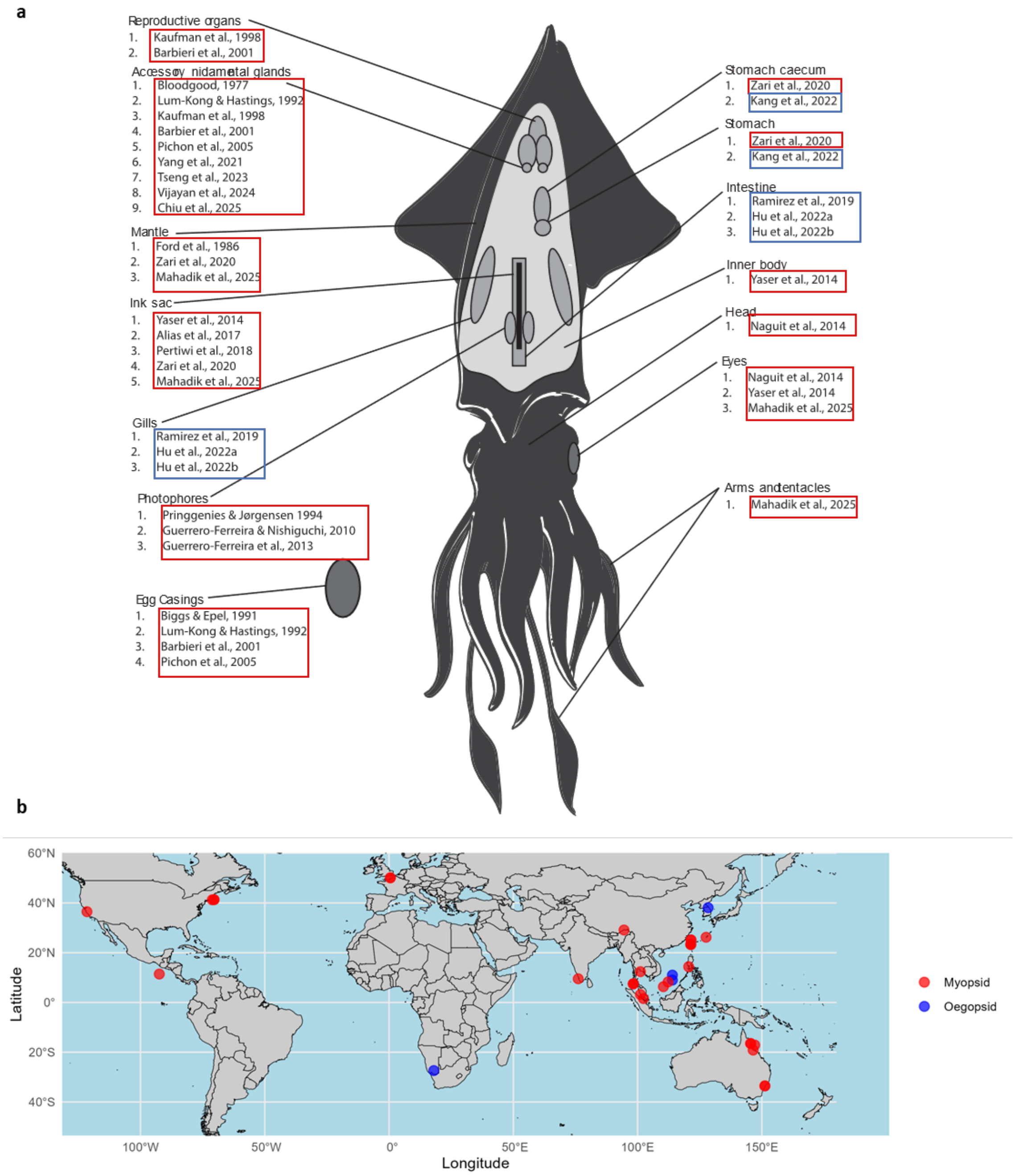


Figure 1.1: Overview of published microbiota studies from squid of the order Teuthoidea. (a) Stylised representation of which body sites were tested for microbes in each publication, myopsid squid in red and the oegopsid squid in blue. (b) The geographic locations of where the squids were sampled around the world, split into myopsids (red) and oegopsids (blue).

1.2 Myopsid squids

Myopsid squid microbiota have only been investigated within the family Loliginidae. Most published research on loliginids has concentrated on the accessory nidamental glands and egg casings, with only a few studies examining bioluminescent bacteria in different body sites (Figure 1.1a).

1.2.1 Accessory nidamental glands and egg casings

Loliginid squids host symbiotic bacteria in the accessory nidamental glands (ANG), which are part of the female reproductive system (Bloodgood, 1977). Female myopsid reproductive maturity is marked by a colour change in the ANG, from colourless/white to red/orange (Bloodgood, 1977; Barbieri et al., 2001). This colour shift results from symbiotic bacteria containing carotenoid pigments within the tubules of the ANG (Bloodgood, 1977; Barbieri et al., 2001). It is hypothesised that the ANG offers a protective bacterial biofilm, preventing fouling and predation on squid egg casings, due to the layered arrangement and high bacterial density between each layer of the casing (Barbieri et al., 2001). Bacterial colonisation of the ANG organs in loliginid squids is not passed directly from mother to offspring. Instead, it occurs through a slow horizontal transmission as the ANG reaches sexual maturity (Kaufman et al., 1998; Barbieri et al., 2001). While the ANG likely plays a role in transferring bacterial communities to egg casings, the presence of distinct bacterial populations in the egg casings—absent in the ANG—suggests additional microbial interactions (Barbieri et al., 2001).

Key studies concerning the ANG of myopsid squids highlight the diversity and characteristics of the bacterial populations confined within these glands (Table 1.1). A total of eight studies, to date, have documented the ANG and egg casings of myopsid squids, with Bloodgood (1977) being the earliest and Chiu et al. (2025) being the most recent. There has been a notable increase in the precision of bacterial identification as technology has advanced over the years; this development has led to differences in observed description of taxa obtained through microscopic identification, traditional cell culturing methods, and more recently, 16S rRNA gene sequencing from cultured bacteria or directly from specimens. Bloodgood (1977) described morphologically distinct bacterial populations within different tubules of the ANG, where the tubules harboured either rod- or coccoid-shaped bacteria, with no apparent overlap between these populations. Specimens of sexually mature *D. pealeii* were dissected, and their associated bacterial populations were examined microscopically. A phase contrast microscope was used to examine the white and red contents of the ANG tubules, revealing bacteria with rod- and coccoid-shaped morphologies, with approximately 10% demonstrating active motility (Bloodgood, 1977). Barbieri et al. (2001) further investigated both mature and immature female specimens of *D. pealeii*. Their findings

indicated that the ANG of *D. pealeii* was predominantly inhabited by Alphaproteobacteria, notably *Pseudoalteromonas* and *Shewanella*, which were also found to dominate the ANG and egg casings of *D. opalescens* (Barbieri et al., 2001, citing personal communication with D. Epel and M. Kaufman). No luminescence was observed in either the ANG or the egg casings; however, the luminescent bacterium *A. fischeri* was identified through 16S rRNA gene analysis of cultured isolates, alongside several non-bioluminescent bacterial strains of *Vibrio* (Barbieri et al., 2001). However, *Vibrio* was not considered an abundant genus within the ANG or egg casings of *D. pealeii.*

Research on the reproductive microbiota of *Doryteuthis* and *Uroteuthis* has offered valuable insights into the bacterial diversity within these squid genera. A study by Vijayan et al. (2024) found that bacteria isolated from these sites showed both antibacterial and antifungal properties. The most abundant bacterial taxa in the ANG of *D. pealeii* were Rhodobacteraceae and Alphaproteobacteria, with Bacteroidia being the most abundant in *D. pealeii* but either absent or negligible in *U. duvaucelii* and *D. opalescens* (Vijayan et al., 2024). This highlights a clear difference in the ANG and egg casing microbiota across species within the loliginid family. Notably, the location and year of collection did not significantly affect the microbiota composition of the ANG and egg casing (Vijayan et al., 2024). Further examination of *D. opalescens* also showed a dense bacterial population in the egg casings and ANG (Biggs & Epel, 1991; Kaufman et al., 1998). Additionally, the ANG of *D. opalescens* had a high abundance of Alphaproteobacteria and Gammaproteobacteria, but notably lacked Bacteroidia and Flavobacteriia (Vijayan et al., 2024). Juvenile specimens of *D. opalescens* primarily hosted Gram-positive bacteria, whereas adults were dominated by Gram-negative bacteria (Kaufman et al., 1998). These findings emphasise the variability of bacterial communities across different species within the same family and between different life stages.

Pichon et al. (2005) investigated the bacterial diversity within the ANG and egg casings of several loliginid squid species, including *Loligo, Uroteuthis, Sepioteuthis,* and *Loliolus.* While *S. lessoniana* and *Loligo vulgaris* embryos were found to be sterile, their egg casings contained a dense population of bacteria, also present in the ANG of mature females of the same species (Kaufman et al., 1998; Barbieri et al., 2001; Pichon et al., 2005). *Vibrio* and *Shewanella* were identified exclusively from the egg casings of *S.*

lessoniana, while *Agrobacterium*, *Roseobacter*, and *Rhodobium* were observed in egg casings of both *S. lessoniana* and *L. vulgaris*. The ANG microbiota of *S. lessoniana* was shown to shift gradually during female reproductive maturation (Yang et al., 2021), a finding further confirmed by Tseng et al. (2023). In their study, four distinct bacterial morphologies were observed across all four squid genera: small coccoid, large coccoid, rod-shaped Gram-negative bacteria, and a large, rod-shaped Gram-positive bacterium. DNA sequencing revealed an abundance of *Roseobacter*, *Agrobacterium*, *Silicibacter*, *Stappia*, *Rhodobium*-like bacteria, *Vibrio*, *Shewanella*, and *Pseudoalteromonas* in the ANG of these species. These results further emphasise the reproductive-specific bacterial communities present in the ANG and egg casings of loliginid squid. Organ-specific microbes have also been reported in hydrothermal vent crabs (Zhang et al., 2017; Osman & Weinnig, 2022).

Yang et al. (2021) investigated the bacterial communities within the ANG of female *S. lessoniana*, demonstrating how environmental conditions and the surrounding seawater bacterial diversity influence their composition. The ANG of *S. lessoniana* mainly consisted of Pseudomonadota, Bacteroidetes, Actinomycetota (previously known as Actinobacteria), and Bacillota (formerly known as Firmicutes), with Gammaproteobacteria, Alphaproteobacteria, and Bacteroidia being the most prevalent classes. Notably, Pseudomonadota and Bacteroidetes increased in abundance as reproductive maturity and ANG development progressed, while *Mycoplasma* and *Lactobacillus* decreased in abundance with increased reproductive maturity. Yang et al. (2021) also observed that Verrucomicrobia were prominent during the early stages of ANG maturation but were replaced by other bacterial groups, such as Gammaproteobacteria, Alphaproteobacteria, and Bacteroidia, as maturation continued. The distinct colouration of sexually mature ANG in *S. lessoniana* was therefore linked to an increase in the abundance of Flavobacteriales and Alphaproteobacteria within the ANG microbiota.

Table 1.1: Main studies investigating the microbiota of the accessory nidamental glands and egg casings of myopsid squids.

Study	**Squid species**	**Sample site**	**Main reported bacterial taxa**	**Targeted region**
Barbieri et al., 2001	*Doryteuthis pealeii*	Accessory nidamental glands and egg casings	*Roseobacter, Shewanella* and *Pseudoalteromonas*	Universal prokaryotic primers (UP)
Yang *et al.,* 2021	*Sepioteuthis lessoniana*	Accessory nidamental glands	Pseudomonadota, Actinomycetota, Bacteroidetes, and Bacillota	V3- V4 Region
Bloodgood, 1977	*Doryteuthis pealeii*	Accessory nidamental glands	Not specified	Cultured
Kaufman et al., 1998	*Loligo opalescens*	Accessory nidamental glands and egg casings	Not specified	Cultured
Pichon et al., 2005	*Loligo vulgaris, Sepioteuthis lessoniana, Uroteuthis duvaucelii, Uroteuthis chinensis, Uroteuthis edulis, Loligo forbesii, Loliolus uyii, and Loliolus beka*	Accessory nidamental glands and egg casings	*Agrobacterium* and *Silicibacter* *Vibrio, Shewanella, Roseobacter, Rhodobium*	27F-1385R pairs (respectively E scherichia coli position 9: 5′-GAGTTTGATCCTGGCTCA-3′ and position 1385: 5′-CGGTGTGTRCAAGGCCC-3′

1.2.2 *Vibrio* and *Photobacterium*

Two bacterial genera have been the primary focus of numerous cephalopod microbiome studies: *Vibrio* and *Photobacterium*. They are both known to contain bioluminescent species and Gram-negative bacteria commonly found in marine environmental samples (Calogero et al., 2022). *Photobacterium* has been documented as widely distributed across various marine environments, including the deep sea (Urbanczyk et al., 2011). Additionally, members of the genus *Vibrio* are recognised as core microbiota taxa in several marine molluscs, such as the gastropod *Dicathais orbita*, European abalone *Haliotis tuberculate,* and in bivalves including *Mytilus edulis* and *Crassostrea gigas* (Romalde et al., 2014; Ngangbam et al., 2015; Le Roux et al., 2016; Offret et al., 2019; Destoumieux-Garzón et al., 2020). Some studies have investigated the bioluminescent

bacteria present in the light organs and other tissues of coastal myopsid squids of the family Loliginidae (Table 1.2). These bacteria are primarily from the genera *Vibrio* and *Photobacterium*. Due to scientific interest in their bioluminescent properties, various body sites of loliginid squids have been explicitly tested for bioluminescence.

Given the current focus on bioluminescence, *Vibrio* and *Photobacterium* are among the most studied symbiotic bacteria in cephalopods. This includes non-'true’ squids, like the bobtail squid, which has a symbiotic relationship with *A. fischeri*, as well as some ‘true’ squids of the family Loliginidae, which also show bacterial bioluminescence (Guerrero-Ferreira & Nishiguchi, 2010; Guerrero-Ferreira et al., 2013; Nyholm & McFall-Ngai, 2021). For instance, some *Loligo* species have been found to host bioluminescent bacteria, mainly *V. harveyi*, in their light organs (Guerrero-Ferreira & Nishiguchi, 2007; Alias et al., 2017). Likewise, bacteria from the *Vibrio* genus dominate the light organs of *U. chinensis* (Guerrero-Ferreira & Nishiguchi, 2010). In 2013, Guerrero-Ferreira et al. isolated and sequenced bacteria from the light organs of four loliginid squid species (*U. chinensis, U. etheriogei, U. noctiluca,* and *U. duvaucelii*), mainly identifying *Vibrio* and *Photobacterium* as the bioluminescent taxa.

Bioluminescent bacteria have also been identified in the ink sacs of various loliginid squid species. Alias et al. (2017) cultured and sequenced *Vibrio* spp. from *Loligo* spp. ink sacs, where symbiotic bioluminescent bacteria were also located in the light organs attached to the ink sacs. This suggests a potential vertical transmission of bacteria between these structures, as similar findings were also reported by Yaser et al. (2014), who noted an abundant presence of *P. leiognathi*. Additionally, *U. duvaucelii* (Pertiwi et al., 2018), *Uroteuthis* spp., and *Loliolus* spp. (Zari et al., 2020), and *S. lessoniana* (Zari et al., 2020) also contained *Photobacterium* in their ink. Moreover, *S. lessoniana*, when inoculated on luminescent agar, also contained *Vibrio* (Zari et al., 2020).

Swabs taken from the vitreous sac of *Uroteuthis* sp. and other loliginid squid eyes revealed the presence of bioluminescent bacteria from the *Photobacterium* genus (Naguit et al., 2014; Yaser et al., 2014). Additionally, the gut microbiota of *Loliolus beka* and *U. edulis* were primarily composed of *Photobacterium*, along with other genera such as *Aliivibrio*, *Physichrilyobacter*, and *Mycoplasma* (Kang et al., 2022). In the digestive tracts of certain fish species, *Photobacterium* seems to assist in the digestion of chitin

(Itoi et al., 2006), and it is thought to perform a similar function in juvenile squids, many of which consume a diet rich in crustaceans, whose exoskeletons are composed of chitin (Portela et al., 2014). Ford et al. (1986) examined the external mantle surfaces of *Lolliguncula brevis* and found *Vibrio* to be the most prevalent genus among Gram-negative bacteria and *Bacillus* to be the most abundant genus among Gram-positive bacteria. Naguit et al. (2014) also identified several *Photobacterium* strains from swabs taken inside the mantle of *Uroteuthis* sp. These findings highlight the widespread presence of *Photobacterium* and *Vibrio* across various squid species and their body sites' microbiota, including photophores, ink, eyes, gut, and external surfaces.

Table 1.2: Presence of Photobacterium (P) and Vibrio (V) reported in loliginid squid body sites. (-) means not analysed.

Squid Taxon		Ink	GI	Outer mantle	Eye	Light Organ	ANG/Egg Casings	Reference
Doryteuthis	*D. pealeii*	-	-	-	-	-	V	Barbieri et al., 2001; Pichon et al., 2005
Loligo	*L. forbesii*	-	-	-	-	-	V	Lum-Kong & Hasting, 1992; Pichon et al., 2005
	L. vulgaris	-	-	-	-	-	V	Pichon et al., 2005
Loliolus	*L. beka*	-	P	-	-	-	V	Pichon et al., 2005; Kang et al., 2022
	L. uyii	-	-	-	-	-	V	Pichon et al., 2005
Lolliguncula	*L. brevis*	-	-	V	-	-	-	Ford et al., 1986
Sepioteuthis	*S. lessoniana*	P, V	P, V	P, V	-	-	V	Pichon et al., 2005; Zari et al., 2020
Uroteuthis	*U. chinensis*	P	P	P, V	-	V	V	Pichon et al., 2005; Guerrero-Ferreira et al., 2013
	U. duvaucelii	P, V	V	P, V	P	V	V	Pichon et al., 2005; Guerrero-Ferreira et al., 2013; Naguit et al., 2014; Pertiwi et al., 2018
	U. edulis	-	P, V	-	-	V	V	Pichon et al., 2005; Kang et al., 2022
	U. etheriogei	-	-	-	-	V	V	Guerrero-Ferreira et al., 2013
	U. noctiluca	V	-	-	-	V	V	Guerrero-Ferreira et al., 2013

1.3 Oegopsid squids

To date, only three oegopsid squid species, *Sthenoteuthis oualaniensis* (mid-form and dwarf-form), *Todarodes sagittatus*, and *Todarodes pacificus*, have had their microbiota investigated. These studies have focused on the microbial communities within their digestive tracts and, in two cases, also their gill tissues (Ramírez et al., 2019; Hu et al., 2022a, 2022b; Kang et al., 2022).

1.3.1 Digestive tract microbiota

While some commonalities have been observed among the three ommastrephid species studied to date, notable species- and sex-specific differences in their microbiota remain evident. In *S. oualaniensis, Mycoplasma, Lactobacillus,* and the *Bacteroidales* S24-7 group were predominant in both mature and immature males and females of mid- and dwarf-form squids, as reported by Hu et al. (2022a; 2022b). Additionally, *Photobacterium* and the BD1-7 clade were abundant in the intestines of immature males and mature females of mid-forms, as well as in both mature and immature females and mature males of dwarf-forms. *Vibrio* was also identified as an abundant genus in immature female dwarf-forms (Hu et al., 2022a). Conversely, the BD1-7 clade and *Mesorhizobium* were more prominent in mature males, while *Vibrio* and Cardiobacteriaceae were observed in immature males of mid-form *S. oualaniensis* (Hu et al., 2022b). These findings suggest that mid-form male *S. oualaniensis* exhibited a broader diversity of abundant bacterial genera than other groups.

Overall, *Mycoplasma* remained the most abundant genus in the intestines of all *S. oualaniensis* mid-forms sampled by Hu et al. (2022b), as well as in *T. sagittatus* (Ramírez et al., 2019) and *T. pacificus* (Kang et al., 2022). A statistically significant negative correlation was found between the mantle length of *S. oualaniensis* and the abundance of Mycoplasmatota (*Mycoplasma*) (Hu et al., 2022b); however, it was positively correlated with Bacillota and Bacteroidetes. The abundance of *Mycoplasma* in *T. pacificus* is also thought to be influenced by the squid's body size, with larger individuals harbouring higher levels of *Mycoplasma* in their gut (Kang et al., 2022). *Mycoplasma* has been observed to be an obligate symbiotic core bacterial taxon in the gut microbiota of most studied marine organisms (such as jellyfish, Viver et al., 2017; cuttlefish, Gentes et al., 2023; lobster, Meziti et al., 2010; hadal amphipod species, Chan et al., 2022; and

many salmonoid species, Star et al., 2013; Heys et al., 2020; Bozzi et al., 2021; Cheaib et al., 2021a; 2021b; Rasmussen et al., 2021). This is thought to be due to its ability to metabolise ammonia (Rasmussen et al., 2021). The gut and stomach caecum of *T. pacificus* were also dominated by *Acrobacter* (Kang et al., 2022). Hu et al. (2022a; 2022b) concluded that the diversity observed in the bacterial communities of the intestines of mid-form and dwarf-form *S. oualaniensis* can be directly linked to the growth, feeding behaviour, and differences in sex and reproductive maturity of the squid.

1.3.2 Gill microbiota

The microbial populations present in the gills of marine invertebrates are known to be markedly influenced by various external marine environmental factors (Jin et al., 2017; Duan et al., 2020; Hu et al., 2022a; Hu et al., 2022b). Consequently, the microbiota of squid gills is expected to reflect the characteristics of the surrounding marine environment (Kuang et al., 2020; Hu et al., 2022a; Hu et al., 2022b). In *S. oualaniensis*, the predominant bacterial phyla identified in mature female and male specimens, as well as immature males, were Pseudomonadota, Bacillota, and Bacteroidetes (Hu et al., 2022a; 2022b). The principal genera observed in the gills of both dwarf- and mid-form *S. oualaniensis* across sexes included the BD1-7 clade, *Lactobacillus*, and *Bacteroidales* S24-7 group (Hu et al., 2022a; 2022b). Notably, *Photobacterium* was also a significant genus in immature females, as well as in both mature and immature males of mid-form squids, and both mature male and female dwarf-form squids (Hu et al., 2022; 2022b). Conversely, immature female dwarf-form *S. oualaniensis* were dominated by *Vibrio* (Hu et al., 2022a). Among all the mid-form *S. oualaniensis* samples, the BD1-7 clade emerged as the most abundant bacterial genus, a finding consistent with its prominence in numerous marine environments (van de Water et al., 2018; Yu et al., 2021; Hu et al., 2022b). Furthermore, gill swabs from two *T. sagittatus* specimens showed a high abundance of *Mycoplasma* (Ramírez et al., 2019). These results showcase the bacterial community composition of the gill microbiota of ommastrephid squids.

1.4 Research objectives

This thesis aims to conduct an initial survey of microbial diversity in oegopsids by progressively exploring community composition within individuals, then among conspecifics, congenerics, and more distantly related squids. To do this, I will observe and report the microbial composition within and among species, genera, and families across different anatomical sites, ages, sexes, body chemistry and geographic regions (N = 500 in total). I will optimise a standard method for extracting and purifying microbial DNA from deep-sea squids, then will examine the composition of different squid bacterial microbiota using 16S rRNA gene amplicon sequencing. Amplicon sequencing enables comparisons of broad changes in taxonomic composition across different conditions (Sogin et al., 2006). It increases the likelihood of capturing less-abundant species through dense sampling of any given community (Mock & Kirkham, 2012). The same core sequencing workflow will be applied to different subsets of samples to address the different research questions (Figure 1.2). This will provide insight into squid lifecycles, ecology, predation and physiology.

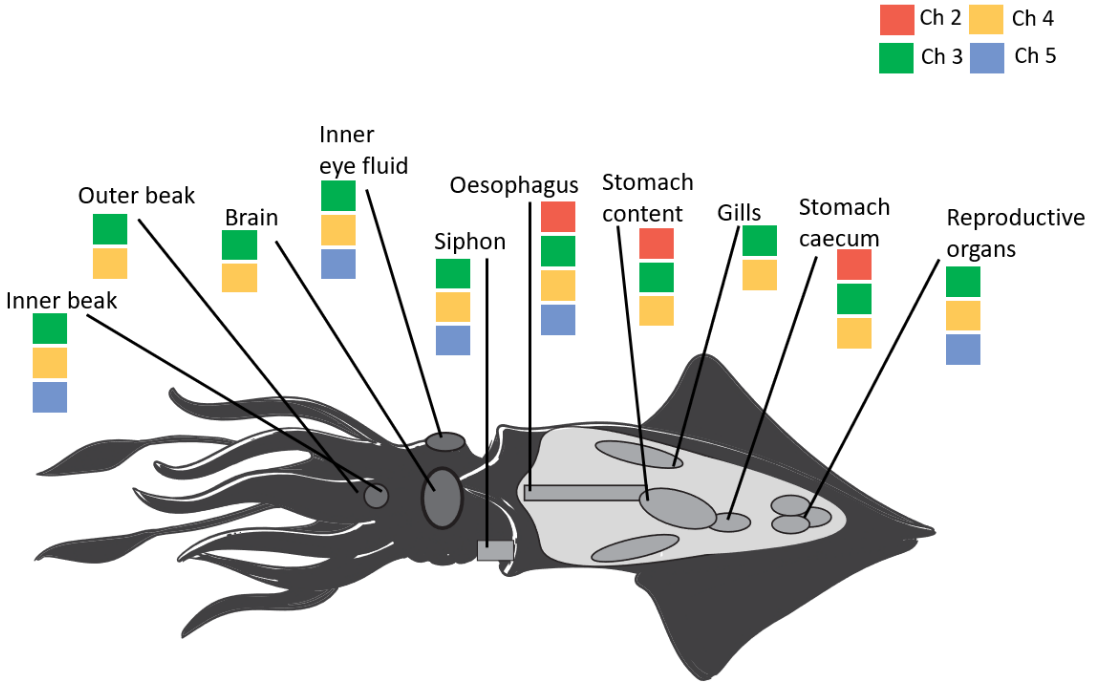


Figure 1.2: Stylised illustration of a deep-sea oegopsid squid and the ten sampled body sites studied in this thesis and the research chapter they relate to.

Research question one: What is the microbial community structure and variability within the digestive tract of a single deep-sea squid species, and is there a difference between sex (focus: NZ Southern arrow squid, *Nototodarus sloanii*)?

Chapter two will investigate variability among individuals within a single deep-sea squid species. A subset of three body sites was sampled in duplicate across ten *N. sloanii* of similar size and geographic origin, representing both sexes (five males and five females; total sample N = 60). This will give an indication of the variation between individuals of the same and opposite sex within the same species (Figure 1.3). The selected sites and number of squid chosen will allow me to determine the number of repetitions needed of individuals per site in the later research questions.

Research question two:

a. How do the microbiota compare between congeneric deep-sea squid species (focus: *Nototodarus* and *Todarodes* spp.)?
b. How do host-associated microbial communities compare within a deep-sea squid family (focus: Ommastrephidae)?

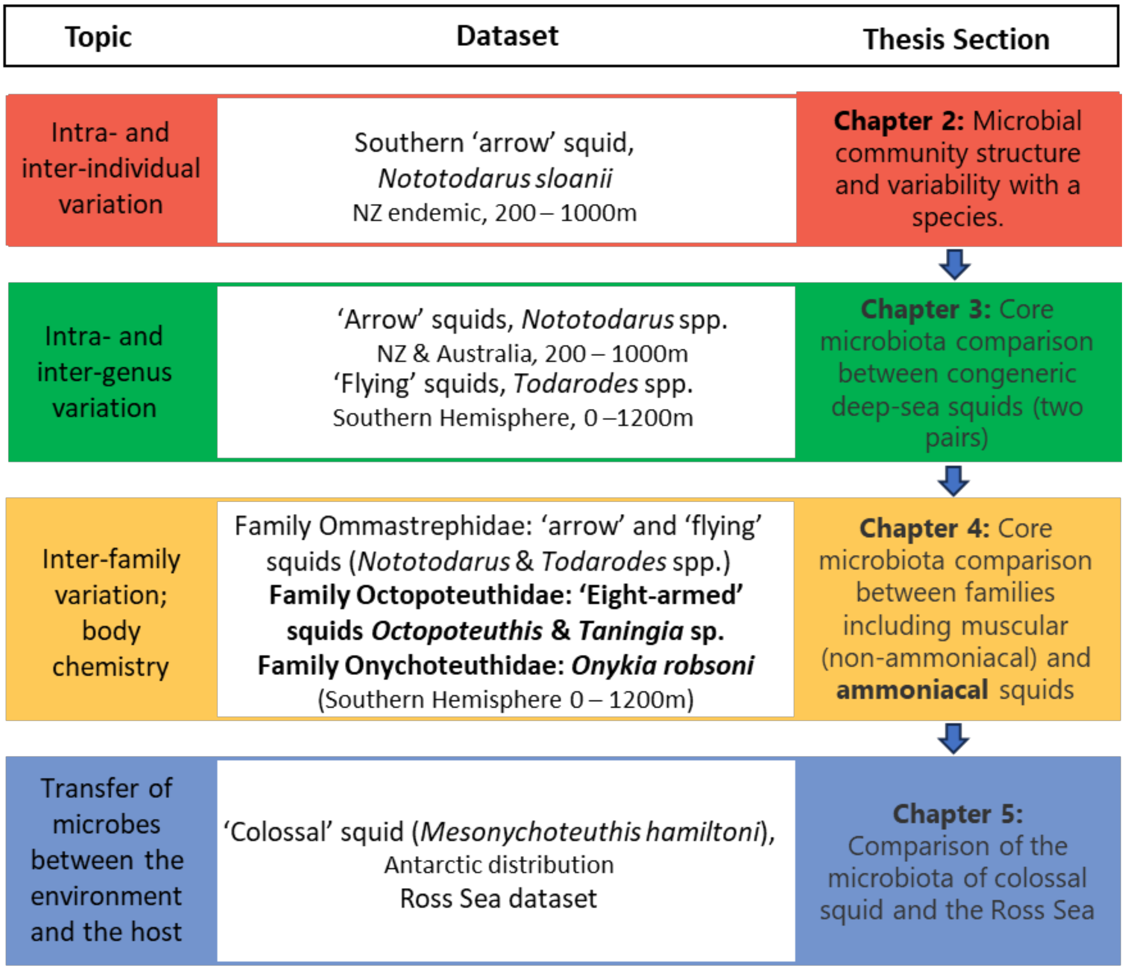
Once intraspecies variability is established (research question one), I will investigate intra-and inter-genus variability in the microbiota of deep-sea squids in chapter three. The intra- and inter-genus variation of *Todarodes* and *Nototodarus* spp. will be examined at ten body sites swabbed in duplicate across twelve squid, three of each selected species (*N. sloanii, N. gouldi*, *T. angolensis, T. filippovae;* total sample N = 240). The increased number of body sites was chosen to examine physiological and ecological differences within and among squids. This will allow for the investigation of variability between two pairs of congeneric species and among genera within the same family (Figure 1.3).

Research question three: How does the microbiota compare among deep-sea squids with different body chemistry (non-ammoniacal Ommastrephidae and ammoniacal Octopoteuthidae and Onychoteuthidae)?

To investigate variability among deep-sea squids with different body chemistry in chapter four, ten different body sites will be sampled across eight squid species. Four of these are considered muscular, non-ammoniacal squids, the same used in research question two (*N. sloanii, N. gouldi, T. angolensis,* and *T. filippovae*) and four ammoniacal squids (*Octopoteuthis* sp. nov. (“fenestra” sensu Kelly 2019), *Taningia danae, T. fimbria,* and *Onykia robsoni*) (Figure 1.3).

Research question 4: What does the microbial composition of the 'colossal' squid (*Mesonychoteuthis hamiltoni*) look like?

In chapter five the microbial compositions of six body sites of the female colossal squid will be observed and compared to a 2023 Ross Sea seawater microbiota study from the National Centre of Biotechnology Information database (Figure 1.3). Tissue samples from different body sites will be collected and analysed to reveal the microbial communities present in the squid and how they differ among body sites, and will be compared with a Ross Sea dataset to examine possible horizontal transfer of bacteria between the squid and its habitat.



Topic	Dataset	Thesis Section
Intra- and inter-individual variation	Southern 'arrow' squid, *Nototodarus sloanii* NZ endemic, 200 – 1000m	**Chapter 2:** Microbial community structure and variability with a species.
Intra- and inter-genus variation	'Arrow' squids, *Nototodarus* spp. NZ & Australia, 200 – 1000m 'Flying' squids, *Todarodes* spp. Southern Hemisphere, 0 –1200m	**Chapter 3:** Core microbiota comparison between congeneric deep-sea squids (two pairs)
Inter-family variation; body chemistry	Family Ommastrephidae: 'arrow' and 'flying' squids (*Nototodarus* & *Todarodes* spp.) **Family Octopoteuthidae: 'Eight-armed' squids *Octopoteuthis* & *Taningia* sp.** **Family Onychoteuthidae: *Onykia robsoni*** (Southern Hemisphere 0 – 1200m)	**Chapter 4:** Core microbiota comparison between families including muscular (non-ammoniacal) and **ammoniacal** squids
Transfer of microbes between the environment and the host	'Colossal' squid (*Mesonychoteuthis hamiltoni*), Antarctic distribution Ross Sea dataset	**Chapter 5:** Comparison of the microbiota of colossal squid and the Ross Sea

Figure 1.3: Flow chart of the progression of research questions and associated target squid taxa in this thesis.

Chapter 2 The Digestive Tract Microbiota of Female and Male *Nototodarus sloanii*

2.1 Abstract

Aotearoa's endemic southern 'arrow' squid (*Nototodarus sloanii*) is one of two species harvested as the country's largest cephalopod fishery, but much about its life history and ecology remains to be learnt. This chapter aimed to investigate the microbial community structure and variability within the digestive tract of a single deep-sea squid species, and whether there is an observable difference between sex. Studying the variability in the microbiota of the digestive tracts of female and male *N. sloanii* using 16S rRNA gene amplicon sequencing would further our understanding of this ecologically and commercially important deep-sea squid species. Ten individuals of both sexes (females N = 5 and males N = 5) were sampled along three sections of their digestive tract (the oesophagus, stomach content, and the stomach caecum). Significant differences in microbial diversity across digestive tract sites were not observed, allowing me to pool the samples into a single dataset representing each individual when comparing between sexes. A first glance at *N. sloanii*'s digestive tract microbiota showed a lack of sex-derived differences and a high abundance of *Brachybacterium* and *Mycoplasma*, which may be linked to help with pathogen prevention and nitrogen assimilation.

2.2 Introduction

Ommastrephidae are one of the most widely distributed, abundant, ecologically and commercially important squid families (Roper et al., 2010). Various species can be found throughout the oceans, from sub-Antarctic waters to sub-Arctic waters, with the majority having a complex spatial distribution, subdivided into feeding areas and reproductive zones (Roper et al., 2010). Most are also recognised as being among the fastest-growing squids, with females typically recorded reaching larger sizes than males, and the majority having a one-year life cycle (Roper et al., 2010). The southern 'arrow' squids, *Nototodarus sloanii*, are ommastrephid squids found in the South Pacific Ocean around Aotearoa New Zealand (hereafter Aotearoa). Arrow squids (composed of *N. sloanii* and *N. gouldi* for fisheries purposes) are the most abundant cephalopod catch in

Aotearoa, with annual landings of 14.332t in 2022/2023 (MPI, 2023). The endemic *N. sloanii* is found in the southern part of Aotearoa's Exclusive Economic Zone (Jackson et al., 2000), where it is considered a key species in pelagic ecosystems due to its vast abundance and role as both predator and prey (Lischka et al., 2020).

Animal-associated microbes can tell us about the host's health and metabolic functions (Ottman et al., 2012; Fan & Pedersen, 2021; Bates et al., 2023). Because of arrow squids' economic and ecological importance, it is necessary to learn more about this species, starting with their microbiota, as it can tell us about their health and about their ecosystem. Studying the digestive tract microbiota can reveal important insights into the host organism's health and immune system, and give us a snapshot into its diet (Corbari et al., 2012; Fan & Pedersen, 2021). The importance of microbes in regulating gene expression in the digestive tract through the promotion of nutrient metabolism and immune response control has been demonstrated in fish (Rawls et al., 2004; Bates et al., 2006). *Nototodarus sloanii* primarily forage in the mesopelagic layers (200–1000m), predominantly preying on mesopelagic fishes, crustaceans, and other cephalopods (Dunn, 2009; Pethybridge et al., 2012). They have been shown to have sex-specific dietary profiles, with females relying more on crustaceans and other cephalopods (Dunn, 2009). This may have implications for their digestive tract microbiota composition, which in turn often plays a crucial role in the breakdown of food, absorption of nutrients, and the health of the host (Iehata et al., 2015). Potential differences in microbiota between sexes have been investigated in various marine organisms, such as isopods (Wenzel et al., 2018), lobsters (Rusanova et al., 2025), octopuses (Iehata et al., 2015), corals (Wessels et al., 2017), elephant seals, and other marine mammals (Nelson et al., 2012). The observed differences in microbial diversity between host sexes are hypothesised to result from variations in diet, size dimorphism, habitat, and metabolic demands (Gao et al., 2018; Nelson et al., 2012; Bates et al., 2023). Notably, the male Chilean octopus (*Octopus mimus*) was found to have significantly higher microbial alpha diversity than the female, which was thought to be due to differences in diet (Iehata et al., 2015). This has also been reported in terrestrial and marine vertebrates (Stoffel et al., 2020; Zhu et al., 2020). However, several studies investigating animal microbiota have also observed a lack of sex-specific differences. For instance, the digestive tract microbiota of Chinese adult alligators lacked sexual dimorphism (Hu, M.Y. et al., 2022), and the skin microbiota of humpback whales showed no sex-specific differences (Apprill et al., 2014). No research, to date, has been conducted on whether/how sexual dimorphism influences the microbiota of cephalopods in the Southern Ocean.

The limited studies published to date on cephalopod microbiota have indicated that microbes play a crucial role in the physiology of squids and their closely related taxa, such as in bioluminescence and egg-survival strategies of Hawaiian bobtail squids (Kerwin & Nyholm, 2017; Douglas, 2019). The egg survival strategies rely on the symbiotic bacteria associated with the bobtail squid's reproductive system; these microbes are detectable in the environment and stable in the host and its developing eggs. These bacteria are also secreted in an egg jelly protective coat, where they prevent fouling by pathogens (Vijayan et al., 2022). Most squid studies have focused on coastal squids, and only four studies to date have been conducted on deep-sea squids (Ramírez et al., 2019; Kang et al., 2022; Hu et al., 2022a; Hu et al., 2022b). These studies have concentrated on the digestive tract and gills of oegopsid squids. No studies have been conducted on squid from the *Nototodarus* genus, and only Kang et al. (2022) have looked at sex-derived differences in digestive tract microbiota in oegopsids. The digestive tracts of *Sthenoteuthis* and *Todarodes* spp. have been reported to contain an abundance of *Mycoplasma*; this is thought to be due to its ability to utilise ammonia in the gut of predator species (Ramírez et al., 2019; Rasmussen et al., 2021; Kang et al., 2022; Hu et al., 2022a; Hu et al., 2022b).

This study reports the bacterial communities found in the digestive tract of *N. sloanii* for the first time, increasing our understanding of the physiology of Aotearoa cephalopods. A sex-driven difference in the digestive microbiota of *N. sloanii* is thought to occur due to differences in diet previously observed (Dunn, 2009).

2.3 Methods

2.3.1 Sample collection

Samples of *N. sloanii* were collected in the oceans around Aotearoa by Earth Sciences New Zealand (formerly NIWA) under permit during routine fishery surveys around Te Waipounamu (South Island), at GPS coordinates: 43.4° S and 177.4° E, and stored at

– 20°C until processed. To investigate inter-individual variability within a single deep-sea squid species, ten *N. sloanii* specimens of similar size and geographic origin were sampled, representing both sexes (five males and five females). For each squid, three sites along the digestive tract (stomach, stomach caecum, and oesophagus) were sampled in duplicate (N = 60).

2.3.2 16S rRNA gene amplicon sequencing

The squids were defrosted, ventrally dissected, and tissue snips of the three body sites were collected and placed in sterile 1.5 mL microcentrifuge tubes and kept at –20°C until DNA extraction. DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany), following the manufacturer's protocol with a slight sample specific optimisation as follows: a portion of the sample tissue from each site was placed in screw-capped, conical-bottomed polypropylene tubes, suspended in 20 µL of proteinase K (10 mM; Thermo Fisher Scientific) and 200 µL of phosphate buffer. Then, the sample was homogenised with silica-zirconia beads (2.5 mm and 0.1 mm in diameter) on a FastPrep-24™ Classic bead-beating grinder and lysis system (MP Biomedicals) for 1 minute at 5.5 m/s. Additionally, a 15-minute incubation period with 5 µL of RNase A (10 mg/mL; Thermo Fisher Scientific GeneJET) at 30°C was added after the final elution step to enhance PCR amplification. The DNA extractions were stored at –80°C until PCR was performed.

The V3-V4 region of the 16S rRNA gene was amplified through PCR using the 341F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CCTACGGGNGGCWGCAG** -3′) and 805R (5′- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GACTACHVGGGTATCTAATCC** -3′) primers with Illumina overhang adapters (primer sequences in bold; Klindworth et al., 2013) for bacterial amplification. The thermal cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes. Successful amplifications were verified on a 2% agarose gel electrophoresis (Muyzer & Smalla, 1998). A negative control per run was used in both the DNA extraction and PCR amplification steps to check for contamination during the process. Before 16S rRNA gene library preparation, each pair of duplicates were pooled to equal volume, resulting in a total of 30 samples, 15 per sex.

The indexing PCR and sequencing were carried out by Auckland Genomics (University of Auckland, New Zealand). For this, the sequencing provider attached a unique combination of Nextera XT dual indices (Illumina Inc., USA) to the DNA from each sample to enable multiplex sequencing of 192 samples per run. Sequencing was performed on an Illumina MiSeq instrument using V3 2-by-300-bp chemistry.

2.3.3 Data processing

The fastq data files generated from our amplicon sequencing were processed using the DADA2 pipeline in R 4.5.0 (R Core Team, 2021) to construct an Amplicon Sequence Variants (ASVs) table (Callahan et al*.,* 2016; ver 1.36.0). The raw fastq files were trimmed using the command filterAndTrim with predetermined maxEE values (truncLen(280,240), maxEE(2,3)). Chimeric sequences were also identified and removed before the final ASVs were produced. The SILVA v138.1 database was then used for the taxonomic classification of the bacterial sequences (Yilmaz et al*.,* 2014). The decontam package in R (Davis et al., 2017; ver 1.29.0) was used to remove contaminant ASVs through the contaminant prevalence method (threshold = 0.5), to identify sequences that were more prevalent in the negative controls than in the positive controls as contaminants (Davis et al., 2017). The decontaminated ASVs were further processed with the removal of all chloroplast, mitochondrial, and non-bacterial sequences, as well as the ASV sequence that was identified as belonging to the known contaminant species, *Ralstonia pickettii* (5′-TGGGGAATTTTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCCGGAAAGAAATGGCTCTGGTTAATACCTGGGGTCGATGACGGTACCGGAAGAATAAGGACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTCCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTGTGCAAGACCGATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTGGTGACTGCACGGCTAGAGTGTGTCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAGATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGATAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACA-3′). I then created both a rarefied ASV table using the ‘rarefy_even_depth’ command from the phyloseq package (McMurdie & Holmes, 2013; ver 1.53.0) to rarefy to even depths of 1000 reads, and a cumulative sum scaling normalised ASV table using the ‘cumNorm’ command from the metagenomeSeq package (Paulson et al., 2013; ver 1.51.0). During the rarefaction processing, 14 out of 30 samples were removed due to insufficient reads: four oesophagi (one female, three male), three stomachs (one female and two male), and seven stomach caecum (three female and four male).

The microbiota as represented by the ASVs were measured primarily by using alpha and beta diversity estimates, and were visualised as relative abundances of taxonomic groups based on the proportion of reads returned, using the following R packages: phyloseq for alpha diversity (McMurdie & Holmes, 2013; ver 1.53.0), ggplot2 for plotting (Wickham, 2016; ver 4.0.0), and vegan for beta diversity (Oksanen et al., 2025; ver 2.7-1). Fourteen samples did not result in a visible band on the agarose gel electrophoresis. They were still sent for sequencing and used for beta diversity, but they were removed during the rarefaction data processing. Shannon's diversity index, which measures both species richness and evenness (Ortiz-Burgos, 2016), was used to estimate alpha diversity from the rarefied data. The significance of the difference between the samples' total microbiota diversity was determined using a Mann–Whitney *U* test (McMurdie & Holmes, 2013). The raw ASV table was used to calculate beta diversity with the use of Aitchison dissimilarity distance matrix (Martino et al., 2019) with the 'vegdist' command and visualised in a Principal Coordinate Analysis (PCoA) ordination plot between *N. sloanii* female and male samples, the significance was assessed using a PERMANOVA test using the 'adonis2' command from vegan, and the differences in dispersion were checked with a PERMDISP test using the 'betadisper' and 'permutest(beta)' commands (Bakker, 2024).

2.4 Results

Intraspecies variability in microbial diversity was assessed across ten individuals of *N. sloanii* (female N = 5 and male N = 5) to determine whether sex had a significant effect on the composition of the digestive tract microbiota. A total of 163,640 reads were obtained, and 5734 ASVs were identified from a total of 30 samples. No significant difference in Shannon's diversity was observed between the digestive tract microbiota of the two sexes. This lack of significant difference was consistent across the three body sites sampled. Therefore, I did not make such a distinction when comparing between the sexes (Appendix C Figure 2a). Similarly, no statistical difference in Shannon's diversity between sexes was observed (Mann–Whitney *U* test, W = 18, $P = 0.22$) (Figure 2.1).

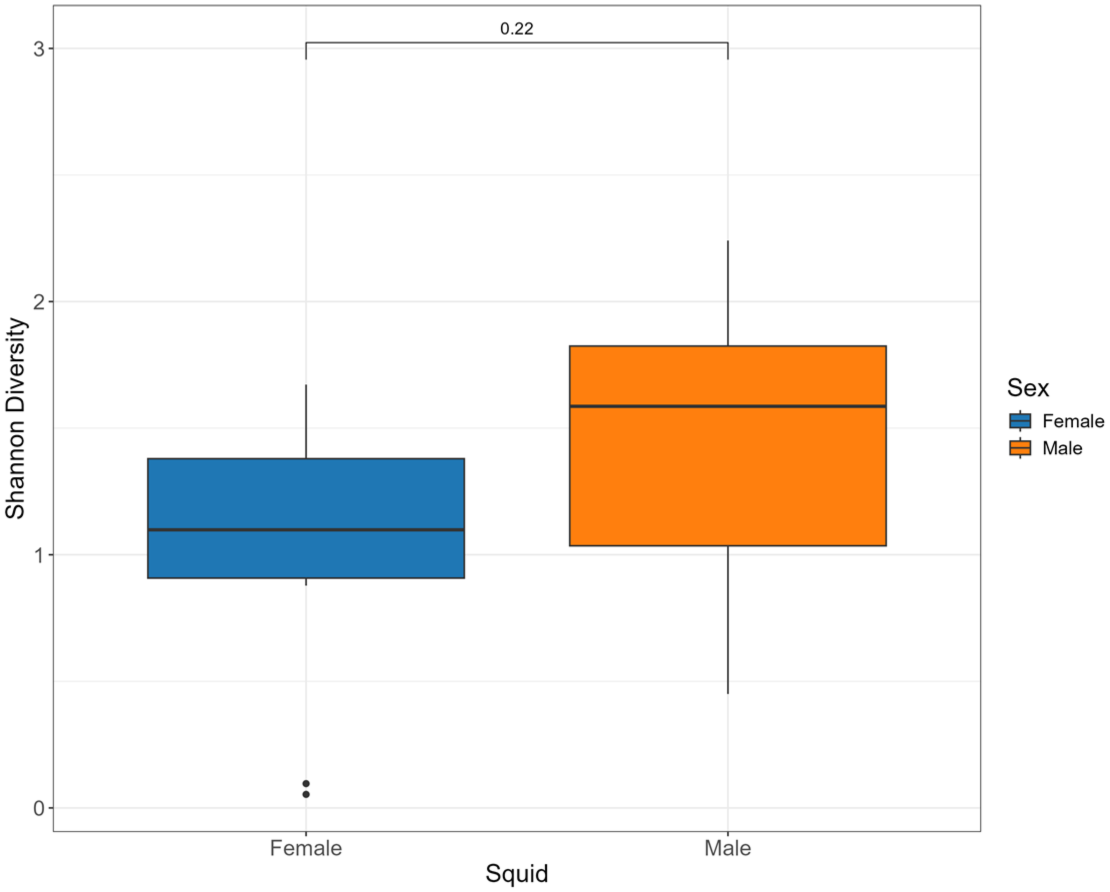


Figure 2.1: Diversity of the microbiota of the digestive tracts of female (blue) and male (orange) N. sloanii*. Shannon index diversity with rarefied data, significance tested with a Mann–Whitney* U *test. Boxes represent the interquartile range (IQR, 25–75%) of the data. Median values are indicated by the bar within each box and whiskers show the values within 1.5 times the IQR.*

No significant separation in the microbial community composition between sexes was observed (PERMANOVA $R^2 = 0.037$, $F_{28} = 1.09$, $P = 0.353$; Figure 2.2a). Direct comparisons between both sexes' digestive tracts showed similar bacterial profiles at the genus taxonomic level (Figure 2.2b). The digestive tracts of the female and male *N. sloanii* consisted primarily of the bacterial genera *Brachybacterium* (41.61% and 53.19% respectively) and *Mycoplasma* (35.99% and 29.14% respectively). However, some differences were observed in the composition of abundant bacteria, notably the female samples exhibited a higher prevalence of the BD1-7 clade (8.38%), which was more than double that in the male samples (4%; Figure 2.2b). The core digestive tract microbiota of both *N. sloanii* sexes, defined as prevalent in more than 50% of samples and with relative abundance greater than 1%, comprised two genera: *Brachybacterium* and *Mycoplasma*.

Although the microbial community compositions of female and male *N. sloanii* were similar, they differed in some minor taxa (Figure 2.2b). *Stenotrophomonas* and *Streptococcus* were observed in the female digestive tract (0.38% and 0.35% respectively) and were not detected in the male samples (Appendix C Table 1).

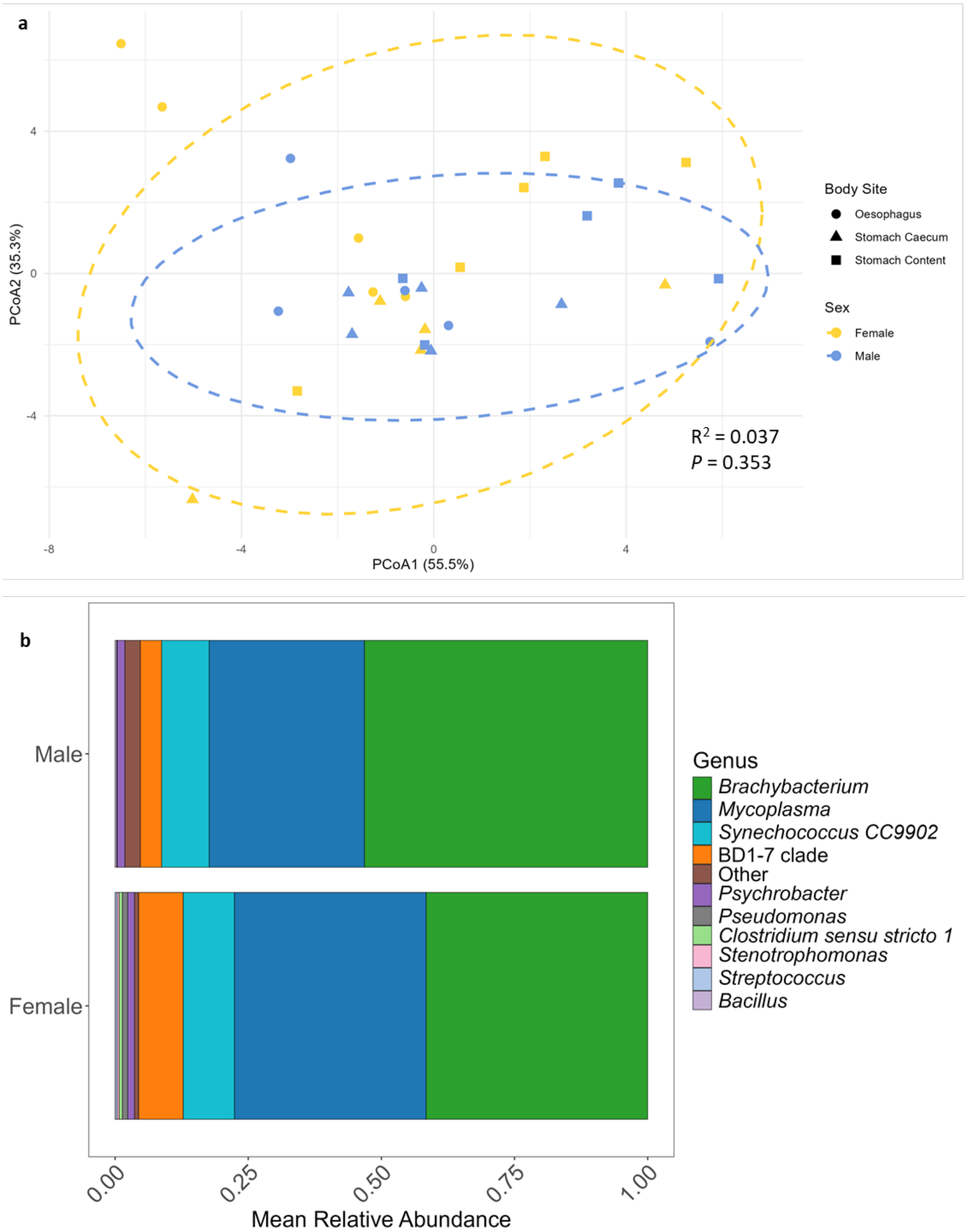


Figure 2.2: Microbial composition of the digestive tract of female and male N. sloanii. *(a) Principal coordinates analysis (PCoA) of Aitchison distances between samples. The coloured dots represent the female and male samples of N. sloanii, with the digestive tract samples identified by shape. With the 95% confidence ellipses assuming multivariate t-distributions. (b) Top ten most abundant bacterial genera within the female and male digestive tracts of N. sloanii, with the other genera grouped into "other".*

2.5 Discussion

This study examined the digestive tract microbiota for the first time in an Aotearoa deep-sea arrow squid, and for the first time worldwide in an ecologically and economically important species of *Nototodarus*. Ten individuals of *N. sloanii* (five female and five male) were used to test for host sex differences in the digestive tract microbiota. The squids were collected at the same depth, location and year, therefore minimising confounding variables.

Several studies on marine invertebrates have examined how sex affects microbiota richness and community composition; both significant and non-significant differences have been reported (Iehata et al., 2015; Wessels et al., 2017; Bates et al., 2023; Rodrigues-Barreto et al., 2024). These differences have been attributed to sexual size dimorphism and/or sex-specific niche selection (Bates et al., 2023). In contrast, for marine vertebrates such as marine mammals, research has also concluded that their gut microbiota are not strongly influenced by sex (Nelson et al., 2012).

Both female and male *N. sloanii* digestive tract microbiota were predominantly dominated by *Brachybacterium* and *Mycoplasma*. *Mycoplasma* has been reported in the gut microbiota of both marine invertebrates and vertebrates as a symbiotic bacterium (Iehata et al., 2015; Ramírez et al., 2019; Hu et al., 2022a; Hu et al., 2022b; Kang et al., 2022; Rodrigues-Barreto et al., 2024). It has been reported to aid in ammonia breakdown and digestion in salmonoids, produce lactic and acetic acids (Bozzi et al., 2021), and increase host health by increasing disease resilience, as a reduction in *Mycoplasma* abundance in the digestive tract has been linked to an increase in pathogenic bacteria (Rasmussen et al., 2021; Scheuring et al., 2022). While these results suggest that it may be a natural member of *N. sloanii*'s digestive tract microbiota regardless of sex, *Mycoplasma's* function in the digestive tract of the squids remains to be investigated more clearly.

Meanwhile, fewer studies have reported the presence of *Brachybacterium* in marine invertebrates, and no studies to date have reported it in cephalopods, much less in deep-sea squids. *Brachybacterium* has been reported in coastal and open oceans (Kim et al., 2015; Kaur et al.,2016; de Castro et al., 2023), manganese nodules in the Pacific Ocean (Wang et al., 2009), Asian sea bass (Orsod et al., 2012), marine sponges (Radjasa

& Sabdono, 2006), and macroalgae (Alvarado & Leiva, 2017). The *Brachybacterium* isolated from Asian sea bass is believed to support the host's antimicrobial defence mechanisms (Orsod et al., 2012). Similarly, the secondary-metabolite-producing *Brachybacterium* found in sponges from Indonesia and macroalgae from Antarctica is thought to have antibacterial activity against other pathogenic bacteria (Radjasa & Sabdono, 2006; Alvarado & Leiva, 2017). While the abundance and function of *Brachybacterium* in the digestive tracts of both female and male *N. sloanii* remains unclear, it is possible that it also serves a beneficial purpose in maintaining host health by helping to counteract pathogenic bacteria present in their food.

The presence of the cyanobacterium *Synechococcus* CC9902 in *N. sloanii* digestive tracts is a first for a cephalopod microbiota study. While it is a relatively well-reported marine bacterium, having been previously isolated in coastal waters (Hamilton et al., 2014) and marine invertebrates (Taylor et al., 2007; Daley et al., 2016; Biessy et al., 2020; Mahadevan & Middlebrooks, 2020; Seiblitz et al., 2025), it is mainly reported as being closely associated with photosynthesis and has been reported to increase organic carbon availability to its host (Taylor et al., 2007; Mahadevan & Middlebrooks, 2020). With the primary purpose of *Synechococcus* CC9902 having been reported as playing a role in photosynthesis, it is possible that it was living opportunistically in the digestive tract of *N. sloanii*, potentially having been in the seawater or prey the squids ingested prior to being caught. Meanwhile, the genus BD1-7 clade, found in both the female and male *N. sloanii* digestive tract microbiota, has been previously reported as an abundant taxon in the gills of oegopsid squids (Hu et al., 2022b). The BD1-7 clade has been reported as an abundant oligotrophic marine bacterium and crucial to the health of its associated hosts, such as cephalopods (Hu et al., 2022b), bryozoans (Figuerola et al., 2025), and gorgonians due to its carbon utilisation rate for various carbon sources (Cho & Giovannoni, 2004). Although the direct function of the BD1-7 clade in the *N. sloanii* digestive tract is unknown, we could potentially infer that it is connected to some form of carbon remineralisation and uptake for the host organism.

The digestive tract microbiota of *N. sloanii* showed no significant sex-specific variability. This was surprising as *N. sloanii* are known for having sex-specific, distinct feeding habits (Dunn, 2009). While niche-specific preferences have been noted for female and male *N. sloanii* due to diet, all squids were caught at the same depth and time, which could

account for the lack of niche-specific sexual difference in the digestive tract microbiota for the sampled individuals at the time of capture. As Jackson et al. (2000) reported, there was a separation of *N. sloanii* stock based on depth. Additionally, it has been reported in other squid species, such as *Moroteuthopsis ingens*, that the difference in female and male diets is due to females eating larger prey and ingesting a greater biomass; however, both sexes consume the same prey species (Jackson et al., 1998). If this is similarly true for *N. sloanii*, it could explain the lack of difference in the microbiota of their digestive tract, as they consume the same type of prey, just in different quantities and sizes.

2.5.1 Limitations

Due to sample availability and attempts to minimise confounding factors such as capture location, time, and depth, ten individual squids were sampled for this study. This is comparable with other studies on squid microbiota, such as Guerrero-Ferreira et al. (2013) and Kang et al. (2022) and appears sufficient for an initial survey of the microbiota. However, some female samples failed to sequence and had to be excluded, thereby reducing the robustness of the statistical analysis. Variations in the microbiota of male and female cephalopods, where observed, have been suggested to result from differences in body mass, diets, and behaviour (Kang et al., 2022). While these results represent a baseline for comparison with other ommastrephid digestive tract microbiota, and with other body sites in *N. sloanii*, in future it would be interesting to explore a greater sample size of *N. sloanii* from different locations, depths, and ontogenetic stages to gain a more complete picture of this species' microbiota across its distribution and lifespan. However, deep-sea specimens for microbial analysis are often limited due to the inaccessibility of samples, and while more work is needed for further in-depth analysis, this is the first study to investigate the microbiota of Aotearoa's cephalopods.

2.6 Conclusion

Brachybacterium and *Mycoplasma* are the primary taxa observed in the digestive tract of both the female and male *N. sloanii*. The gut microbiome plays a key role in food digestion and the release of necessary metabolites for the host organism, and diet is an important factor in shaping the gut microbiome. This suggests that the observed female

and male *N. sloanii*’s diets were composed of similar prey, or of prey that required similar microbial gut compositions.

Future research should explore different anatomical parts, such as the gills, inner eye fluid and reproductive organs, to determine whether sex-specific microbiota differences are observed at other anatomical sites, possibly driven by vertical niche separation in the water column. Studying differences in the microbiota of other deep-sea squid species could reveal whether this is a trend among oegopsid squids or unique to *N. sloanii*. Understanding deep-sea squids’ microbiota could help predict their responses to environmental stressors, such as climate change, and offer valuable insights into their life history in their hard-to-reach habitat.

Chapter 3 Prelude

Investigating the variability in the digestive tract microbiota of female and male *N. sloanii* revealed a high abundance of *Brachybacterium* and *Mycoplasma,* with no observable statistically significant sex-derived differences. This sample size of ten *N. sloanii* and three body sites showed consistent results in bacterial community composition, thus allowing me to study three individuals of four different species of Aotearoa ommastrephid squids: *Nototodarus sloanii, Nototodarus gouldi, Todarodes angolensis,* and *Todarodes filippovae* (N = 12). This was done to explore the variability between congeneric and confamilial species of deep-sea squids. Since there was no difference between the three body sites chosen from the digestive tract of *N. sloanii* in chapter two, the stomach content, stomach caecum, and oesophagus were also pooled into a single digestive tract body site for chapter three. Seven more body sites were added to test for differences in the microbiota within a deep-sea squid species and among four different deep-sea squid species belonging to the same family.

Chapter 3 Intraspecific and Interspecific Variability in Deep-Sea Squid Microbiota (Family: Ommastrephidae)

3.1 Abstract

The microbiota of cephalopods have provided important insights into the life history and ecology of the host organism. Very little is known about the microbiota of ommastrephid squids, and no research has been conducted on the microbiota of *Nototodarus sloanii, Nototodarus gouldi, Todarodes angolensis,* and *Todarodes filippovae*. Twelve squids (three individuals per species) were collected and dissected for a total of 240 samples from ten body sites in duplicate. 16S rRNA gene amplicon sequencing was used to investigate variability in the microbiota between congeneric and confamilial species of deep-sea squids. The microbial community composition within a genus was quite similar. There was no significant difference in alpha and beta diversity among congeneric species and alpha diversity between confamilial species. However, there was a significant difference observed in the beta diversity between squid genera within the same family. *Mycoplasma,* the BD1-7 clade, and *Brachybacterium* were the most abundant taxa observed in the microbiota of all four deep-sea squids investigated. Overall, *Mycoplasma* was primarily reported in the beak samples of *Nototodarus* and *Todarodes*. *Brachybacterium* was noted in the brain, digestive tract, and inner eye fluid samples, and the BD1-7 clade was mainly observed in the gills and reproductive organs of the squids. Investigating the microbiota of deep-sea squids is necessary for a better understanding of their health and the health of their surrounding environment.

3.2 Introduction

The deep sea (depth 200–11,000 m) accounts for ~95% of the total living space on Earth, yet it remains one of the least-researched habitats. In this environment, cephalopods—especially squids—thrive and play key ecological roles as predators and prey (Matsui et al., 1988). Much of our understanding of the ecology and biology of deep-sea cephalopods is inferred from their predators and from the gut contents and morphology of dead specimens collected from fisheries activities through targeted efforts and as bycatch (Cherel, 2020; Xavier et al., 2022). Recently, coastal cephalopod studies have been conducted on their microbiomes, which provided insight into their ecology and physiology (Lutz et al., 2019; Nyholm & McFall, 2021; Vijayan et al., 2024). This would offer a novel way to gain information on deep-sea specimens; however, the majority of microbiota studies to date have been on myopsid squids in shallow coastal ecosystems, and there are currently only four studies published on deep-sea oegopsids (Ramírez et al., 2019; Hu et al., 2022a; Hu et al., 2022b; Kang et al., 2022).

Microbiomes offer the chance to gain insight into the biology of hard-to-observe animals (Sonnega & Sheriff, 2024). Because the microbiota can also be analysed post-mortem, specimens can be either commercially caught or bycaught, which is advantageous for research on deep-sea cephalopods. Microbial symbionts can affect the host organisms in a variety of ways: morphology of organs (McAnulty et al., 2023), immune system development (Taschuk & Griebel, 2012), susceptibility to certain diseases (Honda & Littman, 2012; Burgos et al., 2020), reproduction (Montgomery & McFall-Ngai, 1994), behaviour (Shropshire & Bordenstein, 2016), and spatial movement (Roura et al., 2017). For example, the previously elusive larval migration from the coast to the open ocean of the common octopus (*Octopus vulgaris*) was investigated using the gastrointestinal microbiota (Roura et al., 2017). Although microbes have been successfully used to understand the life history of some host cephalopods (e.g., octopuses, Roura et al., 2017; cuttlefishes, Lutz et al., 2019; and bobtail squids, Kamp et al., 2025), these studies have been limited to coastal species.

Host–microbe interactions have major implications for the health and function of the host organism (Rosenberg et al., 2007; McFall-Ngai et al., 2013; McAnulty et al., 2023). Mutualistic relationships have been observed between cephalopod host species and various microbes, which appear to play important roles in cephalopod bioluminescence, reproductive development, and digestion (Anderson et al., 2014; Kang et al., 2022; McAnulty et al., 2023). The study of cuttlefish microbiota has contributed to our understanding of interactions between hosts and symbionts in the context of cephalopod mariculture (Lutz et al., 2019). One notable example of such a symbiotic association is found in the 'Hawaiian bobtail' (*Euprymna scolopes*), which has a well-documented relationship with the bacterium *Aliivibrio fischeri* (Nyholm & McFall-Ngai, 2021). The Hawaiian bobtail provides nutrients and protection for *A. fischeri*, while the bacterium emits light, providing countershading that helps reduce the squid's visibility to predators (Lasa & Romalde, 2021). Similarly, in other cephalopod species such as

Octopus mimus and *Doryteuthis pealeii*, certain bacterial groups such as Alphaproteobacteria and Gammaproteobacteria are crucial to reproductive processes and the defence of eggs against fouling (Barbieri et al., 2001; Iehata et al., 2016; Kerwin & Nyholm, 2017; Lutz et al., 2019). While bacteria provide insight into the evolution and physiology of cephalopods, very little information is known about the microbiota in deep-sea oegopsids.

One of the most widely distributed (from the sub-Arctic to sub-Antarctic waters) and abundant deep-sea oegopsid families is the Ommastrephidae (Hoving et al., 2014). The majority of the ommastrephids have a complex spatial distribution and are subdivided into feeding areas and reproductive zones (Roper et al., 2010). As with most other oegopsid squids, ommastrephid juveniles are found in the upper pelagic levels, and the adults are found in the deeper mesopelagic zone and at the bottom of the continental slope (Villanueva, 1992). This family contains ecologically and commercially important squid genera (Roper et al., 2010), such as the flying squids (*Todarodes*) and the arrow squids (*Nototodarus*). In Aotearoa's waters, *N. sloanii* and *N. gouldi* are the largest cephalopod fishery, with annual landings of 14.332 t in 2022/2023 (MPI, 2023). The response of arrow squids to climate change, therefore, has economic and ecological implications and is very likely to be influenced by their microbiota, as has been seen in other marine organisms (Lesser et al., 2016; Apprill, 2017; Scanes et al., 2021).

There are three species in the genus *Nototodarus* (ML 420mm; Roper et al., 2010): *N. hawaiiensis, N. gouldi*, and *N. sloanii*. *Nototodarus hawaiiensis* has a patchy distribution in Indo-Pacific waters, but does not occur in Aotearoa's Exclusive Economic Zone (EEZ). The arrow squids found in Aotearoa's EEZ have distinct but overlapping distributions (Roper et al., 2010). *Nototodarus gouldi* occurs in the northern half of Aotearoa's waters and across the Tasman Sea to Australia. The endemic *N. sloanii* is found in the southern part of the EEZ, with a distribution that extends to the Chatham Rise, a submarine ridge known to be a productive zone with high species richness and diversity (Jackson et al., 2000; Bull et al., 2001; Dunn, 2009). *Nototodarus sloanii* is considered a keystone species in pelagic ecosystems due to its vast abundance and role as both predator and prey (Lischka et al., 2020). These squids primarily forage in the mesopelagic layers (200–1000m), predominantly preying on mesopelagic fishes, crustaceans, and other cephalopods (Dunn, 2009; Pethybridge et al., 2012).

The ommastrephid genus *Todarodes* (which currently contains five species) is important for fisheries in Japan (*T. pacificus*), but species in this genus are not commercially fished in Aotearoa. Two large-bodied (up to 540mm ML) *Todarodes* occur in Aotearoa's EEZ: *T. angolensis* and *T. filippovae*. *Todarodes angolensis*, the Angolan flying squid, has a sub-Antarctic circumpolar distribution, which overlaps with the most southern distribution of *T. filippovae* (Braid, pers. comm.). There has been historical confusion and debate about the identity and distribution of these two species (Roper et al., 2010). However, within Aotearoa, *T. filippovae* occurs within and north of the subtropical convergence, while *T. angolensis* has a more southern distribution (although there is some overlap on the Chatham Rise and Campbell Plateau). Examining the microbes of these species could help us determine whether physiology or location is a driving factor in their symbiotic bacterial communities.

This study aims to use microbial communities to provide insight into the life history and ecology of deep-sea oegopsids. This chapter focuses on two genera of ommastrephids from Aotearoa waters: the 'arrow' squids (*N. sloanii and N. gouldi)* and the 'flying' squids (*T. filippovae* and *T. angolensis).* The study investigates the microbiome of these species using 16S rRNA gene amplicon sequencing. I aim to investigate: (1) how the microbiota compare between two pairs of congeneric species (*Nototodarus* spp. and *Todarodes* spp.); and (2) how host-associated microbial communities compare within a deep-sea squid family (comparing all four ommastrephid species).

3.3 Methods

3.3.1 Sample collection

Squid samples (*N. sloanii, N. gouldi, T. angolensis,* and *T. filippovae*; Table 3.1) were collected off the coast of Aotearoa by Earth Sciences New Zealand (formerly NIWA) under their permits during routine fishery surveys around Te Waipounamu (South Island) and stored at –20ºC (Figure 3.1). Samples were collected in the austral summer, except for *N. gouldi*, which were all collected during the austral winter. All samples were frozen at sea after capture and maintained until thawed for the first time (for sampling), thereby maintaining cold chains to preserve the *in-situ* microbiota.

Table 3.1: Biological and geographic information for specimens used in this study.

Squid Species	Squid ID	Location	Mantle Length(mm)	Weight(g)	Water Depth(m)	Latitude	Longitude
Todarodes angolensis	TAG1	sub-Antarctic	375	2000	700	49.5 S	168.6 E
Todarodes angolensis	TAG2	sub-Antarctic	380	2000	700	49.0 S	167.1 E
Todarodes angolensis	TAG3	sub-Antarctic	416	2000	952	46.9 S	170.3 E
Todarodes filippovae	TSQ1	sub-Antarctic	440	2000	662	51.8 S	167.9 E
Todarodes filippovae	TSQ2	Chatham Rise	487	2600	871	42.6 S	175.4 E
Todarodes filippovae	TSQ3	Chatham Rise	495	2400	885	43.2 S	174.2 E
Nototodarus sloanii	NOS11	Chatham Rise	242	416	314	43.2 S	175.5 E
Nototodarus sloanii	NOS12	Chatham Rise	296	625	314	43.2 S	175.5 E
Nototodarus sloanii	NOS13	Chatham Rise	232	551	314	43.2 S	175.5 E
Nototodarus gouldi	NOG1	West Coast South Island	275	523	333	41.1 S	171.0 E
Nototodarus gouldi	NOG2	West Coast South Island	285	753	333	41.1 S	171.0 E
Nototodarus gouldi	NOG3	West Coast South Island	265	531	333	41.1 S	171.0 E

Three individuals per species (*N. sloanii, N. gouldi, T. angolensis,* and *T. filippovae*; N = 12) were chosen as previous work on *N. sloanii* revealed relatively low variation between individuals, including those of different sexes (chapter two). Because specimens were opportunistically collected, selection based on sex was not possible. As a result, all specimens (except for a single *N. gouldi*) were female. For each specimen, ten body sites were sampled in duplicate: stomach caecum, stomach contents, oesophagus, inner and outer beak, surfaces, gills, brain, inner fluid of the eye, siphon, and reproductive organs (N = 240). Samples representing the digestive tract (stomach caecum, stomach contents, and oesophagus) were later pooled to equal volume, which resulted in a total of eight body sites for data analysis.

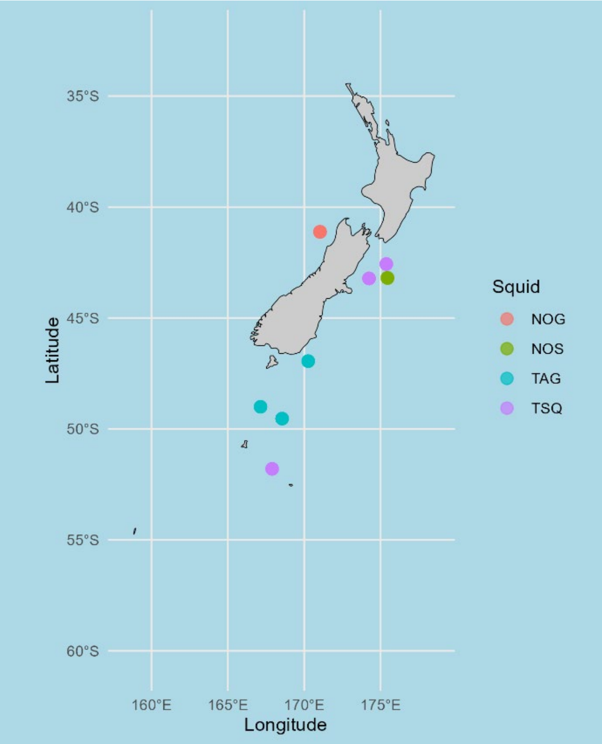


Figure 3.1: Sampling locations for squid specimens used in this study. NOG = N. gouldi*, NOS =* N. sloanii, *TAG =* T. angolensis*, TSQ =* T. filippovae *(R package rnaturalearthdata ver 1.0.0; South et al., 2024).*

3.3.2 16S rRNA gene amplicon sequencing

The DNA extraction and 16S rRNA gene sequencing was performed following the same protocol and modifications as in section 2.3.2. For this dataset, 15 samples failed to show any visible amplification in the gel and gave less than 1 ng/µL of PCR product for sequencing. All samples were sent for sequencing, but these 15 samples were removed during the data processing steps due to insufficient reads. As in section 2.3.2 before 16S rRNA library preparation, each pair of duplicates was pooled to equal volume, resulting in a total of 96 samples, 24 per species.

3.3.3 Data processing

Data processing was conducted following the same protocol as in section 2.3.3, with the following modifications: 15 samples were removed after the filtering stage as they had zero reads post-filtering, and during the rarefaction step, 15 additional samples were lost due to insufficient reads for the alpha diversity. A Mann–Whitney *U* test was used to compare Shannon's alpha diversity index between species of the same genus and genera of the same family (Ilstrup, 1990). A Principal Coordinates Analysis (PCoA) using Aitchison dissimilarity distance matrix with the 'vegdist' command from vegan (Oksanen et al., 2025; ver 2.7-1) was employed on the raw data to visualise beta diversity (Martino et al., 2019), with significance tested between species and genera using a PERMANOVA test using the 'adonis2' command from vegan; the robustness of the PERMANOVA results was verified with a PERMDISP test using the 'betadisper' and 'permutest(beta)' commands also from vegan (Bakker, 2024). The impact of geographic location on the microbiota was visualised using the top ten relative abundance of proportion of reads returned to describe the bacterial community present, as the small sample size prevented a robust statistical analysis.

3.4 Results

Across the four ommastrephid squid species, 16S rRNA gene amplicons were sequenced from 96 samples representing eight body sites, of which 66 samples were successfully sequenced. 4,820,120 reads were obtained, and 5734 ASVs were identified.

3.4.1 Overall microbial richness

No significant difference was seen in Shannon's diversity estimates between congeneric species pairs (Mann–Whitney *U* test: *Nototodarus*, W = 180, *P* = 0.98; *Todarodes*, W = 215, *P* = 0.61) or between confamilial genus pairs (Mann–Whitney *U* test: W = 632, *P* = 0.058; Figure 3.2).

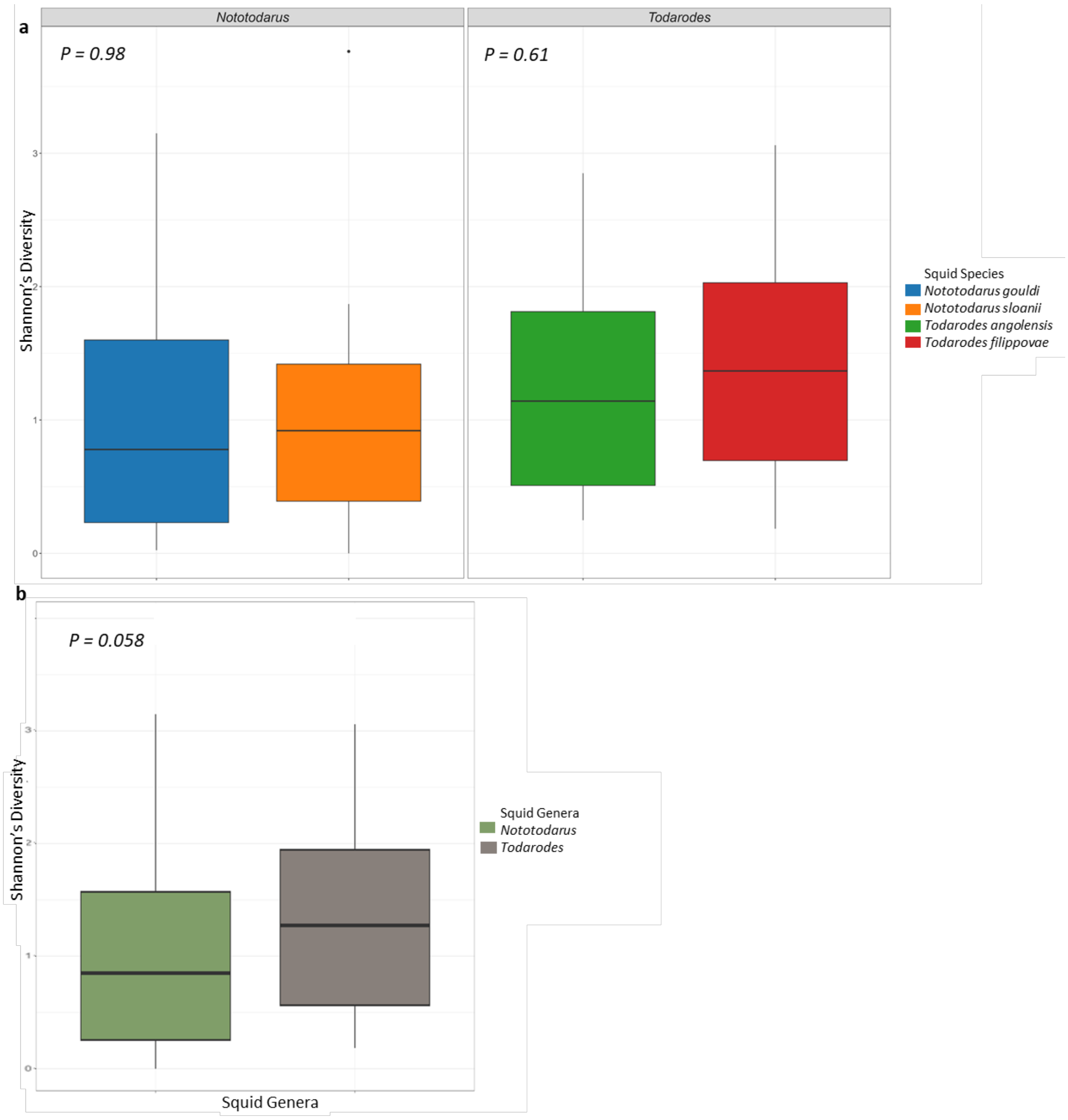


Figure 3.2: Shannon's diversity index with a Mann–Whitney U *test for significance (a) among species within the same genera and (b) between genera within the same family. Boxes represent the interquartile range (IQR, 25–75%) of the data, with median values indicated by the bar within each box, and whiskers show values within 1.5 times the IQR.*

3.4.2 Microbial community composition

No significant difference in beta diversity was observed between *T. angolensis* and *T. filippovae* (PERMANOVA $R^2 = 0.004$, $F_{47} = 0.172$ and $P = 0.893$; Figure 3.3a), nor was there a difference between *N. sloanii* and *N. gouldi* (PERMANOVA $R^2 = 0.008$, $F_{59} = 0.522$ and $P = 0.642$; Figure 3.3b). There was considerable overlap in the 95% confidence ellipses between the two *Nototodarus* species; however, *N. sloanii* exhibited more dispersion. Although the ellipses also overlap with *Todarodes*, *T. angolensis* clustered within *T. filippovae* when using 95% confidence ellipses (Figure 3.3a).

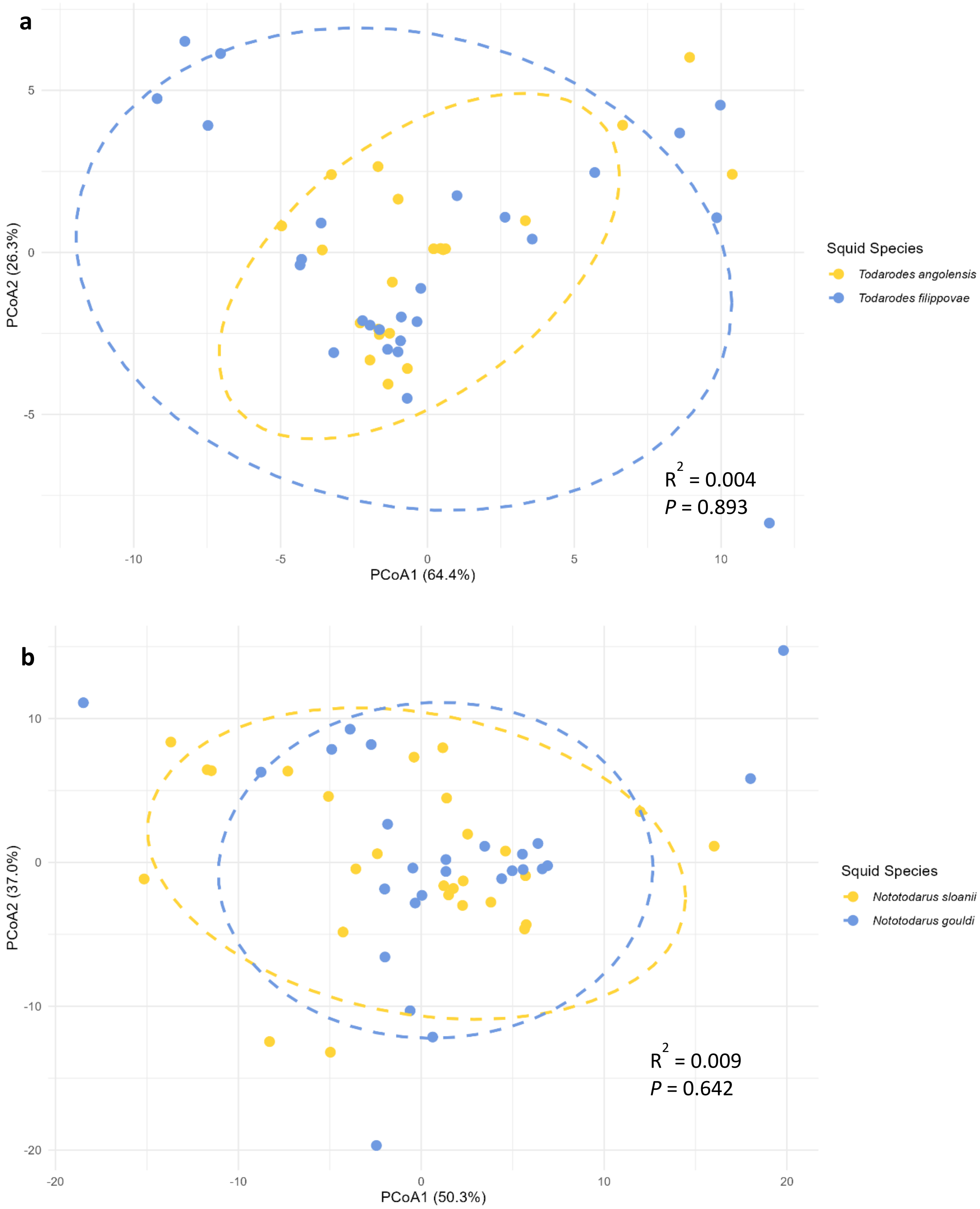


Figure 3.3: Principal coordinates analysis (PCoA) of Aitchison distances between samples, with the 95% confidence ellipses assuming multivariate t-distributions. (a) beta diversity between Todarodes angolensis *and* T. filippovae*; and (b) beta diversity between* Nototodarus gouldi *and* N. sloanii.

3.4.3 Microbial taxa observed

Overall, the whole dataset consisted of five bacterial genera with >1% mean relative abundance: *Brachybacterium*, *Mycoplasma*, BD1-7 clade, *Pseudoalteromonas*, and *Aurantivirga. Brachybacterium* was observed in the digestive tract, brain, and inner eye fluid of all four squids at differing abundances. The digestive tracts of *T. filippovae* and *T. angolensis* were the most heavily dominated by *Brachybacterium* (over ~80%) compared to *N. sloanii* and *N. gouldi,* which both had less than 50% (Figure 3.4). All four species also had a high proportion of *Brachybacterium* in the brain and inner eye fluid. Although *T. angolensis* and *N. gouldi* had the highest relative abundance of *Brachybacterium* in the brain, they had the lowest relative abundance in their inner eye fluid (Figure 3.4). The relative abundance of this bacterium in *T. filippovae* and *N. sloanii* was relatively similar between the brain and inner-eye fluid (Figure 3.4).

Mycoplasma was recovered in all squid species in this analysis. The inner and outer beak surfaces in *N. sloanii*, *N. gouldi,* and *T. angolensis* were dominated by *Mycoplasma* (>88%; Figure 3.4), as were those of *T. filippovae* but at a lower relative abundance (<62%). *Mycoplasma* was also observed in *Nototodarus* digestive tracts, with *N. gouldi* showing over twice the abundance (Figure 3.4). The lower abundance of *Mycoplasma* (5.80%) in the gills of *N. gouldi* was driven by a single individual (Appendix D Figure 7). Low abundances were also observed in the reproductive organs of *N. gouldi* (13.91%) and the siphon of *T. filippovae* (16.10%).

The BD1-7 clade was the third most abundant taxon sequenced in all squids. This clade was observed in high abundance (above 85%) in the gills of all four squid species. Although the BD1-7 clade was of average abundance (less than 50%) in the reproductive organs of most squid species, it was reported as having a much greater abundance in *N. sloanii* (97.02%). The digestive tract of *N. sloanii* contained a relatively higher abundance of BD1-7 (20.35%) compared to other reported squids, which was mainly driven by a single individual (Appendix D Figure 7). The most abundant taxon observed in the siphons of *Todarodes filippovae* was the BD1-7 clade, making up ~25% of the relative abundance (Figure 3.4).

Unique bacteria were recovered from various body sites of some squid species. The inner eye fluid of *N. gouldi* contained a high abundance of *Pseudoalteromonas* (46.85%),

and very low levels of *Psychrobacter*. *Psychrobacter* was the second most abundant bacterium in the inner eyes of *Todarodes*. In *T. angolensis*, the abundance of *Psychrobacter* (20.80%) was representative of all sequenced individuals, while the abundance in *T. filippovae* was driven by a single individual. Similarly, *Aurantivirga*, reported in the inner beak and outer beak of *T. filippovae* (~30%), was driven by a single individual whose beak microbiota was heavily dominated by *Aurantivirga* (> 95%). The female reproductive organs were the most diverse site (with taxa grouped as 'other') for most species except for *N. sloanii*, which had the highest diversity found in the brain (~10%). The female reproductive organs of both *T. filippovae* and *T. angolensis* contained the highest diversity (>45%) of the reported taxa for each species. Low levels of *Photobacterium* were reported (<1%) across samples, with the exception of the *T. angolensis*'s siphon (21.92%), which was primarily driven by the high abundance observed in a single specimen.

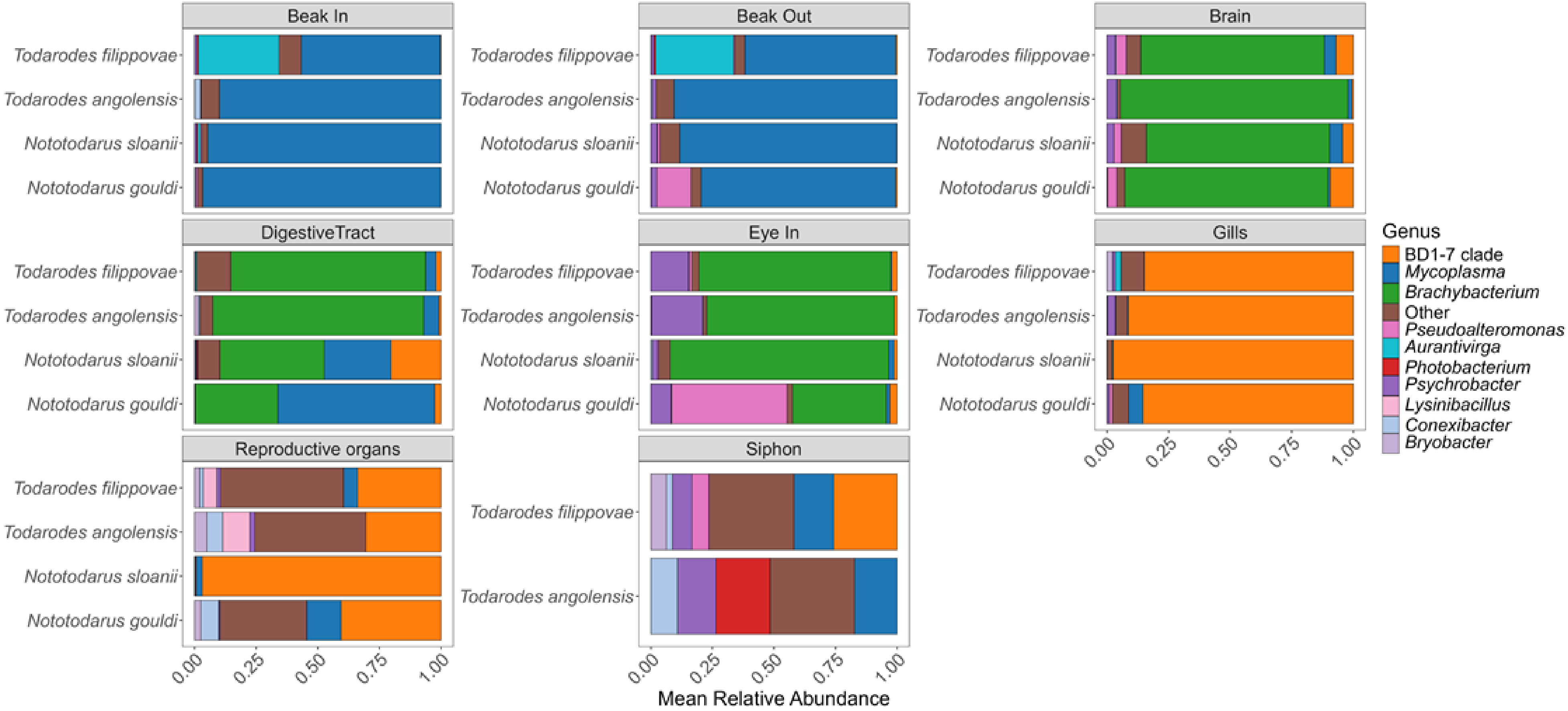


Figure 3.4: Top ten most abundant bacterial genera within the eight body sites of N. sloanii, N. gouldi, T. angolensis*, and* T. filippovae*, with the other genera grouped into "other". Only* Todarodes *spp. had sequences for the siphon.*

3.4.4 Microbial composition between genera of the same family

The microbial diversity between *Nototodarus* and *Todarodes* was observed to be significantly different. This diversity was possibly driven by differences in dispersion (PERMANOVA $R^2 = 0.028$, $F_{108} = 3.067$ and $P = 0.039$), which was seen in the close central clustering of *Todarodes* within the larger, more spread-out *Nototodarus,* as seen by the 95% confidence ellipses (Figure 3.5). However, dispersion was unequal between groups, meaning that the significant difference between genera of the same family could be due to dispersion differences and/or community differences (PERMDISP $F_{108} = 5.228$, $P = 0.012$). The gill samples, regardless of genus, were separate from the rest of the body sites.

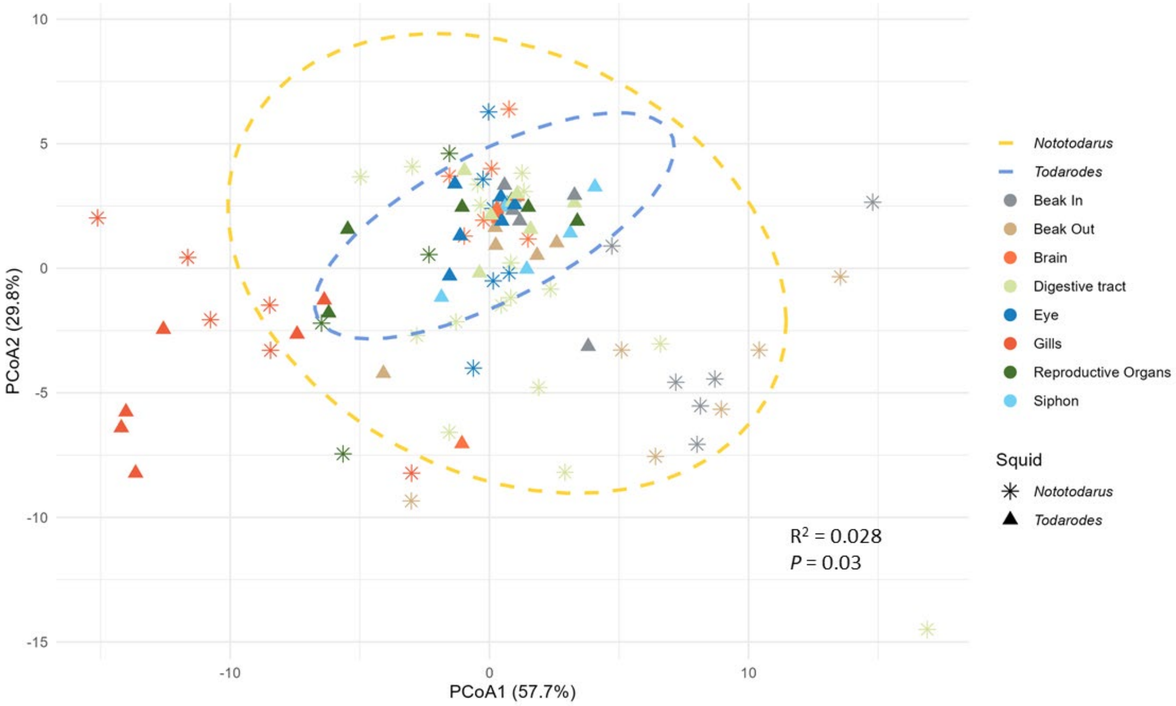


Figure 3.5: Principal coordinates analysis (PCoA) of Aitchison distances between samples. The shapes represent the two genera, Todarodes *and* Nototodarus*, with the different body sites represented by colour. The ellipses represent 95% confidence intervals assuming multivariate t-distributions for each genus.*

In both *Todarodes* and *Nototodarus,* the most abundant bacteria varied noticeably by body site, and some similarities were observed between squid genera, but also some notable differences. The inner and outer beaks of *Todarodes* and *Nototodarus* were dominated mainly by *Mycoplasma* (>69%). The notable abundance of *Aurantivirga* in *Todarodes* (~20%) was driven by its high presence in *T. filippovae*, while the abundance

of this bacterium was below 1% in the beaks of *Nototodarus*. The outer beak of *Nototodarus* had a higher abundance of *Pseudoalteromonas* (~7%) compared to *Todarodes* (0.27%; Figure 3.6). A high abundance of *Brachybacterium* was found in the digestive tract, inner eye, and brain of *Todarodes* and *Nototodarus* (Figure 3.6). However, while *Brachybacterium* made up more than 80% of the digestive tract of *Todarodes,* it was only ~38% of the taxa found in *Nototodarus'* digestive tract. The rest of the microbial diversity in the digestive tract of *Nototodarus* was mainly attributed to *Mycoplasma* (45.36%) and the BD1-7 clade (11.46%; Figure 3.6). The BD1-7 clade was abundant in the gills of *Nototodarus* and *Todarodes* (>88). Although the BD1-7 clade also dominated the female reproductive organs of *Nototodarus* (~55%), this body site was mainly made up of taxa from the 'other' classification in *Todarodes* (~50%).

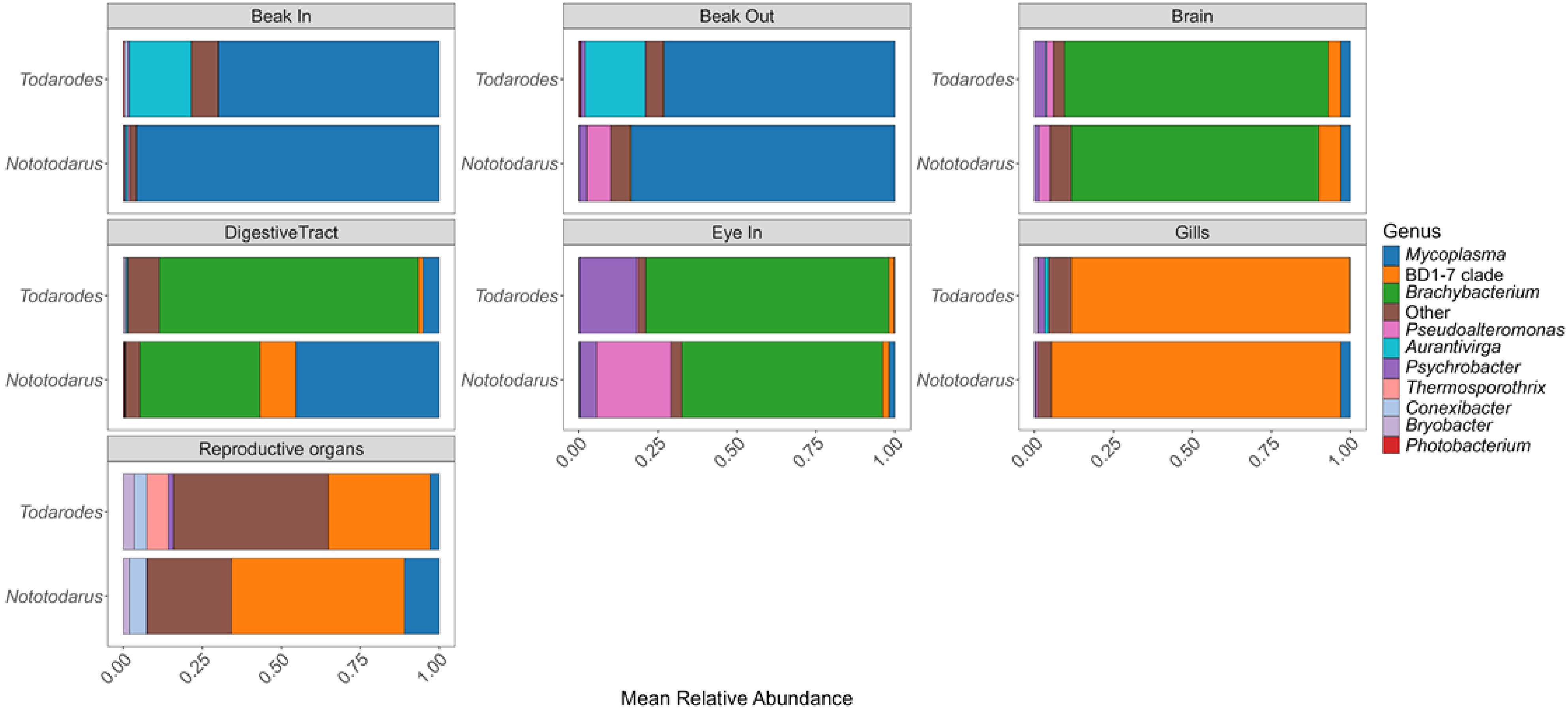


*Figure 3.6 Top ten most abundant bacterial genera within the seven shared body sites between the two genera (*Nototodarus *and* Todarodes*), with the other genera grouped into "other".*

3.4.5 Impact of geographic location on microbial composition

While there wasn’t enough data for statistical testing, a description of the bacterial community composition of *T. filippovae* between geographical locations was conducted. There was a clear difference in microbial composition between *T. filippovae* from the Chatham Rise (N = 2) and the sub-Antarctic (N = 1). Both the Chatham Rise and the sub-Antarctic digestive tract samples were primarily composed of *Brachybacterium*. In contrast, the sub-Antarctic beak samples were mainly composed of *Mycoplasma* (>96%), and the Chatham Rise beak samples were mainly composed of *Aurantivirga* (>47%), with *Mycoplasma* being the next most abundant taxon (>36%; Figure 3.7).

The inner eye fluid from the Chatham Rise was mainly composed of *Brachybacterium* (94.93%), while the sub-Antarctic sample was mostly composed of a mix of *Brachybacterium* (~44%) and *Psychrobacter* (~43%). The brain samples from both locations were primarily composed of *Brachybacterium* (>70%). Although the BD1-7 clade was seen in brain samples from both locations, it was higher in the sub-Antarctic (16.29%) compared to the Chatham Rise (2.48%; Figure 3.7). The brain samples from the Chatham Rise contained unique bacteria (*Aurantivirga*, *Mycoplasma*, *Pseudoalteromonas*, and *Psychrobacter*) that were not recovered from the sub-Antarctic samples (Figure 3.7). The Chatham Rise gill samples were dominated by the BD1-7 clade (98.79%), while the sub-Antarctic sample had a lower abundance of the BD1-7 clade (57.16%) and also contained *Aurantivirga*, *Bryobacter*, and *Psychrobacter*.

The sub-Antarctic reproductive organ sample had a high amount of *Thermosporothrix* (~40%), which was not detected in any of the Chatham Rise body sites (Figure 3.7). The Chatham Rise reproductive organs microbiota were mainly the BD1-7 clade (~45%), which was found in low abundance in the sub-Antarctic sample (~13%) where *Thermosporothrix* dominated instead. The sub-Antarctic reproductive organ sample contained 16.00% *Acidibacter*, which was not among the top 10 taxa for the Chatham Rise reproductive organ samples. However, the Chatham Rise samples contained *Bryobacter* (3.09%) and *Psychrobacter* (1.99%), which were not among the top 10 taxa in the sub-Antarctic sample.

The Chatham Rise siphon samples were primarily composed of the BD1-7 clade (37.82%), with *Mycoplasma* as the second most abundant taxon (17.04%). In contrast,

the sub-Antarctic siphon sample was more diverse, with only 1.87% of BD1-7- clade, and was primarily composed of *Bryobacter* (18.85%), *Mycoplasma* (14.22%), and *Alicyclobacillus* (14.10%), with 32.18% grouped into 'other' (Figure 3.7). Unique bacteria were found in the siphon samples from the two regions; the sub-Antarctic siphon sample contained *Alicyclobacillus, Bryobacter,* and *Thermosporothrix,* while the Chatham Rise samples contained *Pseudoalteromonas* and *Acidibacter* (Figure 3.7).

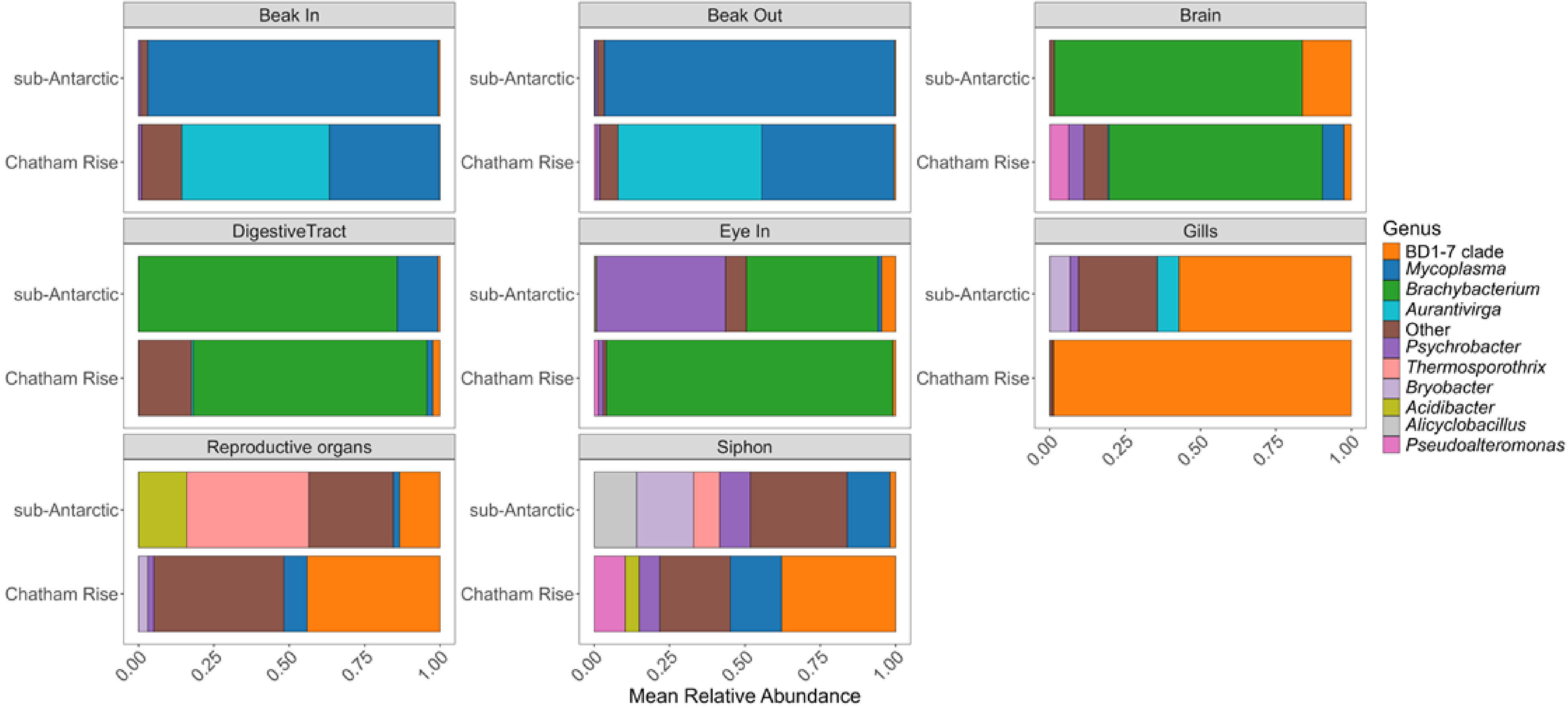


Figure 3.7: Top ten most abundant bacterial genera within the eight shared body sites of the deep-sea squid species Todarodes filippovae *between the two sampling locations of the sub-Antarctic and the Chatham Rise, with the other genera grouped into "other".*

3.5 Discussion

This is the first study to assess the differences in microbiota between congeneric species of the ommastrephid family. Herein, differences were found in the microbiota among the eight different body sites of the four species, as well as within and between genera. No significant differences were observed in the alpha diversity, measured with Shannon's diversity index, either within a genus or between genera. No significant differences were observed in the beta diversity among species of the same genus; however, there was a significant difference observed in the beta diversity between genera. Locality and seasonality may have impacted the microbial diversity. Specimens for each species were collected from a single site, except for *T. filippovae*, which was collected from the Chatham Rise and the sub-Antarctic, and showed noticeable differences in the microbial diversity between sites.

3.5.1 Body sites

The difference found in the abundance and diversity of digestive tract microbes could be linked to their varying habitat depths, which expose them to different prey. An organism's diet has been seen to be fundamental to its microbial gut composition (Liu et al., 2016; Wang et al., 2016; Ngugi et al., 2017; Osman & Weinnig, 2022; Kang et al., 2022). Although *Mycoplasma* and *Brachybacterium* were detected in most body sites in the present study, these bacteria are known to help break down and assimilate various forms of nitrogen in other marine organisms' digestive systems and protect against pathogens (Orsod et al., 2012; Rasmussen et al., 2021). Ommastrephids are known to prey on myctophid fish, crustaceans, and cephalopods (Dunn, 2009; Pethybridge et al., 2013; Clough et al., in prep). In the present study, *Nototodarus* spp. were caught at depths of ~300 meters, while *Todarodes* spp. were caught at 600–1000 meters. *Todarodes* beaks contained *Aurantivirga*, which was driven by the *T. filippovae* specimen from the sub-Antarctic. Previously, this bacterium has been isolated in the deep-sea waters of the Pacific Ocean (Song et al., 2015).

This is the first report of the reproductive organ microbiota of oegopsid squid. The female reproductive organs of the specimens in the present study were mostly dominated by the BD1-7 (which was found in a very high abundance in *N. sloanii*). In all species (other than *N. sloanii*), this body site showed the highest diversity. Some studies

have reported the microbiota of the accessory nidamental glands and egg casings of coastal myopsid squids from the family Loliginidae (Bloodgood, 1977; Barbieri et al., 2001; Yang et al., 2021; Vijayan et al., 2024). These studies have shown that there is a difference in the microbiota between species, but that location and year of collection did not affect the microbial composition of the reproductive organs (Vijayan et al., 2024), in contrast with the apparent geographic differences observed in *T. filippovae* in this study. Both the myopsid squid *Sepioteuthis lessoniana* and the oegopsid squids *T. filippovae, N. sloanii,* and *N. gouldi* reproductive organs report *Mycoplasma* (Barbieri et al., 2001; Yang et al., 2021). Apart from *Mycoplasma,* there seems to be an observable difference in the studied microbiota between myopsid and oegopsid squids.

The microbes *Brachybacterium* and *Mycoplasma* were recovered from all four species in the present study. These microbes are associated with nitrogen remineralisation, which could be linked to mobility (Tak et al., 2018; Rasmussen et al., 2021). The high mobility range of *T. angolensis* and other ommastrephid squids, such as *Todaropsis eblanae*, suggests that they have access to various nitrogen sources, and symbiotic bacteria could act as a trophic link between different marine organisms and habitats (Kremer et al., 2025). As members of the Ommastrephidae, *Todarodes* and *Nototodarus* are also powerful swimmers and mobile squid (Roper et al., 2010), but future research is needed on the link between their mobility and symbiotic bacteria. *Brachybacterium* was recovered from the digestive tract, brain, and inner eye fluid of both genera. *Nototodarus* and *Todarodes* squid have similar light absorption and bandwidth in the spectral curve of their eyes (Howard et al., in prep), which could explain the similarities in their inner eye fluid microbial compositions, which was dominated by *Brachybacterium*.

3.5.2 Impacts of sampling

Differences in season during sampling could have impacted the microbiota that was recovered. All samples were recovered in the austral summer, except for *N. gouldi* samples, which were all recovered during the austral winter. Because *Todarodes* and *Nototodarus* both live for approximately one year and have multiple spawning cohorts (Roper et al., 2010), the specimens captured at different sites would most likely have been born at different times. Although the impact of seasonality on cephalopod microbiota has not been previously evaluated, variation in the seasonality of seawater microbiota (but not annual temporal variation) has been reported (Ferrera et al., 2024; Priest et al., 2025). It is unclear whether the differences in the microbiota of *N. gouldi* were influenced by seasonal differences in the timing of specimen capture. Future studies should investigate the impact of seasonality and further assess the impact on geographic location of oegopsid squids' microbiota.

The location where specimens were captured could also have impacted the microbiota recovered from different body sites. All specimens of *N. sloanii* and two specimens of *T. filippovae* were collected from the Chatham Rise and a single *T. filippovae* specimen was collected from the sub-Antarctic. The microbiota recovered from *T. filippovae* differed markedly between sites. The Chatham Rise is a submarine ridge off the east coast of Aotearoa, which is known for being a relatively productive zone with a higher species richness and diversity compared to the sub-Antarctic (Bull et al., 2001; Dunn, 2009).

Thermosporothrix, a thermophilic bacterium (Yabe et al., 2016), was a unique microbe found in the reproductive organs and siphon of the sub-Antarctic *T. filippovae* sample. The productivity of these regions is impacted by various oceanic conditions, including temperature, salinity, nutrients, and currents (the southland current and the sub-Antarctic water; Rintoul, 2000). These differences could impact the diversity of ocean microbes and the available prey for predators, which would impact the diet of local squids (Moloney et al., 2011). Pethybridge et al. (2013) observed that differences in lipid content of the digestive gland among individuals of *T. filippovae* were linked to changes in ecosystem structure, including prey composition and spatial and temporal changes in oceanographic conditions. However, this is all speculative due to the small sample size of three individuals and a much larger sample size would be needed, as well as testing with different species in different locations, to support these hypotheses further.

3.6 Conclusion

Although differences were found between species and genera in the Ommastrephidae, these were only observed to be statistically significant between genera. The implications of the differences found herein between specimens are obscured by the current lack of available information on the observed bacteria. Future studies should focus on the functions and oceanic distribution (both geographically and vertically) of bacteria, so that more concrete links can be drawn between bacteria and their hosts. Future

research on squid microbiota should also include the collection of seawater alongside host specimens to help determine whether location-based differences in squid microbiota—such as those in the sub-Antarctic *Todarodes*—are reflecting local ambient bacteria or bacteria within food webs. Further studies on the function of the bacteria will also allow us to determine whether the functions performed by the different bacteria within the deep-sea squid's microbiota are the same and therefore serve the same purpose. Capturing baseline microbial data from hosts and their environment will be essential in understanding impacts to oceanic systems in our changing world.

Chapter 4 Prelude

In chapter three, microbial community composition was observed to be quite similar among four ommastrephid squid species (*Nototodarus sloanii, Nototodarus gouldi, Todarodes angolensis,* and *Todarodes filippovae*). However, depending on body site, there were some notable observable differences between genera within the same family, and an observable statistically significant difference in the microbial diversity. In chapter four, the four squid species from chapter three were grouped together due to their ‘muscular’ composition and lack of ammonia as a buoyancy mechanism and compared to four new deep-sea oegopsid squid species that are known for being ammoniacal: *Taningia danae*, *Taningia fimbria*, *Onykia robsoni*, and *Octopoteuthis*. Therefore, in chapter four, the microbiota of eight different deep-sea oegopsid squids with different body chemistry (ammoniacal vs non-ammoniacal) were assessed. The same eight body sites were used to investigate these species’ microbiota.

Chapter 4 The Role Ammoniacal Vs Non-Ammoniacal Body Chemistry Plays in Determining the Microbiota of Deep-Sea Squids

4.1 Abstract

For deep-sea squids, maintaining a constant position in the water column can be energetically costly. While some maintain their position through constant, active swimming (‘muscular’ squids such as ommastrephids), others achieve neutral buoyancy through specialised ammonium-filled tissues (‘ammoniacal’ squids). Research on cephalopod microbiota have demonstrated that symbiotic microbes are crucial in the development and adaptability of the host’s physiology and life history. Therefore, this study investigated whether the microbiota between four ammoniacal and four non-ammoniacal deep-sea oegopsid squid species was influenced by host body chemistry through 16S rRNA gene amplicon sequencing. A significant difference in microbial diversity was observed between the two squid groups; however, no significant difference in microbial richness was observed. The non-ammoniacal group was reported to have a higher overall abundance of the BD1-7 clade, especially in the gill microbiota. In contrast, the ammoniacal group was observed to have a higher overall abundance of *Mycoplasma*, especially in the digestive tract and gill microbiota. *Onykia robsoni* exhibited the most distinct microbial composition in both outer and inner beak samples. Understanding the microbiota of deep-sea squids and how it differs between ammoniacal and non-ammoniacal squids helps us better understand their physiology and adaptability, as *in situ* observations of deep-sea organisms are costly and rare due to the inaccessibility of the deep ocean.

4.2 Introduction

The ability to regulate buoyancy in the ocean is essential for pelagic marine organisms, enabling them to maintain position and conserve energy. Marine organisms have evolved to maintain their buoyancy in the ocean with a relatively low energy expenditure in several ways, swim bladders (fish), removing denser ions to create a less dense liquid (ctenophores), increasing surface area for increased drag (plankton), low-density oils/waxes (deep-sea sharks, sperm whales), and hydrodynamic planes (spines

in radiolarian plankton, ‘wings’ of pteropods; Molloy & Cowling, 1999). For cephalopods, maintaining a ‘fixed’ position in the water column comes at a great energetic expense (Webber et al., 2000; Seibel et al., 2004). Coastal cephalopods are thought to expend much less energy to maintain their neutral buoyancy; however, increased depth results in a decrease in locomotion and the energetic cost of neutral buoyancy increases. Therefore, having specialised buoyancy mechanisms is crucial for survival (Seibel et al., 2004). Cephalopods have been observed to achieve neutral buoyancy in marine environments through four different methods: gas-filled chambered shells, substitution of sulphate ions in seawater with chloride ions, storage of low-density fats, and special tissues filled with ammonium-rich fluids (Clarke et al., 1979; Seibel et al., 2004). This use of ammonium (which does not compress under pressure) instead of gas-filled chambers allows cephalopods to inhabit much greater depths (Clarke et al., 1979; Pratt & Anderson, 2025).

Oegopsid squids can be divided into two distinct groups based on their density in seawater (Clarke et al., 1979). ‘Muscular’ squids are denser than seawater and must continuously swim to avoid sinking; ammoniacal squids achieve neutral buoyancy by storing ammonium within various body tissues (Clarke et al., 1979; Voight et al., 1995; Pratt & Anderson, 2025). Ammoniacal squids are polyphyletic and this mechanism appears to have evolved in parallel several times (Voight et al., 1995). This is supported by the fact that different deep-sea squid families store ammoniacal fluid in various body tissues, and this distribution can also vary with developmental stage (Clarke et al., 1979). For instance, ‘glass’ squids (family Cranchiidae) have a specific coelomic cavity in which they store their ammoniacal fluid (Clarke et al., 1979; Voight et al., 1995), while some octopoteuthid squids have been reported to have high levels of ammonium in their mantles (Clarke et al., 1979). Clarke et al. (1979) theorised that differences in buoyancy among squid species reflect different lifestyle requirements. An increase in the amount of ammonium in body tissues is correlated with migration to deep waters, suggesting that ammonium levels increase with life stage, as oegopsid squids are known to expand their depth distribution as they grow (Seibel et al., 2004). The ammonium-rich fluid has a lower density than seawater, and one of the primary mechanisms for ammonium buoyancy involves the substitution of sodium ions in seawater with ammonium ions, which are lighter (Clarke et al., 1979; Seibel et al., 2004; Pratt & Anderson, 2025). This

results in different body chemistry between ammoniacal and non-ammoniacal squids, which might be reflected in their microbiota.

Invertebrates can host microbial symbionts either externally or internally, within host cells or specific symbiont cells (Kerney et al., 2011; Petersen & Osvatic, 2018). These symbionts can influence the development of host organs, the immune system (Taschuk & Griebel, 2012), reproduction (Montgomery & McFall-Ngai, 1994), behaviour (Shropshire & Bordenstein, 2016), and vulnerability to certain diseases (Honda & Littman, 2012; Burgos et al., 2020). As a result, microbial symbionts have been observed to evolve, speciate, and reproduce in tandem with their hosts. These types of mutualistic interactions between a host and their microbiota are believed to provide an evolutionary benefit; for example, deep-sea mussels (*Bathymodiolus*) have adapted to survive the extreme conditions of hydrothermal vents and cold seeps in the deep ocean thanks to their symbiotic relationship with horizontally acquired chemosynthetic methanotrophic endosymbionts in their gills (Fujiwara et al., 2000; Kellermann et al., 2012; Szafranski et al., 2014; Lasa & Romalde, 2021). Ammonium-related symbiotic microbes, such as *Mycoplasma,* have been reported in various marine organisms and environments and are crucial to host health (Rasmussen et al., 2023; Zeng et al., 2025). The limited research on cephalopod microbiomes so far suggests that microbes also play a crucial role in the physiology of squids, such as bioluminescence and egg survival in the Hawaiian bobtail 'squid' *Euprymna scolopes* (Kerwin & Nyholm, 2017; Douglas, 2019).

In cephalopods, microbial studies to date have primarily focused on commercially abundant or easily reared species, such as octopuses, cuttlefish, and bobtail 'squids'. Mutualistic relationships have been observed between these species and various microbes, which appear to play important roles in cephalopod bioluminescence, reproductive development, and digestion. One example of such a symbiotic association is found in *Octopus mimus* and *Doryteuthis pealeii*, where certain bacterial groups, such as Alphaproteobacteria and Gammaproteobacteria, play crucial roles in the reproductive system and defence of eggs against fouling (Barbieri et al., 2001; Iehata et al., 2016; Kerwin & Nyholm, 2017; Lutz et al., 2019). Additionally, investigations of the gastrointestinal microbiota of the common octopus (*Octopus vulgaris*) have provided

valuable insights into the previously elusive larval migration from the coast to the open ocean (Roura et al., 2017).

This study will investigate the microbiota of eight squid species, comprising four ammoniacal and four non-ammoniacal species, to determine whether there are significant differences in the microbial associates of deep-sea squids based on variations in their body chemistry. Thus, these observations may provide novel insights into the physiology, ecology, and behaviour of these deep-sea squids. The eight species of squid are *Onykia robsoni, Octopoteuthis* sp. nov. (“fenestra” sensu Kelly 2019)*, Taningia danae,* and *Taningia fimbria,* which are well-known ammoniacal squids, while the ommastrephid squids *Nototodarus sloanii, Nototodarus gouldi, Todarodes angolensis, and Todarodes filippovae* are non-ammoniacal and need to swim to maintain their position in the water column (Clarke et al., 1985). In Octopoteuthidae, ammonium storage is in the mantle and gelatinous layer, whereas in Onychoteuthidae, its location is currently unknown (Pratt & Anderson, 2025).

4.3 Methods

4.3.1 Sample collection

Samples of *O. robsoni, Octopoteuthis*, *N. sloanii, N. gouldi, T. angolensis,* and *T. filippovae* were collected in Aotearoa New Zealand waters by Earth Sciences New Zealand (formerly NIWA) under permit during routine fisheries surveys around Te Waipounamu (South Island) and stored at –20°C (Figure 4.1a). The *T. danae* and *T. fimbria* specimens were collected by scientific observers in waters off Huia, Tāmaki Makaurau (Auckland) and Whakaari (White Island), respectively. All samples were caught, frozen for preservation, and sampled at the first thawing, thus maintaining cold chains to preserve the *in-situ* microbiota. The microbial variation of ammoniacal and non-ammoniacal squids was examined by swabbing ten body sites (Figure 4.1b) in duplicate across 20 squids, three of each selected species (*O. robsoni, Octopoteuthis, N. sloanii, N. gouldi, T. angolensis,* and *T. filippovae*) and one each for *T. fimbria* and *T. danae,* which are large squids with limited availability of specimens. The ten sites chosen were: the stomach caecum, stomach content, and oesophagus (which were then combined to form the digestive tract), the inner and outer beak, the gills, the brain, the interior fluid of the eye, the siphon, and the reproductive organs. *Taningia fimbria* did not have any brain or siphon samples.

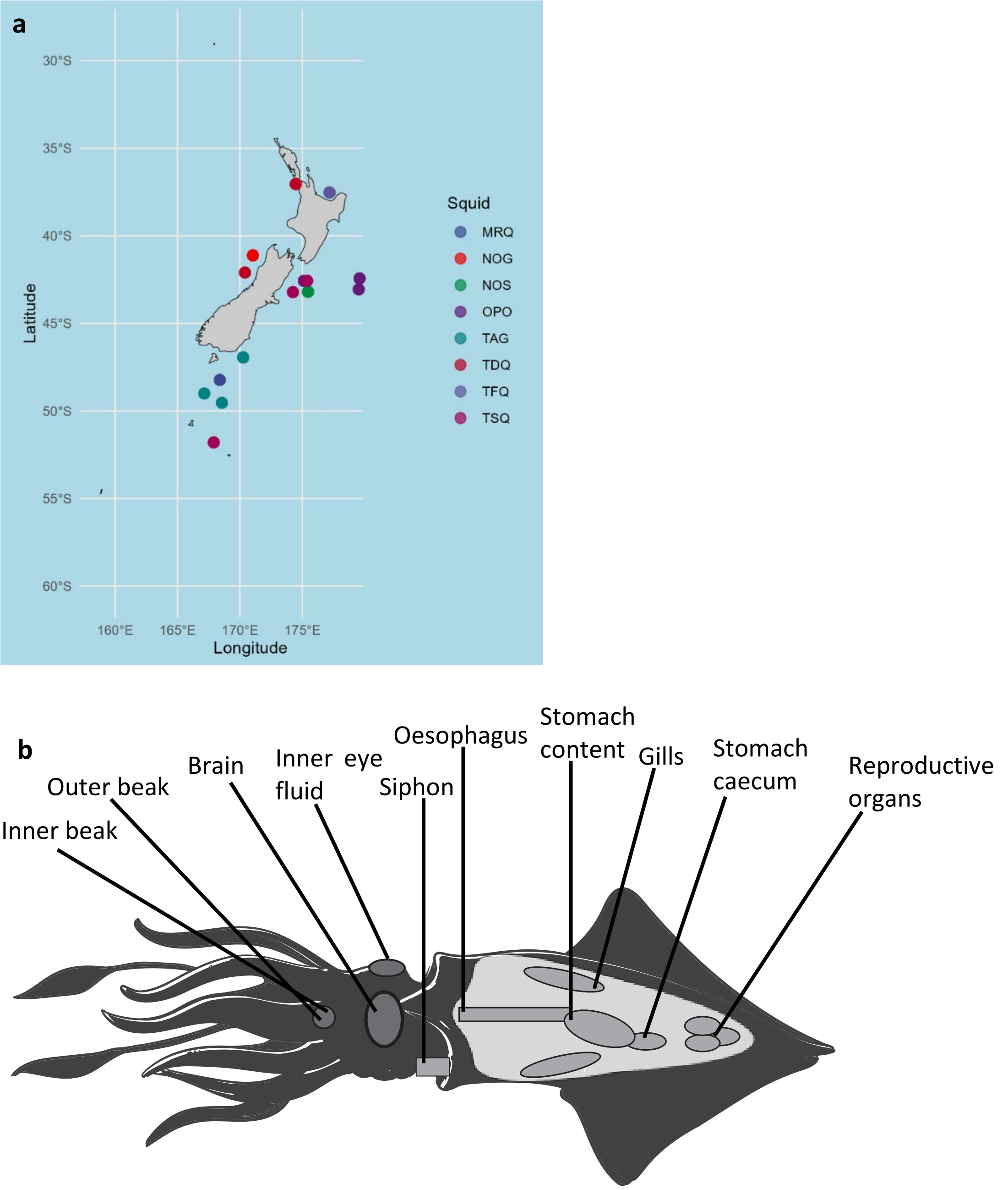


Figure 4.1: (a) Sample collection of oegopsid squids used in this study. MRQ = Onykia robsoni*, NOG =* Nototodarus gouldi*, NOS =* N. sloanii*, OPO =* Octopoteuthis*, TAG =* Todarodes angolensis*, TDQ =* Taningia danae*, TFQ =* T. fimbria*, TSQ =* Todarodes filippovae *(R package rnaturalearthdata ver 1.0.0; South et al., 2024). (b) Representation of the ten body sites sampled in this study.*

4.3.2 16S rRNA gene amplicon sequencing

The same DNA extraction protocol was followed as in section 2.3.2. The V3-V4 region of the 16S rRNA gene was amplified through PCR following the same protocol as in section

2.3.2, with the difference that before 16S rRNA library preparation, each pair of duplicates were pooled to equal volume, resulting in a total of 198 samples.

4.3.3 Data processing

Data processing was conducted following the same protocol as in section 2.3.3, with the following modifications: The error model and ASV calling were performed on a run-by-run basis to account for different empirical relationships between the assigned quality scores and the error rates associated with individual sequencing runs and machines. The ASV count tables from the two runs were then merged into a single table before the chimeric sequences were identified and removed. During the data processing, 26 samples were lost due to insufficient reads. This includes all the siphon samples for the *Nototodarus* squids. Shannon's alpha diversity index was used with a Kruskal–Wallis test and a post-hoc pairwise comparison using Mann–Whitney *U* test to explore microbial diversity differences between ammoniacal and non-ammoniacal groups and among different species within these groups (Ilstrup, 1990). Additionally, a PCoA with Aitchison distance matrix with the 'vegdist' command from vegan (Oksanen et al., 2025; ver 2.7-1) was employed on the raw data to visualise beta diversity (Martino et al., 2019), with significance tested between ammoniacal and non-ammoniacal groups using a PERMANOVA test using the 'adonis2' command from vegan; the robustness of the PERMANOVA results was verified with a PERMDISP test using the 'betadisper' and 'permutest(beta)' commands also from vegan (Bakker, 2024).

4.4 Results

Bacterial communities were successfully characterised using 16S rRNA gene sequences from 172 samples, enabling comparisons across eight body sites in eight squid species. In total, 10,191,709 reads were obtained and 5734 ASVs were identified, with significant differences observed between ammoniacal and non-ammoniacal squid taxa.

4.4.1 Microbial richness

No significant difference in Shannon's diversity index of microbes between ammoniacal and non-ammoniacal groups was observed (Mann–Whitney *U* test, W = 3692, $P = 0.065$; Figure 4.2). No significant difference in microbiota diversity was seen between the different squid genera within the non-ammoniacal group, or within the ammoniacal

group (Kruskal–Wallis test, ammoniacal H = 7.67, *P* = 0.053; non-ammoniacal H = 3.87, *P* = 0.276; Figure 4.2).

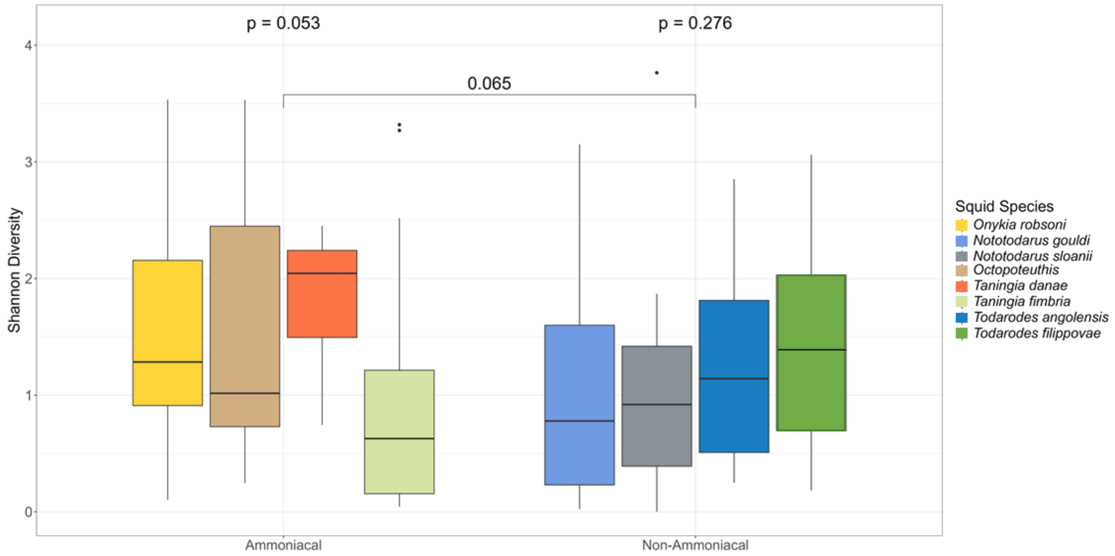


Figure 4.2: Shannon diversity of rarefied data of the microbiota of ammoniacal and non-ammoniacal deep-sea oegopsid squids. Significance within a group was tested with Kruskal–Wallis, and the significance between ammoniacal and non-ammoniacal squids was tested with a Mann–Whitney U *test. Boxes represent the interquartile range (IQR, 25–75%) of the data, with median values indicated by the bar within each box, and whiskers demonstrate the values within 1.5 times the IQR.*

4.4.2 Microbial composition

There was a significant difference observed in the bacterial community composition between ammoniacal (*O. robsoni, Octopoteuthis*, *T. danae,* and *T. fimbria*) and non-ammoniacal (*N. sloanii, N. gouldi, T. angolensis* and *T. filippovae*) squids (PERMANOVA R^2 = 0.03, F_{236} = 7.315, *P* = 0.002; Figure 4.3). However, dispersion was unequal between groups, therefore the significant difference in bacterial community composition could be due to dispersion differences and/or community differences (PERMDISP F_{236} = 6.5508, *P* = 0.008). There was a wider dispersion of *Nototodarus* points than *Todarodes* for the non-ammoniacal squids.

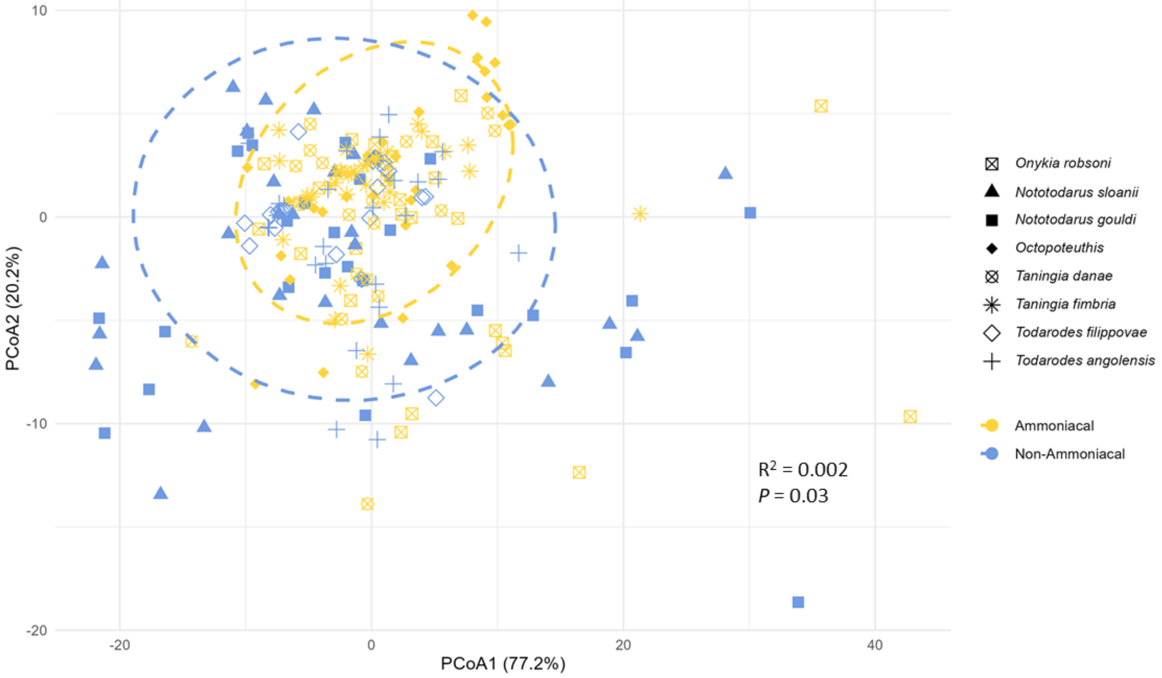


Figure 4.3: Principal coordinates analysis (PCoA) of Aitchison distances between samples, with the 95% confidence ellipses assuming multivariate t-distributions between ammoniacal (yellow) and non-ammoniacal (blue) deep-sea oegopsid squid groups. The shapes represent the individual squid species.

4.4.3 Microbial composition of different body sites

The most abundant genera observed were *Mycoplasma* and *Brachybacterium* in both ammoniacal and non-ammoniacal squids, and the BD1-7 clade mostly in the non-ammoniacal squids' gills and reproductive organs, followed to a lesser extent by *Photobacterium* in the ammoniacal squids*, Pseudoalteromonas* in the non-ammoniacal squids, and *Psychrobacter* in both (Figure 4.4). In the non-ammoniacal squid group, *N. sloanii* and *N. gouldi* siphon samples had zero reads that passed the filtering stage, and two of *N. sloanii*'s reproductive organ samples failed to pass the filtering stage. Siphon and brain samples were also not available for *T. fimbria* due to limited opportunistic sampling.

Mycoplasma and *Psychrobacter* were present in all ammoniacal and non-ammoniacal siphon samples at differing relative abundances. However, *T. angolensis* and *T. danae* also exhibited a high proportion of *Photobacterium* (22% and 67% respectively), while *T. filippovae* and *O. robsoni* contained more than 20% of the BD1-7 clade, and *Octopoteuthis* contained 53% of *Psychrobacter* (Figure 4.4*)*. The most notable difference overall between the ammoniacal and non-ammoniacal squids' siphons was the differing relative abundance of *Mycoplasma* between the two, with the ammoniacal squids having an average of 27% and the non-ammoniacal squids having an average of 17%.

The BD1-7 clade was observed in all four non-ammoniacal squid gills (>85%), and of the ammoniacal squids, only *O. robsoni* was reported to have more than >79% of the BD1-7 clade (Figure 4.4). The rest of the ammoniacal squids had a much smaller relative abundance of the BD1-7 clade in their gills, with *T. fimbria* having none, and instead they had a much higher abundance of *Mycoplasma* (>29%), except for in *O. robsoni*. *Taningia fimbria* also had a high relative abundance of *Brachybacterium* (41%), which was only present in small abundances in *O. robsoni* (3%) and not at all in any of the non-ammoniacal squids. Overall, the ammoniacal squids' gill bacterial communities' composition appeared to be more diverse in taxa than the non-ammoniacal squids, especially *T. danae* and *Octopoteuthis* (Figure 4.4).

The four non-ammoniacal squid species' female reproductive organs had a high abundance of the BD7-1 clade, with *Nototodarus* having a higher percentage than *Todarodes* (Figure 4.4). The BD1-7 clade was also present in three of the ammoniacal squids, but at much lower percentages (<22%), and was absent in *T. fimbria*. *Onykia robsoni* also had the highest abundance of *Acidothermus* (13%) in its reproductive organs. The ammoniacal squids had a much higher percentage of taxa sorted into other (*O. robsoni* had the most >50%) and thus not represented by the top ten genera. *Taningia danae* and *T. fimbria* were the only species that contained *Brachybacterium* in their reproductive organs (30% and 67%, respectively). The ammoniacal squids' reproductive organs were the most diverse compared to the non-ammoniacal squids, which were mainly composed of the BD1-7 clade (Figure 4.4).

Within the ammoniacal squid group, brain samples were not available for *T. fimbria* due to limited opportunistic sampling. Both ammoniacal and non-ammoniacal squid brains were majority *Brachybacterium* (more than 66%, except for *T. danae*, which was composed of only 13% *Brachybacterium*; Figure 4.4). *Mycoplasma* had a higher relative abundance in the brain samples of ammoniacal squids than in the non-ammoniacal squids (>11% and <6% respectively). Meanwhile, the non-ammoniacal squids contained differing amounts of the BD1-7 clade (except for *T. angolensis*), which was not present in the ammoniacal squids above 1%. In the ammoniacal squids, *T. danae* had a high

relative abundance of *Photobacterium* (51%), which was absent in all other squids. Therefore, the overall bacterial communities in the brain samples of both ammoniacal and non-ammoniacal squids had relatively similar compositions.

The eye samples of both the ammoniacal squids and non-ammoniacal squids were primarily composed of *Brachybacterium* (>51%) except for *N. gouldi* eye samples in the non-ammoniacal squids, which were mainly composed of *Pseudoalteromonas* (47%) and *T. danae* in the ammoniacal squids, which had a high percentage of *Photobacterium* (46%). *Photobacterium* was further observed in the eye of *O. robsoni* (5%). The inner eye fluid samples of the non-ammoniacal squids contained *Psychrobacter*, which was not observed in the ammoniacal squids above 2%. *Octopoteuthis* was the only squid with *Roseimarinus* observed in its body sites, with 11% in its inner eye samples (Figure 4.4).

The digestive tracts of both the ammoniacal and the non-ammoniacal squids contained *Mycoplasma* and *Brachybacterium* (Figure 4.4). The ammoniacal squids' digestive tracts were primarily composed of *Mycoplasma* (>55%). In contrast, the non-ammoniacal squids were mainly composed of *Brachybacterium* (>42%), except for *N. gouldi* which was seen to have a high amount of *Mycoplasma* (64%). The BD1-7 clade was observed in all the non-ammoniacal squids' digestive tract samples except for in *T. angolensis*; however, it was only seen in low amounts in *O. robsoni* (12%) in the ammoniacal squids. The inner and outer beaks of both the ammoniacal and non-ammoniacal squids contained *Mycoplasma*. The beak samples of *O. robsoni* had the least amount of *Mycoplasma*, but the most diverse bacterial community composition, both when compared within the ammoniacal squids and the non-ammoniacal squids (Figure 4.4). *Todarodes filippovae* samples contained *Aurantivirga* (>30%), which was not seen in any other non-ammoniacal squid at abundances greater than >2% and only in *Octopoteuthidae* in the ammoniacal squids’ beak samples (3%). The inner beak of *T. fimbria* had a high amount of *Brachybacterium* (47%) reported, while *T. danae* contained a small amount of *Moritella* (8%), not reported in any other squid beak samples. Similarly, *N. gouldi* had a small amount of *Pseudoalteromonas* (14%) present in the outer beak samples, which was not observed above 2% in any other squids’ beak samples. The most notable difference between the non-ammoniacal and ammoniacal squids was that the non-ammoniacal squids contained a small amount of *Psychrobacter* (>1%), which was not seen to be present in the ammoniacal squid samples (Figure 4.4).

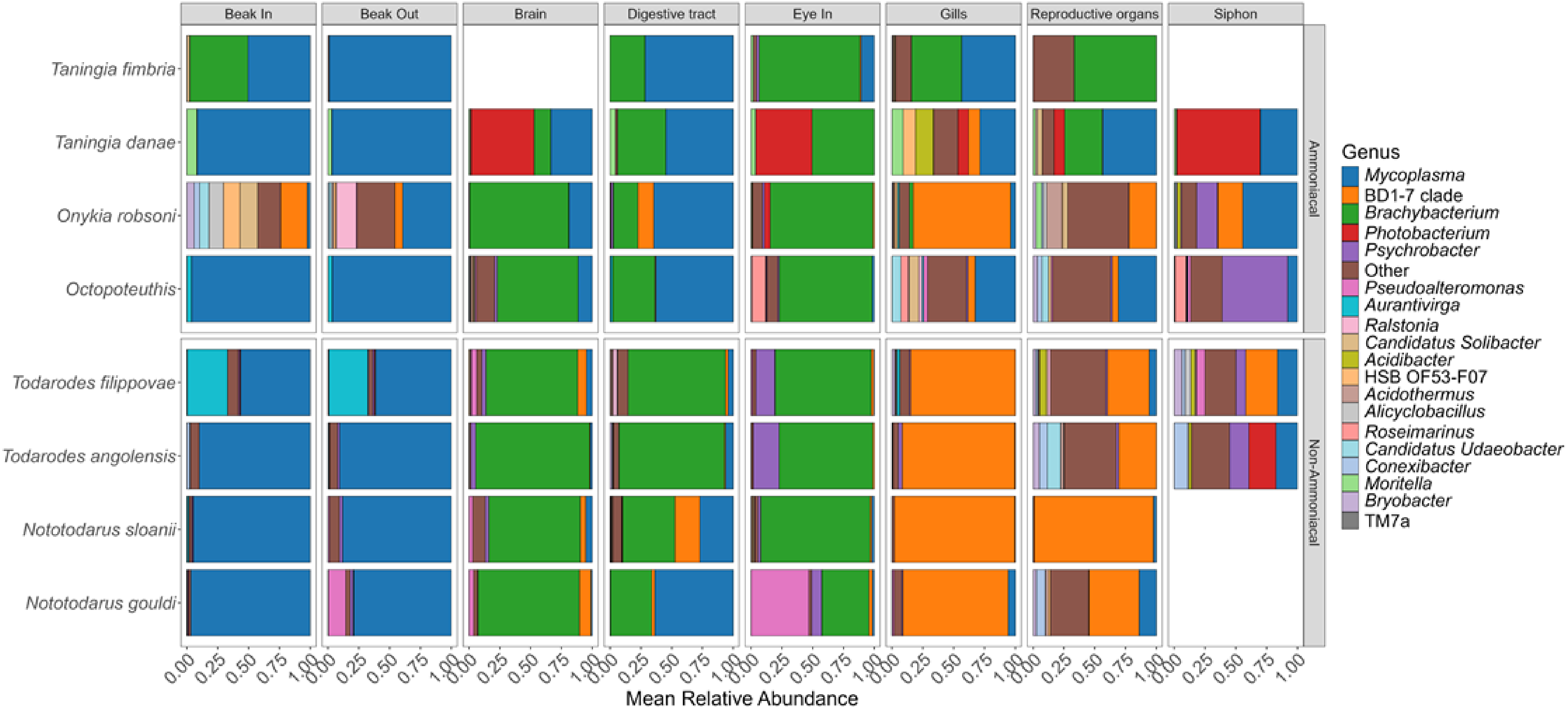


Figure 4.4: The top twenty most abundant bacterial genera within the eight shared body sites of deep-sea squid species, grouped into ammoniacal and non-ammoniacal taxa. Taningia fimbria *did not have any brain or siphon samples collected, while the siphon samples of* N. sloanii *and* N. gouldi *did not pass the filtering stage.*

4.5 Discussion

Few studies have investigated oegopsid squid microbiota, and no studies have compared how ammoniacal and non-ammoniacal squid microbiota could differ due to different body chemistry. In this study, I examined the microbiota of different body sites in two groups of squids, ammoniacal and non-ammoniacal, across eight different species and three different families of squids. Overall, the beak, inner eye fluid, and brain samples were the most similar between the ammoniacal and non-ammoniacal squids; in contrast, the digestive tract, gills, siphon, and reproductive organ samples showed the greatest differences in bacterial community composition between the two groups. *Mycoplasma*, the BD1-7 clade, and *Brachybacterium* were the most abundant taxa observed overall within both the ammoniacal and non-ammoniacal squids.

Brachybacterium is reported here for the first time both in octopoteuthid and onychoteuthid deep-sea squids, and was found in the digestive tract, inner eye fluid, and brain of all samples of observed squids, as well as in *T. fimbria*'s beak, reproductive organs, and gills, *T. danae*'s reproductive organs, and *O. robsoni*'s gills. Other studies have reported it in marine organisms, such as in Asian sea bass (Orsod et al*.,* 2012), marine sponges (Radjasa & Sabdono, 2006), and macroalgae (Alvarado & Leiva, 2017), as well as in different marine environments, both in coastal and open oceans (Kim et al., 2015; Kaur et al*.,* 2016; de Castro et al*.,* 2023), and manganese nodules in the Pacific Ocean (Wang et al., 2009). It was hypothesised to play a role in the host's antimicrobial defence mechanisms and in the production of antibacterial secondary metabolites (Radjasa & Sabdono, 2006; Orsod et al*.,* 2012; Alvarado & Leiva, 2017). While the function of *Brachybacterium* in the ammoniacal and non-ammoniacal squids remains unclear, it could play a beneficial role in helping the host's defence mechanism against pathogenic bacteria, as observed in Asian seabass and marine sponges.

Mycoplasma was observed in most body sites in both the ammoniacal and non-ammoniacal squids, with the lowest relative abundance observed in the inner eye fluid for both groups and in the gills and brain of the non-ammoniacal squids. *Mycoplasma* is considered a symbiotic, beneficial core taxon in ammoniotelic cephalopods, as it is thought to actively metabolise ammonia (Rasmussen et al., 2021, 2023). *Mycoplasma* has been reported as a symbiotic bacterium, helping with ammonia breakdown and

digestion in salmonoids by producing lactic acid and acetic acid (Bozzi et al., 2021); it has also been reported in the digestive tract of various marine vertebrates and invertebrates with the same purpose such as in deep-sea isopods (Wang et al., 2016), European abalone (Gobet et al., 2018), marine bivalves (Akter et al., 2023), salmonoids (Cheaib et al., 2021b; Rasmussen et al., 2023), and six different cephalopod species (Iehata et al., 2015; Ramírez et al., 2019; Kang et al., 2022). This would explain its presence in the digestive tract and beak of all squids, as ammonia is produced during the breakdown of food in a carnivorous diet (Boucher-Rodoni & Mangold, 1995; Seibel et al., 2004; Pratt & Anderson, 2025). The observed higher relative abundance of *Mycoplasma* in the digestive tract of ammoniacal squids could be a result of them having a higher level of ammonia in their tissues, naturally. It has been reported that a decrease in *Mycoplasma* abundance in the digestive tract of salmonids leads to an increase in pathogenic bacteria (Bozzi et al., 2021; Rasmussen et al., 2022; Scheuring et al., 2022). This was also observed in the eastern oyster, where it is thought to reduce parasitic infections by sequestering arginine (Pimentel et al., 2021; Akter et al., 2023). The ammoniacal squids were also found to have higher levels of *Mycoplasma* in their gills, which is where the excess ammonia is diffused out by squids (Boucher-Rodoni & Mangold, 1995).

The gills of the non-ammoniacal squids were reported to be primarily composed of the BD1-7 clade; this was only true of *O. robsoni* in the ammoniacal squids. The reproductive organs of the non-ammoniacal squids were also observed to have a high amount of the BD1-7 clade. The BD1-7 clade, found in varying amounts in at least one squid species belonging to both ammoniacal and non-ammoniacal groups across all body sites, has previously been reported as an abundant taxon in the gills of dwarf-form populations of non-ammoniacal *Sthenoteuthis oualaniensis* squids (Hu et al., 2022a). It is an abundant oligotrophic marine bacterium and is important to the health of its associated hosts, such as cephalopods (Hu et al., 2022a), bryozoans (Figuerola et al., 2025), and gorgonians, due to its carbon utilisation rate across multiple carbon sources (Cho & Giovannoni, 2004). While its purpose in squids is unclear, it could be linked to carbon utilisation. Its greater presence in non-ammoniacal squids could be due to body chemistry, and the ammoniacal squids could have a different microbial associate that serves a similar function.

The highest abundance of *Psychrobacter* was observed in the siphon of *Octopoteuthis*; however, it was reported more in the body sites of the non-ammoniacal squids. *Psychrobacter* tends to be an aerobic and psychrophilic bacterium that is commonly found in both terrestrial and marine environments (Maruyama et al., 2000; Romanenko et al., 2002; Wu, G. et al., 2015). It has been isolated from the gills and skin of various teleosts, Antarctic sea urchins, corals, and marine molluscs, such as the commercial *Octopus maya* (Juni, 1992; Romanenko et al., 2002, 2008; Lo Giudice & Rizzo, 2018; De la Cruz-Leyva et al., 2019). *Psychrobacter* is also widespread in cold Antarctic environments (Bowman et al., 1996, 1997; Romanenko et al., 2002). It is known for hydrolysing organic substrates; therefore, it is thought to contribute to nutrient recycling in marine ecosystems. Its function in the body sites of deep-sea squids is unknown for both non-ammoniacal and ammoniacal squids; however, it was observed in the inner eye fluid of the two *Todarodes* species, which have a more circumpolar distribution compared to the rest of the squid species studied (Roper et al., 2010).

The highest abundance of *Pseudoalteromonas* was seen in the non-ammoniacal squid *N. gouldi* in the inner eye fluid. *Pseudoalteromonas* has been previously isolated from mussels, coastal squids, seawater, holothurians, abalone, tunicates, sponges, and various fishes (Gillan et al., 1998; Ivanova et al., 1998; Holmström et al., 1992; Lovejoy et al., 1998; Barbieri et al., 2001; Skovhus et al., 2004; Bowman, 2007; Ma et al., 2014; Bosi et al., 2017; Offret et al., 2019). It is known to produce active compounds for anti-fouling purposes, such as antibacterial, antifungal, antiviral, and algicidal activities (Skovhus et al, 2004; Peng et al., 2020). In cephalopods, the coastal squid *Doryteuthis pealeii* was observed to have a high concentration of *Pseudoalteromonas* in its egg capsules and accessory nidamental glands, serving as an anti-fouling defence (Barbieri et al., 2001). *Pseudoalteromonas* is a marine, ecologically abundant, and important bacterium due to its ability to influence biofilm formation, produce an anti-fouling substance, and influence marine invertebrate larval settlement and metamorphosis (Holmström et al., 1992). It is thought to be an important member of the ocean microbial loop and may be used by its host organism as a defensive agent (Dobrestov et al., 2006; Bowman, 2007; Peng et al., 2020). Its exact purpose in the inner eye fluid of *N. gouldi* is unknown.

Other less abundant but notable microbial taxa observed in the ammoniacal and non-ammoniacal squids were *Aurantivirga*, *Acidothermus*, *Moritella*, and *Roseimarinus*. *Aurantivirga* was found in the beak samples of *T. filippovae*; other studies have reported it in phoronids, seawater samples, and octopuses' skin (Song et al., 2015; Holt et al., 2023; Costas-Imbernón et al., 2024; Meunier et al., 2024) as a chitin-degrading bacterium (Meunier et al., 2024). It could serve a similar purpose in *T. filippovae*. Meanwhile, *Acidothermus* was observed in the reproductive organs of *O. robsoni* and has been isolated to date only from a thermal spring and is characterised as both thermophilic and acidophilic (Kristjansson & Alfredsson, 1991; Berry & Normand, 2020). *Moritella,* which was only observed in the ammoniacal *T. danae,* is a psychrophilic bacterium that has been isolated from salmonids in fish farms, hadal amphipods, deep seawater and deep sediment samples, as deep as the Mariana Trench (Nogi & Kato, 1999; Benediktsdóttir et al., 2000; Xu et al., 2003; Chan et al., 2022). *Moritella* is therefore thought to be associated with deep-sea environmental adaptations due to its barophilic functions (Xu et al., 2003; Chan et al., 2021; 2022), and may serve a similar functional adaptation in large-bodied deep-sea squids such as *T. danae*. *Roseimarinus* was only observed in different body sites of ammoniacal *Octopoteuthis*. It has been isolated from different marine environments, including marine sediment in China, in mussels, and the gut of a sea urchin (Wu, W.J. et al., 2015; Li et al., 2019; Ketchum et al., 2021). While higher temperatures have been observed to lead to lower *Roseimarinus* abundance, the bacterium's function in its symbiotic host remains unclear (Ketchum et al., 2021). Although the roles of these more minor microbial taxa are not yet known, there seems to be a difference in community composition between ammoniacal and non-ammoniacal squids, as well as between species within the two groups.

The genus *Photobacterium* was detected for the first time in the inner eye samples of two ammoniacal squids, *T. danae* and *O. robsoni*, as well as in the brain, gills, reproductive organs and siphon of *T. danae* and the siphon of *T. angolensis*. *Photobacterium* is commonly associated with being a luminescent-producing bacteria in marine animals, as seen in loliginid squids and *Neorossia caroli* (Urbanczyk et al., 2011; Calogero et al., 2022). Bobtails in the genera *Rondeletiola* and *Sepiolina* have been observed to contain *Photobacterium* within their light organs (Nishiguchi & Nair, 2003; Nishiguchi et al., 2004; Guerrero-Ferreira & Nishiguchi 2007; Moreira et al., 2014; Moi et al., 2017). It is globally distributed and present in most ocean environments, including the deep sea, as well as in the intestines of marine organisms (Urbanczyk et al., 2011). Variation in the presence of *Photobacterium* species in global oceans and marine environments can be attributed to different environmental stressors, with some species preferring warmer coastal waters and others being more commonly observed in colder, deeper waters (Dunlap et al., 2007; Urbanczyk et al., 2011). In the digestive tracts of some fishes, *Photobacterium* appears to help digest chitin (Itoi et al., 2006) and it may serve a similar function in young squids, many of which have a crustacean-rich diet, whose exoskeletons are made of chitin (Portela et al., 2014). Its function in the body sites of *T. angolensis*, *T. danae* and *O. robsoni* is unknown; however, since these are not known bioluminescent body sites, nor are the photophores of *Taningia* known to house symbiotic bioluminescent bacteria, it is probably not serving as a bioluminescent bacterium.

4.5.1 Future directions

The differences in bacterial taxa observed between the ammoniacal and non-ammoniacal squids could be due to differences in the squids' body chemistry. However, future studies should focus on metagenomic sequencing, as it is possible that, while the bacterial taxa differ, they serve the same function. It would also be important to learn the bacterial functions so we can better understand the host-symbiont relationship and how future environmental stressors could impact it. The location of the bacteria within the squids’ tissues could also allow us to understand better how they are acquired and what their purpose is. Fluorescence *in situ* hybridisation would be an interesting way to investigate this (Frickmann et al., 2017), as where the bacteria are located could affect what their role is.

When doing 16S rRNA gene amplicon sequencing, the rare microorganisms can go undetected. More traditional culture-based methods may be better suited for isolating some microbes (Prakash et al., 2013), so a combination of the two methods could provide a more complete overview of the squids’ microbiota, however this is usually limited by storage of samples. It would also be interesting to get a complete picture of the squid species' microbiota, not just the bacteria; therefore, looking at the fungi and viruses would be an important next step.

Increasing the number of specimens and types of non-ammoniacal and ammoniacal squids to further build this dataset and see whether a more distinct picture emerges of the differences in microbiota between squids with different body chemistry would also support more robust statistical tests and inferences.

Chapter 5 Prelude

After reporting on the digestive tract microbiota of female and male *N. sloanii*, eight distinct body sites microbiota for four different ommastrephid species, and the microbiota of eight body sites for ammoniacal and non-ammoniacal deep-sea oegopsid squids, chapter five investigates the microbiota of a single opportunistically collected colossal squid from the Ross Sea. Samples from six body sites are compared with the microbiota of twenty seawater samples from the Ross Sea to investigate potential horizontal transfer between the environment and the squid. The previous chapters have illustrated that while more specimens are important for a robust in-depth analysis, having a sample size of one still allows for the opportunity to describe the microbiota observed of enigmatic understudied organisms from hard-to-reach habitats like the deep sea. Therefore, this is the first study to report on the colossal squid's microbiota, allowing us to further our understanding of this mysterious species' biology and its habitat.

Chapter 5 The Microbiota of the Colossal Squid

5.1 Abstract

Most of the physiology and ecology of the colossal squid, the heaviest living non-colonial invertebrate, remains unknown. Previous studies on animals' microbiota have been used to infer more about their life history and habitat; this approach may be especially useful for rare and hard-to-access species, such as the colossal squid. This opportunistic study assesses the microbiota of six body sites in the colossal squid and compares it to that of the publicly available Ross Sea microbiota dataset. Both samples underwent 16S rRNA gene amplicon sequencing and were processed together with the DADA2 pipeline on R. A significant difference in microbial composition and richness between the two datasets was observed, with only *Pseudomonas* as an overlapping bacterium. The colossal squid's microbiota was primarily composed of *Mycoplasma*, *Stenotrophomonas,* and *Acidovorax*. In contrast, the Ross Sea microbiota contained mainly *Pseudoalteromonas* and *Alteromonas*. This is the first study to report on the colossal squid's microbiota, increasing our knowledge of this enigmatic species' biology and the environment it inhabits.

5.2 Introduction

The colossal squid (*Mesonychoteuthis hamiltoni*) is the heaviest living non-colonial invertebrate, and much of its basic life history and biology remains a mystery (Vermeij, 2016; Rosa et al., 2017; Remeslo et al., 2019). Although much larger than the other members, the colossal squid belongs to the family Cranchiidae (Voss, 1980), also known as 'glass squids' because of their typically translucent bodies (Rosa et al., 2017). However, unlike most cranchiids, the colossal squid lacks this translucency due to its higher muscle density, further emphasising its uniqueness. The colossal squid is believed to be endemic to the Southern Ocean, with a circumpolar distribution extending from the Antarctic continent to the Sub-Antarctic Front (Rosa et al., 2017; Remeslo et al., 2019). Like most oegopsid squids, the colossal squid juveniles are found in the upper pelagic zone (Rosa et al., 2017), migrating to deeper waters as they grow, and eventually inhabiting depths of up to 2000 meters (Lu & Williams, 1994; Rosa et al., 2017). However, mature females have occasionally been captured by fishing vessels at

shallower water depths than anticipated, suggesting that, like other cranchiid species, female colossal squid might migrate back up to shallower waters to spawn (Rosa et al., 2017). A summer spawning might also be occurring, as a few mated females were observed in early December (Remeslo et al., 2019). As of now, no egg masses have been recorded, and there have been few investigations conducted on colossal squid paralarvae (Rosa et al., 2017); therefore, very little is known about the reproductive biology of this species. Stable isotopic analysis places colossal squids as top predators in the Southern Ocean food chain, probably feeding on lanternfishes, Patagonian toothfish (Yukhov, 2012; Remeslo et al., 2015), sleeper sharks, and squids (Bolstad & O'Shea, 2004; Ibáñez & Keyl, 2010; Jereb & Roper, 2010; Roberts et al., 2011). It is believed that, due to the squids' metabolic restrictions and pressures from gigantism, they are not high-speed predators but rather ambush predators (Rosa & Seibel, 2010; Rosa et al., 2017). However, to date, no direct observations of colossal squid feeding strategies have been made.

Marine microbial symbionts are crucial to marine organisms' survival in diverse ocean environments, including the deep sea (Dubilier et al., 2008; Li et al., 2023). The symbiotic relationship between host and microbe can affect the morphology, immune system (Honda & Littman, 2012; Taschuk & Griebel, 2012; Burgos et al., 2020), reproduction (Montgomery & McFall-Ngai, 1994), and behaviour (Shropshire & Bordenstein, 2016) of the host organism (Li et al., 2023). Microbial symbionts have been found to coevolve, speciate and reproduce in tandem with their hosts (O'Brien et al., 2019). These types of mutualistic interactions between a host and its microbiota are thought to confer an evolutionary advantage. For example, symbiotic bacterial biofilms are fundamental in the settlement behaviour of marine invertebrates such as cnidarians, echinoderms, and polychaetes (Ezenwa et al., 2012; Huang et al., 2012). Invertebrates can host microbial symbionts outside or inside their bodies, within host cells, or within symbiont-specific cells (Kerney et al., 2011; Petersen & Osvatic, 2018). They can acquire microbes either vertically or horizontally (Bright & Bulgheresi, 2010); however, mixed transmission modes, including both vertical and horizontal transmission, have also been observed (Bright & Bulgheresi, 2010; Szafranski et al., 2014). Understanding microbial acquisition processes can provide insights into the behaviour of the organism (Li et al., 2023). With so little being known about the physiology and life history of the colossal squid, learning

more about its microbiota might give us insights into its biology and ecology. Symbiotic marine microbes have been observed as providing the host organisms with fundamentally necessary acclimatisation or adaptation strategies to challenging habitats (O'Connor et al., 2014; Murray et al., 2016). The microbiota of Antarctic soft corals (Webster & Bourne, 2007), sponges (Rodríguez-Marconi et al., 2015; Moreno-Pino et al., 2020), ciliates (Pucciarelli et al., 2014), and ascidians (Riesenfeld et al., 2008) all appear host-specific and necessary for the creation of bioactive molecules for their survival in this extreme Antarctic environment (Murray et al., 2016).

Mutualistic relationships have been observed between coastal myopsid squids and various symbiotic microbes, which appear to play important roles in cephalopod bioluminescence, reproductive development, and digestion (Barbieri et al., 2001; Guerrero-Ferreira & Nishiguchi, 2010; Guerrero-Ferreira et al., 2013; Naguit et al., 2014; Kang et al., 2022; Tseng et al., 2023). For instance, the accessory nidamental gland, a reproductive organ found in the females of coastal squid species, appears to house important symbiotic bacteria explicitly acquired from the outside environment (Vijayan et al., 2022); these microbes can be crucial to the host cephalopod development (Kerwin & Nyholm, 2017). Meanwhile, all the microbiota studies conducted on deep-sea oegopsid squids prior to this thesis have concentrated on the digestive tract and gills of 'flying' squids (family Ommastrephidae): two *Todarodes* and two *Sthenoteuthis* (mid and dwarf form), with *Mycoplasma* (for the digestive tract) and the BD1-7 clade (for the gills) commonly being the most abundant genera mentioned (Ramírez et al., 2019; Kang et al., 2022; Hu et al., 2022a; Hu et al., 2022b). No microbiota studies have been conducted on any members of the Cranchiidae family, which have unique ecological and chemical characteristics, such as a less active lifestyle and ammonium-chloride-filled coelom (Voss, 1980). This could have interesting implications for their symbiotic microbes. The colossal squid is also known for living most of its life at greater depths than the previously studied deep-sea squid species, meaning that it interacts with different deep-sea zones and free-living microbial communities.

This study aims to report the microbial compositions of six distinct body sites of a female colossal squid, an ecologically important member of the Southern Ocean mesopelagic food web, with the hope of better understanding its elusive nature and physiology. Tissue samples from different body sites were collected and analysed to determine the

different bacterial communities present in the squid and how they differ among body areas, as well as being compared to a recently collected, and publicly available dataset of seawater from the Ross Sea to consider the possible horizontal transfer of bacteria between the squid and its habitat.

5.3 Methods

5.3.1 The colossal squid dataset

For this chapter, a previously collated dataset, consisting of 16S rRNA gene sequences of ten samples collected from a colossal squid, covering a total of six different body sites was utilised (Table 5.1). The colossal squid specimen (NMNZ M.316689) was collected at the surface in the Ross Sea, Antarctica, in January 2014, from a fishing vessel with Antarctic toothfish (*Dissostichus mawsoni*) hooked on commercial longlines. The female specimen was netted, brought aboard the FV *San Aspiring*, and frozen at –20°C for transport and storage until examination. The specimen was then thawed at the Museum of New Zealand Te Papa Tongarewa in Wellington, kept in chilled seawater for 18 hours, with examination commencing after the first 12 hours of defrosting, and subsequently fixed and accessioned into the Museum’s collection. External surfaces (beak and siphon) were swabbed early in the defrosting process to minimise contamination. However, the specimen was placed on the deck of the ship before being frozen and then defrosted in a large tank with personnel climbing in and out while wearing protective gear, which could have resulted in sample degradation. Internal surfaces (such as the oesophagus and brain) were swabbed in duplicate using sterile swabs as tissues partially thawed and became accessible. Swabs were stored at –80°C until lysis and DNA extraction. The selected body sites were opportunistically chosen to minimise visible damage to the specimen, facilitate comparison with previous research, and gain further insights into the squid's physiology and life history.

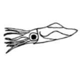
Table 5.1: The body sites swabbed on the colossal squid for microbial analyses.

Body Sites	Number of Swabs
Reproductive Organs	1
Brain	2
Eyes	2
Siphon	1
Beak	2
Oesophagus	2

Swabs from each colossal squid body site were subsampled using sterile scissors and forceps, and approximately 1 cm^3 was placed in 1.5 mL screw-capped conical-bottomed polypropylene tubes, suspended in 270 µL of phosphate buffer and 270 µL of SDS lysis buffer, and homogenised with silica-zirconia (2.5 mm and 0.1 mm diameter) beads on the Vortex-Genie 2 G-560 for 10 minutes at 30,000 rpm. A standard CTAB DNA extraction protocol was applied to all samples, with slight optimisation of the incubation time to one hour and a precipitation period of 48 hours at –20°C (Archer et al., 2015; Chakraborty et al., 2020). The V3-V4 region of the 16S rRNA gene was then amplified through PCR as in section 2.3.2. The DNA libraries were then prepared using Illumina MiSeq V3 600-cycle paired-end chemistry, according to the manufacturer's protocol, a unique combination of Nextera XT dual indices (Illumina Inc., USA) were attached to the DNA from each sample to allow multiplex sequencing of 192 samples per sequencing run. Sequencing was conducted on an Illumina MiSeq instrument using V3 2-by-300-bp chemistry.

5.3.2 The Ross Sea dataset

A publicly available dataset (accession number: PRJNA1266853), consisting of 16S rRNA gene sequences from Incheon National University, comprising 20 seawater samples from the Ross Sea collected in 2023, spanning depths from 0 to 2070 meters, was also utilised for this chapter. This dataset had been generated by extracting DNA from 0.22 mm Sterivex filters using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The 16S rRNA gene V3-V4 region in environmental DNA was amplified, and a Nextera XT Index Kit v2 (Illumina, San Diego, CA, USA) for multiplexing was used for amplicon indexing. The amplicon library was sequenced on the Illumina MiSeq platform using the manufacturer's 2300 bp paired-end



protocol. The dataset was downloaded from the National Centre for Biotechnology Information (NCBI) on 31st July 2025.

5.3.3 Amplicon data processing

I processed the sequencing datasets generated as described above (sections 5.3.1 and 5.3.2) together. Both datasets were processed using the DADA2 (Callahan et al., 2016; ver 1.36.0) pipeline in R 4.5.0 (R Core Team, 2021) as in section 2.3.3, with the following modifications: Raw FASTQ files were filtered (using the filterAndTrim function) with the following parameters: truncLen (270, 210) with a maxEE (4,5) and truncLen (280, 240) and a maxEE of (3,4) for the squid and Ross Sea seawater samples, respectively. The error model and Amplicon Sequence Variant (ASV) calling were performed on a run-by-run basis to account for different empirical relationships between the assigned quality scores and the error rates associated with individual sequencing runs and machines. The ASV count tables from the two runs were then merged into a single table before the chimeric sequences were identified and removed. One sample (duplicate brain sample) was removed from further downstream data processing due to insufficient reads. I then created both a rarefied ASV table using the rarefy_even_depth from the phyloseq package (McMurdie & Holmes, 2013; ver 1.53.0) to rarefy to even depths of 1000 reads, and a cumulative sum scaling normalised ASV table using the ‘cumNorm’ command from the metagenomeSeq package (Paulson et al., 2013; ver 1.51.0).

5.3.4 Statistical analysis

The same statistical analysis was applied as in section 2.3.3 with the raw ASV table used to calculate beta diversity, with R package vegan (Oksanen et al., 2025; ver 2.7-1), with the use of Aitchison distance matrix (Martino et al., 2019), using the command ‘vegdist’ and visualised in a Principal Coordinate Analysis (PCoA) ordination plot between the colossal squid samples and Ross Sea samples.

5.4 Results

5.4.1 Description of colossal squid microbiota

Microbiota were analysed from each of six colossal squid body regions: the beak, oesophagus, reproductive organs, siphon, eye, and brain (Table 5.1). A total of 1,277,076 reads were obtained, and 5734 ASVs were identified from the combined 30 samples.

The microbial communities of the beak and oesophagus were dominated by *Mycoplasma*, accounting for 60.16% and 93.35% of the relative abundance, respectively (Figure 5.3a). At the beak, the following most abundant genera were *Pseudomonas* (22%) and *Acidovorax* (8%); within the oesophagus, no taxon apart from *Mycoplasma* was observed with an abundance >10%, with *Stenotrophomonas* (4%) and *Variovorax* (2%) the next most abundant after *Mycoplasma*. In the brain and eye, *Stenotrophomonas* was most abundant, accounting for 51% and 40%, respectively, and the next most abundant taxa were *Variovorax* (20%) and *Pseudomonas* (16%). At the siphon and reproductive organ sites, *Acidovorax* was most abundant (36% and 29%, respectively), and *Methylphilus* (17%) in the siphon and *Acinetobacter* (27%) in the reproductive organs were reported as having the next highest relative abundances.

5.4.2 Diversity of colossal squid and Ross Sea microbiota

To investigate potential similarity between the colossal squid microbiota and the bacterial communities observed in the Ross Sea, a Ross Sea microbiota dataset was analysed in conjunction with the colossal squid. The Ross Sea dataset exhibited considerably higher Shannon's diversity (Mann–Whitney *U* test, $W = 35$, $P = 0.01$) than the total colossal squid dataset (Figure 5.1).

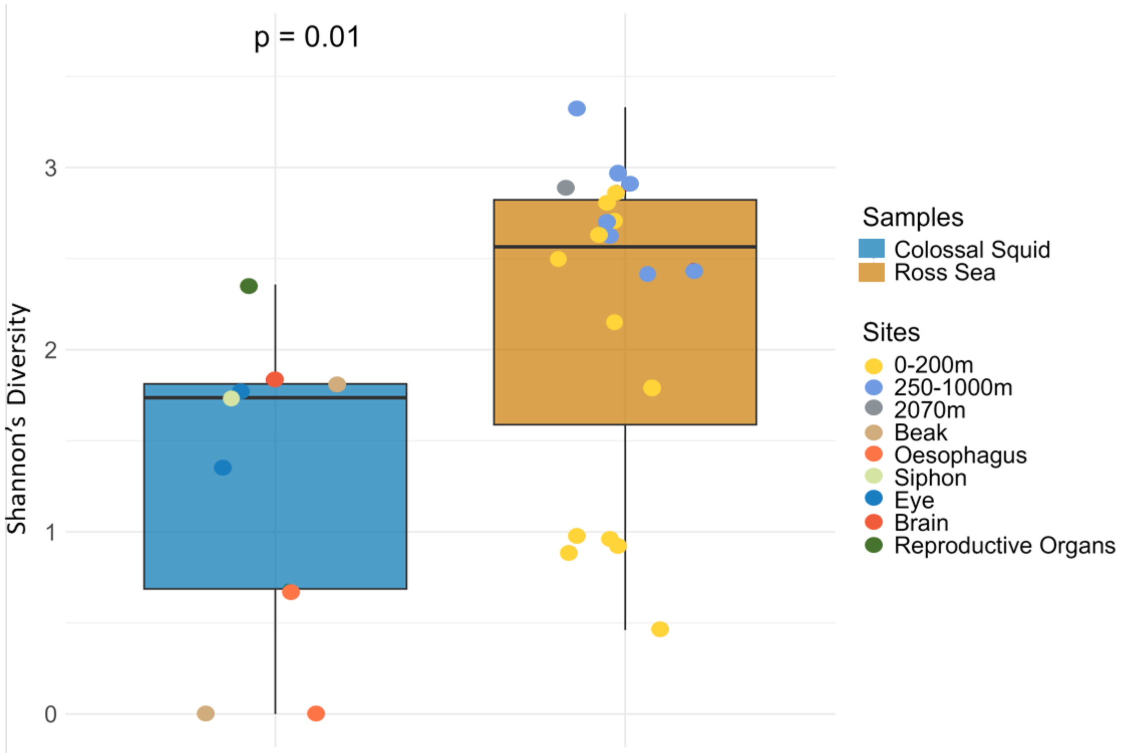


Figure 5.1: Diversity of the colossal squid (blue) and the Ross Sea dataset (brown). Shannon's diversity index with rarefied data, significance observed with a Mann–Whitney U *test. Boxes represent the interquartile range (IDR, 25–75%) of the data. Median values are indicated by the bar within each box, and whiskers show the values within 1.5 times the IQR.*

The colossal squid exhibits a distinct microbiota composition across its various body sites compared to the Ross Sea microbiota depth profile (PERMANOVA: R^2 = 0.271, F_{28} = 10.98, *P* = 0.001; Figure 5.2). However, it is important to note that the beta dispersion was also significant (F_{28} = 19.28, *P* = 0.001), indicating that the dispersion differences between the samples will at least partially explain the PERMANOVA results. Despite this, the samples cluster very clearly by group (colossal squid vs. Ross Sea) in both the centre and the spread of diversity (Figure 5.2). This further illustrates a lack of horizontal bacterial transfer between these colossal squid and Ross Sea datasets, and that microbial communities from the Ross Sea dataset were distinctly different from those found on various body sites in the colossal squid.

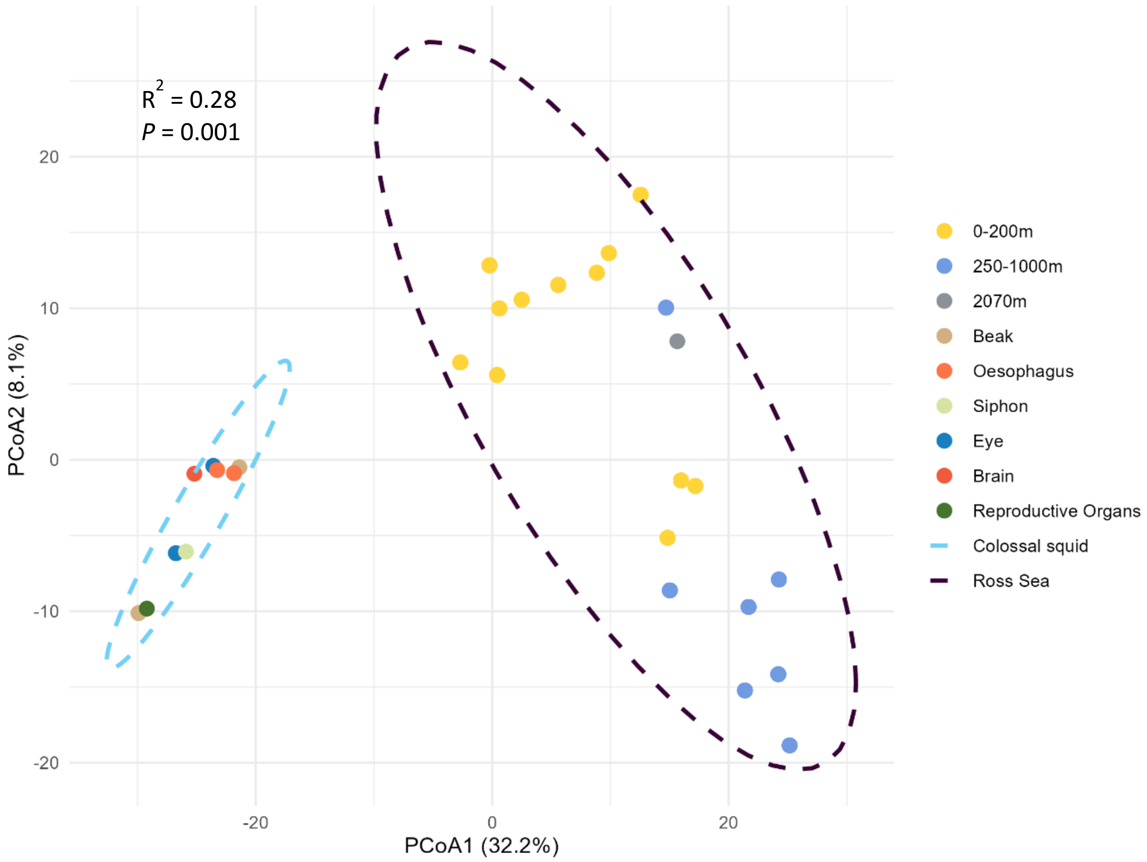


Figure 5.2: Principal coordinates analysis (PCoA) of Aitchison distances between samples. The coloured dots represent the body sites of the colossal squid and the Ross Sea depths. The ellipses represent 95% confidence intervals.

The Ross Sea dataset was separated into three oceanic zones: epipelagic (0-200 meters), mesopelagic (250-1000 meters), and bathypelagic (2070 meters). The closer to the surface, the higher the relative abundance of *Pseudoalteromonas* found (22% at 2070m, 63% in the mesopelagic zone, and 83% in the epipelagic zone). There was a clear difference in the bacteria found in the colossal squid and the Ross Sea samples from all depth strata. The only overlapping genera found between colossal squid and the Ross Sea was the genus *Pseudomonas*, which was observed in the squid's beak (22%), eye (16%), reproductive organs (16%), and siphon (26%) and only appears with more than 1% relative abundance in the bathypelagic Ross Sea sample with a relative abundance of 2% (Figure 5.3a). This was further shown by examining samples with relative abundances greater than 1% and bacterial genera prevalent in at least 5% of the total samples from the colossal squid and the Ross Sea: only one shared core genus, *Pseudomonas*, was identified (Figure 5.3b). When looking at the most abundant genera reported in other research on global oegopsid squids and the overlap in genera with the

colossal squid, *Mycoplasma* was identified as the shared core genus between all squids; however, between other Southern Ocean squid microbiota and colossal squid, *Acidovorax* and *Stenotrophomonas* were also identified as shared genera (Figure 5.3b).

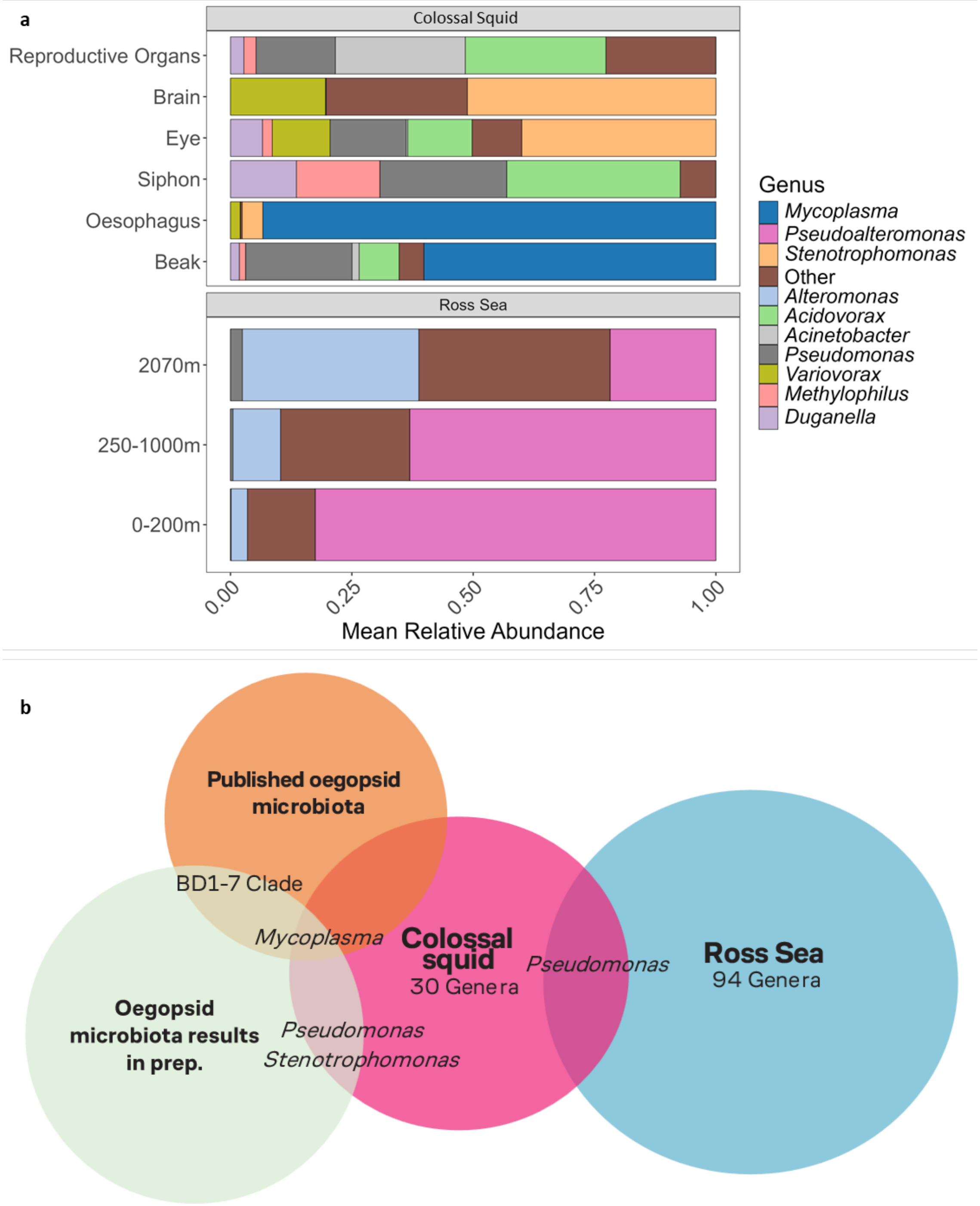


*Figure 5.3: The microbiota of the colossal squid and Ross Sea samples. (a) Top ten most abundant bacterial genera within the colossal squid (*Mesonychoteuthis hamiltoni*) and the Ross Sea datasets. (b) The bacterial genera found in both the colossal squid and Ross Sea datasets, and any observed overlap between them. Overlaps between the most abundant genera observed in other oegopsid squids (published (three ommastrephid species, digestive tract and gills) and chapters two, three, and four (four ommastrephids, one onychoteuthids, three octopoteuthids, ten body sites)) and colossal squid are also shown.*

5.5 Discussion

This study is the first to investigate the microbiota of the colossal squid, an elusive deep-sea squid of the Cranchiidae family, using high-throughput 16S rRNA gene amplicon sequencing and comparing it with a Ross Sea dataset to assess possible microbial horizontal transfer. A significant difference in Shannon's diversity index was observed between the two datasets. The only genus reported to overlap between the Ross Sea and colossal squid datasets was *Pseudomonas.* The colossal squid had a low microbial biodiversity, with 30 genera, of which *Mycoplasma,* a well-known marine symbiotic bacterium, was noted, which also appears in other deep-sea squid digestive tract research (Ramírez et al., 2019; Kang et al., 2022; Hu et al., 2022a; Hu et al., 2022b). Microbiota studies on five species of Antarctic sea anemones also displayed low microbial diversity but substantial phylogenetic novelty (Murray et al., 2016).

In the oesophagus and beak of the colossal squid, a high abundance of *Mycoplasma* was observed, which is a common core taxon reported in many marine organisms (Wang et al., 2016; Rasmussen et al., 2021; Cheaib et al., 2021a; Rasmussen et al., 2023), functioning as either a parasitic or commensal microbe to its host. In salmonid species, *Mycoplasma* has been observed to act as a commensal gastrointestinal microbe, likely due to its ability to exploit ammonia in the gut of most teleosts (Bozzi et al., 2021; Rasmussen et al., 2021). The *Mycoplasma* found in the gut of eastern oyster (*Crassostrea virginica*) is thought to provide a beneficial mutualistic relationship with the host by reducing parasitic infections through sequestration of arginine (Pimentel et al., 2021; Akter et al., 2023). *Mycoplasma* was detected at a high abundance in eastern oysters, blue mussels (Akter et al., 2023), and the stomach microbiota of six different cephalopod species (five myopsid and one oegopsid squid) studied by Kang et al. (2022) and two more oegopsid species reported by Ramírez et al. (2019) and Hu et al. (dwarf form 2022a; and mid form 2000b). Kang et al. (2022) theorised that differences in *Mycoplasma* levels across cephalopods may be linked to their distinct feeding behaviours. This aligns with our preliminary findings, as the highest abundance of *Mycoplasma* was observed in the oesophagus and beak, both parts associated with the gastrointestinal tract. This suggests it might play a role in helping the colossal squid break down and utilise ammonia or arginine from its digestion.

Meanwhile, *Acinetobacter* found in the reproductive organs of the colossal squid is globally distributed and a common, heavy-metal-resistant bacterium found in various marine environments (Simidu et al., 1980; Huddedar et al., 2002; Shete et al., 2015). It appears in various marine mollusc microbiota as a carotenoid-producing bacterium or one with light absorbance similar to carotenoids, as observed in the gut microbiota of the noble scallop (*Chlamys nobilis*; Liu et al., 2020). The only cases in which *Acinetobacter* has been reported in invertebrate reproductive organs to date are in the common bedbug (Bellinvia et al., 2019) and the locust (Lavy et al., 2020). Therefore, it can only be hypothesised that its high abundance in the reproductive organs of the colossal squid may be linked to its carotenoid-like properties, since carotenoids are vital for many marine animals' health as they contribute to camouflage, antioxidation, and are considered essential for reproduction (Maoka, 2011). The symbiotic bacteria reported in myopsid squids' (the only squids for which this has been studied so far) reproductive organs (the accessory nidamental glands and eggs) are different from the ones observed in the colossal squid (Barbieri et al., 2001; Pichon et al., 2005; Yang et al., 2021; Vijayan et al., 2024), which could be due to different reproductive strategies between the orders, such as egg-laying. Myopsid squids attach sessile 'egg mops' to the seafloor (Jackson, 2004), which must resist fouling across their development period, while many oceanic squids (potentially including the colossal) produce pelagic egg masses that are either free floating in the water column or carried and brooded by the female until hatching (Laptikhovsky, 1999; Seibel et al., 2000), with different implications for potentially useful microbial associates. Female coastal cephalopod reproductive maturity is marked by a colour change in the ANG, from colourless to orange (Bloodgood, 1977; Barbieri et al., 2001). This colour shift is due to symbiotic bacteria containing carotenoid pigments within the accessory nidamental glands (Bloodgood, 1977; Barbieri et al., 2001), which may also occur in the colossal squid reproductive organs.

Acidovorax and *Stenotrophomonas* were found to be prominent bacteria in the siphon, reproductive organs, beak, brain and eye of the colossal squid. *Acidovorax* has been recognised as a core taxon in several researched marine organisms, such as in the microbiota of the sea slug (*Elysia chlorotica*; Devine et al., 2012), for nitrate reduction in the gut of sea bream (*Sparus aurata*; Kormas et al., 2014) and appears to facilitate nitrogen absorption in the excretory organs of earthworms (Schramm et al., 2003; Devine et al., 2012). While no research has noted its presence and function in oegopsid squid, it could be performing a similar nitrogen-reducing or absorption function in the colossal squid.

Stenotrophomonas is often regarded as an opportunistic pathogen and has been isolated from both marine and terrestrial environments (Romanenko et al., 2008; Torres et al., 2017). Commensal mutualistic *Stenotrophomonas* has been successfully isolated from six deep-sea invertebrates and demonstrated its production of antifungal and antimicrobial metabolites (Romanenko et al., 2008). Its potential function in the colossal squid remains unknown. However, it could be linked to its secondary metabolite production, which may help keep the brain and eyes free of parasitic microbes.

An interesting discovery is the relatively high abundance of the genus *Methylophilus* found in the siphon of the colossal squid. *Methylophilus* bacteria, as the name suggests, utilise methanol or other methane-like compounds as energy and carbon sources (Lin et al., 2021). They have previously been identified in the gills of three different bivalve species; however, their function has not been reported (Wood & Kelly, 1989). While no links have previously been drawn in the symbiotic relationship between squid and *Methylophilus*, a mutualistic energy-beneficial relationship may be occurring between the microbe and its host, the colossal squid. Meanwhile, *Variovorax*, found in the squid's brain, eye and oesophagus, is recognised for its sulphur and phosphate metabolism in nutrient-limited oceans (Naim et al., 2014; Wang et al., 2025). While the Ross Sea is not known for being a sulphur or phosphate limited sea (Rivaro & Ianni, 2025), it may still contribute to increasing the nutrient uptake in the colossal squid. *Duganella,* for which this is the first report in cephalopod microbiota, has instead been previously seen in marine environments to produce ‘violacein’, which is noted as a natural algicide against harmful algae (Cai et al., 2024). This could account for its high abundance in the colossal squid, where it might act as a commensal symbiont against disease-causing pathogens.

The only common taxon between the tested colossal squid body sites and Ross Sea depth samples was *Pseudomonas*. In comparison, other myopsid squid studies have reported *Pseudoalteromonas*, observed in the Ross Sea samples, within the accessory nidamental glands and eggs of different loliginid squid (Pichon et al., 2005). Previous

marine microbial studies have recognised *Pseudomonas* for its high bioactive potential, owing to its production of various antibacterial compounds (Hwang et al., 1989; Isnansetyo & Kamei, 2009). Marine *Pseudomonas* has been identified in diverse ecosystems, including deep-sea and extreme environments, as well as in association with various marine organisms, such as in the hypobranchial gland of Tyrian purple-producing marine gastropods (Isnansetyo & Kamei, 2009; Ngangbam et al., 2015). It has also been reported as a core taxon in different cephalopod body sites, including the posterior salivary gland of blue-ringed *Octopus maculosus* (Hwang et al., 1989). In the colossal squid, *Pseudomonas* was found in all tested body sites except the oesophagus, and in very low abundance in the brain. While its function remains unconfirmed, the colossal squid may use *Pseudomonas* for similar antibacterial compound production and nutrient remineralisation as seen in other marine molluscs (Isnansetyo & Kamei, 2009).

The results discussed are based on a single female colossal squid specimen from 2014, from which six body sites were opportunistically sampled, some in duplicate and some only once, as well as a 2023 Ross Sea dataset. It is therefore important to emphasise that this is primarily a description of the bacterial taxa I found in the colossal squid's different body sites, and that more specimens and samples would be required to draw significant statistical conclusions. The statistics were only applied between the colossal squid and Ross Sea datasets to indicate a preliminary difference in their microbial compositions and a potentially minute horizontal transfer of bacteria.

The Ross Sea samples were sequenced in 2023, nearly 10 years after the colossal squid was captured and swabbed. This temporal difference could contribute to the low overlap in taxa between the two datasets. However, ocean microbial datasets have shown that temporal variation in microbial diversity and abundance in ocean environments is seasonal rather than annual (Ferrera et al., 2024; Priest et al., 2025). While the temporal dynamics of microbial communities in the Ross Sea are understudied, the colossal squid and the Ross Sea samples were both collected in January, therefore reducing the likely impact of seasonal dynamics. Regardless, more samples for both the Ross Sea and colossal squid are necessary to strengthen the observations made in this study. However, large colossal squid are rarely available for laboratory study, and the logistics of defrosting and preserving such specimens are complex. While future samples will no doubt reveal additional information, conducting the first microbial analysis on the colossal squid represented a unique and exciting opportunity.

5.6 Conclusion

The colossal squid microbiota was observed to be a unique microbial community with low diversity in comparison to its environment, the Ross Sea. *Methylophilus* was reported for the first time in a cephalopod, in the siphon microbiota of the colossal squid. A significant statistical difference between the colossal squid and Ross Sea dataset was observed both with Shannon's diversity index and Aitchison distance-based beta diversity. The present dataset was opportunistically collected over a short time window to minimise damage to the specimen, resulting in low sample numbers and limited access to some body sites. In the future, should the opportunity arise, I recommend collecting a more complete set of duplicate samples from all anatomically distinct body sites, ideally in parallel with other cranchiids and Antarctic squids for comparison, as well as simultaneous sampling of squids and their surrounding environment. This will allow for a better understanding of the importance of horizontal transfer from the surrounding marine environment in deep-sea squid. Likewise, including additional data from further squids would allow us to investigate the intra-species variation in their microbiota. Overall, although much remains to be investigated, this study reports the first microbiota data of the colossal squid and its potential implications for its physiology, allowing us to build our knowledge of these enigmatic deep-sea creatures.

Chapter 6 Conclusions and Overall Trends Observed

Microbial communities in the ‘dark ocean’ (meso-bathypelagic) are estimated to be responsible for at least one third of biological carbon production in the ocean (Arístegui et al., 2005; Corinaldesi, 2015). Microbial symbionts have been found to evolve, speciate and reproduce in tandem with their hosts (Dick, 2019; Sahu & Minj, 2025). These types of mutualistic interactions between hosts and their microbiota are thought to confer an evolutionary advantage (Osman & Weinnig, 2022). For example, yeti-crabs (*Kiwa hirsuta*) successfully survive in extreme environments on hydrothermal vents and cold seeps in the deep ocean due to having chemosynthetic bacteria on their claws that they eat to supplement their diet (Thurber et al., 2011). However, despite the significance of host–symbiont interactions, our knowledge regarding the elaborate symbiotic relationships between most invertebrates and their microbiota remains limited (Petersen & Osvatic, 2018).

Despite the considerable economic, scientific, and ecological importance of deep-sea squids, there is an overwhelming lack of data on their biology and ecology. This PhD thesis aimed to provide novel insights into deep-sea squid physiology and ecology by investigating the microbial communities living within and on these cephalopods through the use of 16S rRNA gene amplicon sequencing. To date, no research has been conducted on the microbiota of deep-sea squids in the Southern Ocean. This project sequenced samples collected from nine different squid species within five distinct families, all belonging to the same order, to create a dataset of deep-sea squids’ microbiota that will be publicly available pending publication of the resulting manuscripts. The data were first used to assess intraspecific variability in the microbial community structure of the digestive tract between *Nototodarus sloanii* female and male individuals. Once the intraspecies variability was established, I investigated the intra- and inter-genus variability in microbiota, as well as variability among deep-sea squids with different body chemistry (ammoniacal vs non-ammoniacal). An opportunistic collection of samples from the colossal squid and a Ross Sea seawater samples dataset also allowed for a preliminary assessment of the potential role of the surrounding environment on the squid's microbiota. This provided insight into the squids’ physiology, diets, and how changes in oceanic conditions due to climate change could affect their health.

6.1 Overall trends in deep-sea squids' microbiota (Order: Oegopsida)

The microbiota of eight different body sites from nine different deep-sea squid species belonging to four different families (Cranchiidae, Ommastrephidae, Onychoteuthidae, Octopoteuthidae) within the order Oegopsida were investigated in this thesis for the first time. *Mycoplasma, Brachybacterium,* the BD1-7 clade*, Psychrobacter,* and *Pseudoalteromonas* are the most common bacteria across body sites among all squid species studied, except for the colossal squid, which shared only *Mycoplasma* among those top five bacterial taxa. *Mycoplasma* is observed to be primarily associated with the digestive tract and beak of all squid species. Kang et al. (2022) found that an increase in mantle size significantly correlated with an increase in *Mycoplasma* abundance in the gut. *Onykia robsoni’s* inner beak sample is the exception as it is composed of a much wider variety of taxa, with the BD1-7 clade being slightly the most prominent. The ammoniacal squids (*T. danae, T. fimbria, O. robsoni,* and *Octopoteuthis*) and *N. gouldi* in the non-ammoniacal squids had a high abundance in the digestive tract; instead, the rest of the non-ammoniacal squids had a higher relative abundance of *Brachybacterium* in the digestive tract. *Brachybacterium* was also reported mainly in the brain and inner eye fluid for all squid species (except for colossal squid). *Taningia fimbria* was also observed to contain a high amount in its inner beak, gills, and reproductive organs, which differed from what was reported in *T. danae,* whose gills were reported to have a much higher bacterial community diversity. The BD1-7 clade was the most abundant bacterium in the gills of the ommastrephid squids and in *O. robsoni*. However, except for *T. fimbria,* all squid species were reported to have >5% of the BD1-7 clade in the gills. Although *Pseudoalteromonas* and *Psychrobacter* are also reported as recurring bacteria, they were found to be more closely associated with *Nototodarus*, *Todarodes*, and *Octopoteuthis*, respectively. Invertebrates’ microbiota have been seen to not only be host specific but also organ specific (Ohwada et al., 1980; Osman & Weinnig, 2022), while true for some squid species and their body sites, there is a common microbial composition within certain body sites and among squid species.

Across all squid species excluding *O. robsoni*, the beaks were observed to harbour similar bacterial communities with varying relative abundances. For all squid species except the colossal squid, this consistency per body site is also observed in the brain and inner eye fluid, as well as ommastrephid gills. In contrast, for all squid species except *T. danae*, the siphon and reproductive organs are among the most diverse body sites, with less distinction between ammoniacal and non-ammoniacal squids. The gill samples in the ammoniacal squids are also quite diverse among squid species, without a clear pattern. Overall, when looking at the complete microbiota between the ammoniacal and non-ammoniacal squids, the ammoniacal squids contain more *Mycoplasma*, and the non-ammoniacal squids contain more of the BD1-7 clade (Figure 6.1b). The relative abundance of the BD1-7 clade seen in the ammoniacal squids' total microbiota is primarily driven by its presence in the microbiota of *O. robsoni* (Figure 6.1a).

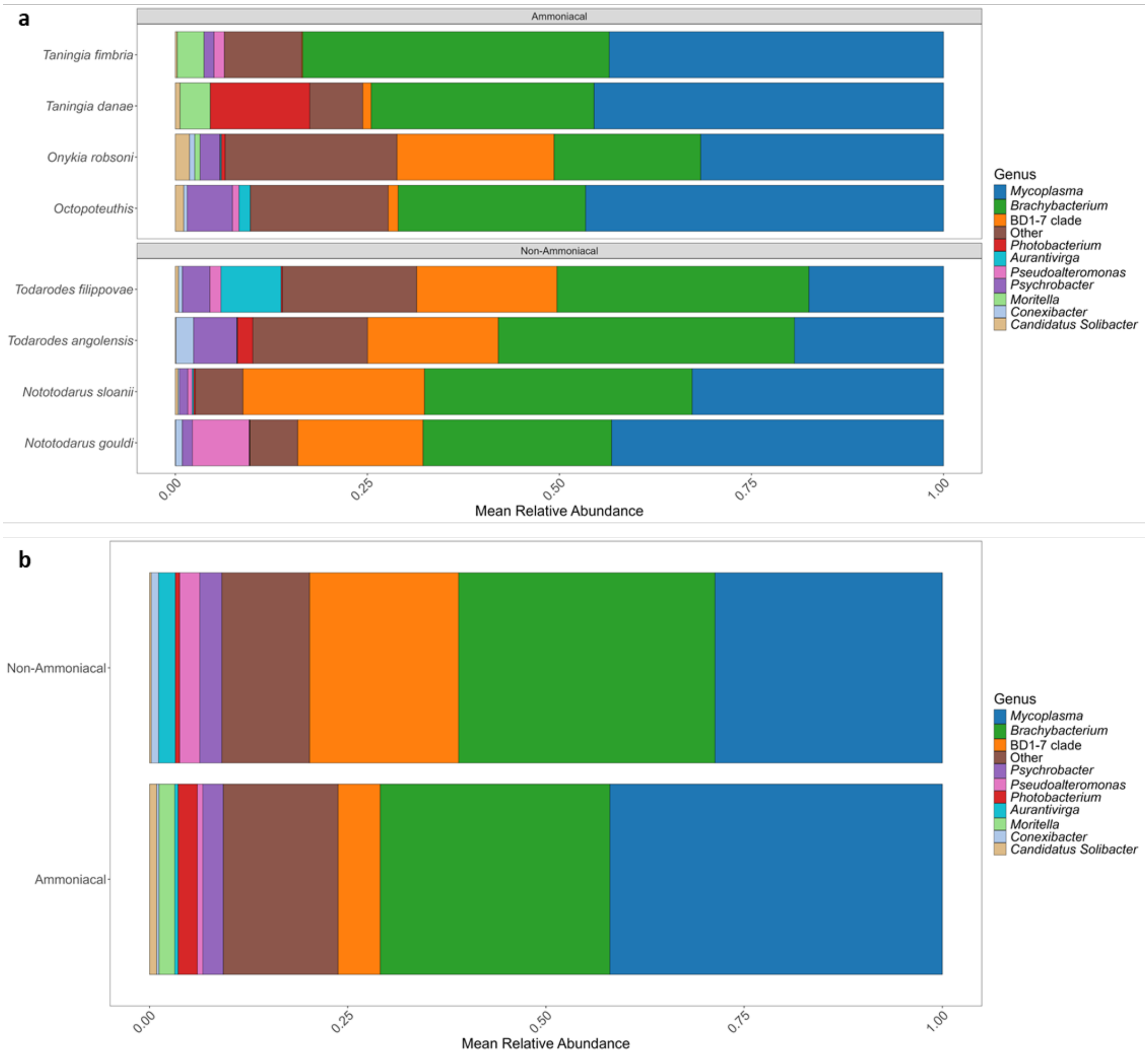


Figure 6.1: The top ten most abundant bacterial genera from chapter four, both (a) the overall microbiota of the eight squid species and (b) the overall microbiota of the ammoniacal and non-ammoniacal squids grouped together.

6.2 Important trends among body sites

An emerging trend observed is that the microbiota of the inner eye fluid and the brain of all nine squid species tend to be similar. This could be attributed to the brain being anatomically positioned closely behind the eyes, and therefore, there may be some vertical migration of bacteria between the two organs. The rest of the eye's microbiota should also be sequenced, and fluorescent in situ hybridisation should be conducted in the future to see where the bacteria are present within the eye and brain tissues, and if the overlapping taxa are seen to be present in the outer tissues that connect. The deep-sea squids analysed in this thesis have eyes that develop outward from brain tissue (Koenig et al., 2016); this could also explain the shared bacterial taxa observed between the two body sites. The function of the bacteria would also be important to assess, as although they are reported as the same taxa, they might serve different functions in different organs. The same match in bacterial community composition is also possibly seen in the gills and reproductive organs; however, it is less clear due to the higher diversity of the bacterial community in one or both of these organs for most squid species studied in this thesis.

The digestive microbiota of three species of deep-sea oegopsid squids (*S. oualaniensis* [dwarf and medium form], *T. pacificus,* and *T. sagittatus*) have been previously reported in published literature. The gill microbiota of *S. oualaniensis and T. sagittatus* have also been previously reported. While the gill microbiota of *S. oualaniensis* is consistent with the ommastrephid deep-sea squids investigated in this thesis (Hu et al., 2022b), the gill microbiota of *T. sagittatus* was reported to have a high abundance of *Mycoplasma* (Ramírez et al., 2019), which is more similar to the microbial composition of the octopoteuthid squids. The ommastrephid digestive and gill microbiota reported in this thesis exhibit microbial taxa similar to those reported in the available published literature; however, *Brachybacterium* in the digestive tract microbiota was reported here for the first time. Most previous microbiota studies in ‘true’ squids have been conducted on coastal myopsid squids.

The only studies of myopsid squids' microbiota are of loliginids; most studies have focused on the microbiota of the accessory nidamental glands or on bioluminescent bacteria present on and in the squid. *Vibrio* is commonly reported in multiple myopsid squids' microbiota; however, it is not seen in any of the top ten most relatively abundant taxa in the nine species of oegopsid squid studied in this thesis. The other primary bioluminescent bacterium studied in myopsid bacterial research is *Photobacterium*, which occurs in only some body sites in some oegopsid squids, such as in *T. danae* and *T. angolensis* and *S. oualaniensis. Doryteuthis pealeii* was observed to contain *Pseudoalteromonas* in its accessory nidamental glands and egg casings (Barbieri et al., 2001; Pichon et al., 2005), which, while not reported in the reproductive organs of the oegopsid squids, was found in other body sites, such as in the beak and inner eye fluid samples of *N. gouldi*. There seems to be a distinct difference in the microbiota of the oegopsid and myopsid squids, for the specific body sites from which a direct comparison can be drawn.

The majority of deep-sea organisms' symbiotic microbial communities are distinct from those of their shallow-water counterparts (Kennedy et al., 2014). This is probably due to microbes having specific functions that alter host organisms' physiology, behaviour, and ecology, thereby broadening the environmental habitats they can survive in (Osman & Weinnig, 2022). For instance, hydrothermal vent crabs (*Austinograea* sp.) have distinctly different microbiota from when compared to shallow-water crab species (*Portunus trituberculatus* and *Eriocheir sinensis*), which allows them to thrive in hypoxic and low-temperature conditions (Zhang et al., 2017). The microbiome of deep-sea animals can help them survive and thrive in the deep-sea (Osman & Weinnig, 2022). Research on deep-sea microbiomes is very often limited by insufficient replication for further analysis due to the inaccessibility of samples (Osman & Weinnig, 2022).

6.3 Initial findings on location as a possible driver of differences in microbial composition

This is the first analysis of the microbiota of the same species at different locations of a Southern Ocean cephalopod (*Todarodes filippovae*). This was conducted opportunistically on three individuals (Chatham Rise, N = 2; sub-Antarctic, N = 1), and a descriptive difference was noted in the microbiota of *T. filippovae*. A much more thorough investigation is necessary to support these initial findings by increasing sample sizes and testing whether the same is true for other species. It would also be interesting to note whether the difference in microbiota is reflected by the ocean, by collecting seawater samples alongside the squids and therefore being able to assess how much of the squids' microbiota is shared with their immediate surrounding environment and if there is a geographical difference in the ocean's microbiota as well. Due to the use of opportunistic samples, only the microbiota of the colossal squid could be compared to seawater samples from its habitat, the Ross Sea. The bacterial genus *Pseudomonas* was the only observed taxon in both datasets; however, another bacterial taxon, *Pseudoalteromonas*, which is reported in all the Ross Sea depth samples, was also observed in several deep-sea squids investigated in this thesis. Interestingly, it was the *T. filippovae* Chatham Rise samples that housed *Pseudoalteromonas,* not the sub-Antarctic ones. Previous research has identified pronounced differences in bacterial assemblages across depths in the Ross Sea (epipelagic, mesopelagic, and bathypelagic), with bacterial abundances decreasing with depth (Celussi et al., 2009). Our investigations have shown a decrease in *Pseudoalteromonas* and an increase in *Alteromonas* with depth.

Low horizontal transfer of bacteria between the colossal squid and its environment is possible; however, much more data would be needed to support this claim. Also, while the colossal squid was caught in the Ross Sea, its geographic distribution is the whole of the Southern Ocean, and thus, it could have a microbial composition more similar to that of another part of the ocean; the horizontal transfer, if occurring, could be during distinct life-stages when the squid is inhabiting different marine habitats; more sampling would be needed to confirm this. It is important that, when future opportunities arise to study the microbiota of deep-sea squid specimens, seawater samples are also collected, thereby allowing us to build a more robust dataset.

6.4 Novelty

This is the first study on the microbiota of deep-sea squids in the Southern Ocean to date. This is also the first study to examine the microbiota of squids belonging to the families Octopoteuthidae, Cranchiidae, and Onychoteuthidae and observe whether there is a difference in the microbiota between ammoniacal and non-ammoniacal squids. As of the current published research, this is also the first study to report on the microbiota of the inner eye fluid, beak, brain, reproductive organs, and siphon of any oegopsid squids worldwide. This is the first report of *Brachybacterium*, *Thermosporothrix,* and *Byrobacter* in a mollusc to date; meanwhile, *Roseimarinus* and *Moritella* are the first reports in cephalopods and have previously only been isolated from bivalves (Li et al., 2019; González-Aravena et al., 2024). The bacterium *Alicyclobacillus*, observed only in sub-Antarctic samples of *T. filippovae*, has not been reported in any other cephalopods and has currently only been isolated from submarine volcanic microbiota in marine environments (Lee et al., 2024). However, the sub-Antarctic samples of *T. filippovae* are based on only one specimen, further sampling would be needed before any conclusions can be confidently drawn.

This is also the first study to report on preliminary differences in deep-sea squid microbiota based on location. While differences were seen in the location-based microbiota of *T. filippovae,* a larger sample size would be necessary to draw more robust conclusions, as well as looking at different species and families of oegopsid squid to see if this is also true for them or if it is something specific to *T. filippovae*, which I do not think it is. Assessing the microbiota of the water in which the squids are found would also help determine whether the differences in microbiota are reflected in the environment. Increasing sample size is difficult due to the elusive nature of deep-sea squids; however, it is necessary to deepen our understanding of the interactions between squids and their microbiota and of the potential host-based drivers of microbial selection.

No differences in the digestive microbiota of female and male *N. sloanii* were observed in this thesis. This was surprising as *N. sloanii* has been reported to exhibit sexually driven dimorphic differences in feeding strategies (Dunn, 2009). However, this could be due to the individual squids used having been caught at the same depth, time, and location and therefore potentially having the same prey availability during that time. Also, different feeding strategies might mean the same prey taxa at different sizes or proportions, which might affect the abundance of particular bacterial taxa observed, but not the composition of taxa observed. It is also unknown when the digestive microbiota is established in deep-sea oegopsids; therefore, the juveniles of *N. sloanii* may exhibit a less sexually driven dimorphic feeding strategy, and the digestive tract microbiota seen in adults may be an artefact of the juvenile stage. No studies to date have examined the microbiota of deep-sea squid juveniles, how they may change ontogenetically and what role this may play in determining the microbiota of mature individuals. Investigating the microbiota of mature female and male deep-sea squids, as well as their paralarvae and juveniles, would also help us better understand the vertical and horizontal transmission of bacteria of their microbiota, allowing us to better understand the link between squid and the ocean by assessing the importance of the marine environment and its health to the microbiota composition of different deep-sea squid species.

6.5 Future directions

The ocean microbiota has been observed to exhibit seasonal changes in microbial composition but not annual changes (Priest et al., 2025). It would be interesting to see if this is also true for the deep-sea squids: do they reflect the oceanic changes that surround them, how much of their microbiota is driven by the seasonal availability of food, if there is a seasonal change in microbiota is this more prevalent in immature

squids than in mature squids, would this be more applicable to smaller deep-sea squids or scavengers like vampyroteuthids? However, if this is the case, the different microbes isolated may share the same functions. Metagenomic sequencing of the microbiota of deep-sea squids would be a necessary next step toward better understanding the symbiotic relationship between the host and its bacteria. It would also allow us to assess whether the observed differences in reported bacterial taxa are only taxonomic and whether they serve the same functional purpose. It would also be necessary to look into whether their microbiota consists of marine fungi and viruses, which it most likely does, to build a complete picture of what millions of other organisms make up our enigmatic squids.

Besides increasing sampling efforts or using broader metagenomic sequencing, several more advanced methods could significantly improve our understanding of the functions and spatial distribution of microbes across different body parts. In the future, we could also investigate spatial metabolomics to understand how the microbes affect the chemistry of their host in the body site they are found in, and also other more distant parts of the host's body (Bauermeister et al., 2022). This could reveal microbe-specific contributions to host physiology that cannot be inferred from compositional data alone. Stable isotope probing in microbial ecology could also help identify specific single-cell activities linked to a bacterium in a host organism's microbiota (Alcolombri et al., 2022). Culture-based methods have been shown to be better suited for isolating certain microbial taxa (Prakash et al., 2013); therefore, using a combination of methods would provide a more complete picture of the squids’ microbiota.

6.6 Conclusion

Over the past 40 years, research into squid-associated microbial communities has steadily progressed, especially with the advent of high-throughput sequencing technologies in the 2000s, which have markedly improved our understanding and analytical capabilities. In myopsid squids, microbiota studies have mainly focused on the accessory nidamental glands and the genera *Photobacterium* and *Vibrio*, primarily because of their bioluminescent properties. In contrast, published research on oegopsid squids has largely concentrated on the gastrointestinal tract. While much remains to be explored, this thesis presents the first microbiota data for nine Southern Ocean deep-sea squid species across ten different body sites, broadening our knowledge of these mysterious creatures. The microbiota of deep-sea squids offers insights into their physiology and ecological roles—something that is hard to do in situ due to their hard-to-reach habitats. Additionally, understanding these microbial communities is critical for gaining broader insights into ocean health, as squids can act as bioindicators because of their migratory behaviour across various ocean zones and rapid response to environmental changes. Collecting this initial baseline microbial data from the squids and comparing it with their environment is therefore essential to better understanding anthropogenic impacts on ocean systems and their inhabitants, helping us predict how they might respond to external environmental stressors.

References

Akter, S., Wos-Oxley, M.L., Catalano, S.R., Hassan, M.M., Li, X., Qin, J.G. and Oxley, A.P. 2023. Host species and environment shape the gut microbiota of cohabiting marine bivalves. *Microbial Ecology*, vol. 86, no. 3, pp. 1755-1772, https://doi.org/10.1007/s00248-023-02192-z

Alcolombri, U., Pioli, R., Stocker, R., Berry, D. 2022. Single-cell stable isotope probing in microbial ecology. *ISME communications*, vol. 2, no. 1, p.55, https://doi.org/10.1038/s43705-022-00142-3

Alias, R., Elangovan, J., Rathy, H.M., Zaid, M.T.M., Mohtar, S.H., Yunus, Y.M., Saadun, R., Lani, M.N. 2017. Isolation and identification of luminescent glowing bacteria in marine squid, *Loligo* sp. from Remis beach, Kuala Selangor, Malaysia. *Journal of Built Environment, Technology and Engineering,* vol. 3, pp. 58-65.

Alvarado, R., & Leiva, S. 2017. Agar-degrading bacteria isolated from Antarctic macroalgae. *Folia Microbiologica,* vol. 62, pp. 409–416, https://doi.org/10.1007/s12223-017-0511-1

Amaral-Zettler, L., Artigas, L.F., Baross, J., Bharathi, L., Boetius, A., Chandramohan, D., Herndl, G., Kogure, K., Neal, P., Pedrós-Alió, C., Ramette, A. 2010. 'A global census of marine microbes', in *Life in the world's Oceans: Diversity, Distribution and Abundance*, Oxford: Blackwell Publishing Ltd. pp.223-245.

Amplicon, P. C. R., Clean-Up, P. C. R., Index, P. C. R. 2013. *16S Metagenomic Sequencing Library Preparation*. Available online at: https://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

Anderson, F.E., Bergman, A., Cheng, S.H., Pankey, M.S., Valinassab, T. 2014. Lights out: the evolution of bacterial bioluminescence in Loliginidae. *Hydrobiologia* vol. 725, pp. 189–203, https://doi.org/10.1007/s10750-013-1599-1

Apprill, A. 2017. Marine animal microbiomes: toward understanding host–microbiome interactions in a changing ocean. *Frontiers Marine Science,* vol. 4, https://doi.org/10.3389/fmars.2017.00222

Apprill, A., Robbins, J., Eren, A.M., Pack, A.A., Reveillaud, J., Mattila, D., Moore, M., Niemeyer, M., Moore, K.M., Mincer, T.J. 2014. Humpback whale populations share a core skin bacterial community: towards a health index for marine mammals? *PLoS One*, vol. 9, no. 3, e90785, https://doi.org/10.1371/journal.pone.0090785

Archer, S.D., McDonald, I.R., Herbold, C.W., Lee, C.K., Cary, C.S. 2015. Benthic microbial communities of coastal terrestrial and ice shelf Antarctic meltwater ponds. *Frontiers in Microbiology*, vol. 6, p.485, https://doi.org/10.3389/fmicb.2015.00485

Arístegui, J., Duarte, C.M., Gasol, J.M., Alonso-Sáez, L. 2005. Active mesopelagic prokaryotes support high respiration in the subtropical northeast Atlantic Ocean. *Geophysical Research Letters* vol. 32, https://doi.org/10.1029/2004GL021863

Bakker, J.D. 2024. PERMDISP. *Applied Multivariate Statistics in R*. https://uw.pressbooks.pub/appliedmultivariatestatistics/chapter/permdisp/

Barbieri, E., Paster, B.J., Hughes, D., Zurek, L., Moser, D.P., Teske, A., Sogin, M.L. 2001. Phylogenetic characterization of epibiotic bacteria in the accessory nidamental gland and egg capsules of the squid *Loligo pealei* (Cephalopoda: Loliginidae). *Environmental Microbiology*, vol. 3, pp. 151–167, https://doi.org/10.1046/j.1462-2920.2001.00172.x

Bates, J.M., Mittge, E., Kuhlman, J., Baden, K.N., Cheesman, S.E., Guillemin, K. 2006. Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation. Developmental biology, vol. 297, no. 2, pp. 374-386, https://doi.org/10.1016/j.ydbio.2006.05.006

Bates, K.A., Higgins, C., Neiman, M., King, K.C. 2023. Turning the tide on sex and the microbiota in aquatic animals. *Hydrobiologia,* vol. 850, pp. 3823–3835, https://doi.org/10.1007/s10750-022-04862-4

Bauermeister, A., Mannochio-Russo, H., Costa-Lotufo, L.V., Jarmusch, A.K., Dorrestein, P.C. 2022. Mass spectrometry-based metabolomics in microbiome investigations. *Nature Reviews Microbiology*, vol. 20, no. 3, pp.143-160, doi: 10.1038/s41579-021-00621-9

Bellinvia, S., Johnston, P.R., Reinhardt, K., Otti, O. 2019. Bacterial communities of the reproductive organs of virgin and mated common bedbugs, *Cimex lectularius*. *Ecological Entomology*, vol. 45, pp. 142–154, https://doi.org/10.1111/een.12784

Benediktsdóttir, E., Verdonck, L., Spröer, C., Helgason, S., Swings, J. 2000. Characterization of *Vibrio viscosus* and *Vibrio wodanis* isolated at different geographical locations: a proposal for reclassification of *Vibrio viscosus* as *Moritella viscosa* comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, vol. 50, no. 2, pp. 479-488, https://doi.org/10.1099/00207713-50-2-479

Berry, A.M.,& Normand, P. 2020. '*Acidothermus*', in: Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley & Sons, Ltd, pp. 1–3, https://doi.org/10.1002/9781118960608.gbm00039.pub2

Biessy, L., Pearman, J.K., Smith, K.F., Hawes, I., Wood, S.A. 2020. Seasonal and spatial variations in bacterial communities from tetrodotoxin-bearing and non-tetrodotoxin-bearing clams. *Frontiers Microbiology,* vol. 11, https://doi.org/10.3389/fmicb.2020.01860

Biggs, J. & Epel, D. 1991. Egg capsule sheath of *Loligo opalescens* Berry: structure and association with bacteria. *Journal of Experimental zoology*, vol. 259, no. 2, pp.263-267, https://doi.org/10.1002/jez.1402590217

Bloodgood, R.A. 1977. The squid accessory nidamental gland: Ultrastructure and association with bacteria. Tissue and Cell 9, 197–208, https://doi.org/10.1016/0040-8166(77)90016-7

Bolstad, K.S. & O'Shea, S. 2004. Gut contents of a giant squid *Architeuthis dux* (Cephalopoda: Oegopsida) from New Zealand waters. *New Zealand Journal of Zoology*, vol 31, no. 1, pp.15-21, https://doi.org/10.1080/03014223.2004.9518354

Bosi, E., Fondi, M., Orlandini, V., Perrin, E., Maida, I., de Pascale, D., Tutino, M.L., Parrilli, E., Lo Giudice, A., Filloux, A., Fani, R. 2017. The pangenome of (Antarctic)

Pseudoalteromonas bacteria: evolutionary and functional insights. *BMC Genomics,* vol. 18, no. 93, https://doi.org/10.1186/s12864-016-3382-y

Boucher-Rodoni, R., & Mangold, K. 1995. Ammonia production in cephalopods, physiological and evolutionary aspects. *Marine and Freshwater Behaviour and Physiology*, vol. 25, pp. 53-60, https://doi.org/10.1080/10236249409378907

Bowman, J.P. 2007. Bioactive Compound Synthetic Capacity and Ecological Significance of Marine Bacterial Genus *Pseudoalteromonas*. *Marine Drugs* vol. 5, pp. 220–241, https://doi.org/10.3390/md504220

Bowman, J.P., Cavanagh, J., Austin, J.J., Sanderson, K. 1996. Novel *Psychrobacter* species from Antarctic ornithogenic soils. *International Journal of Systematic and Evolutionary Microbiology*, vol. 46, no. 4, pp. 841-848, https://doi.org/10.1099/00207713-46-4-841

Bowman, J.P., Nichols, D.S., McMeekin, T.A. 1997. *Psychrobacter glacincola* sp. nov., a halotolerant, psychrophilic bacterium isolated from Antarctic sea ice. *Systematic and Applied Microbiology*, vol. 20, no. 2, pp. 209-215, https://doi.org/10.1016/S0723-2020(97)80067-7

Bozzi, D., Rasmussen, J.A., Carøe, C., Sveier, H., Nordøy, K., Gilbert, M.T.P., Limborg, M.T. 2021. Salmon gut microbiota correlates with disease infection status: potential for monitoring health in farmed animals. *Animal Microbiome*, vol. 3, no. 1, pp. 1-17, https://doi.org/10.1186/s42523-021-00096-2

Bright, M., & Bulgheresi, S. 2010. A complex journey: transmission of microbial symbionts. *Nature Review Microbiology,* vol. 8, pp. 218–230, https://doi.org/10.1038/nrmicro2262

Bull, B., Livingston, M.E., Hurst, R., Bagley, N. 2001. Upper-slope fish communities on the Chatham Rise, New Zealand, 1992–99. *New Zealand Journal of Marine and Freshwater Research*, vol. 35, no. 4, pp. 795-815, https://doi.org/10.1080/00288330.2001.9517043

Burgos, H.L., Burgos, E.F., Steinberger, A.J., Suen, G., Mandel, M.J. 2020. Multiplexed competition in a synthetic squid light organ microbiome using barcode-tagged gene deletions. *mSystems*, vol. 5, no. 6, pp. 10-1128, https://doi.org/10.1128/msystems.00846-20

Cai, G., Yang, X., Yu, X., Zheng, W., Cai, R., Wang, H. 2024. The novel application of violacein produced by a marine *Duganella* strain as a promising agent for controlling *Heterosigma akashiwo* bloom: Algicidal mechanism, fermentation optimization and agent formulation. *Journal of Hazardous Materials,* vol. 466, pp. 133548, https://doi.org/10.1016/j.jhazmat.2024.133548

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, vol. 13, no. 7, pp. 581-583, https://doi.org/10.1038/nmeth.3869

Calogero, R., Rizzo, C., Arcadi, E., Stipa, M.G., Consoli, P., Romeo, T., Battaglia, P. 2022. Isolation and identification of luminescent bacteria in deep sea marine organisms from Sicilian waters (Mediterranean Sea). *Journal of Marine Science and Engineering*, vol. 10, no. 8, p. 1113, https://doi.org/10.3390/jmse10081113

Cerf-Bensussan, N., & Gaboriau-Routhiau, V. 2010. The immune system and the gut microbiota: friends or foes? *Nature Reviews Immunology,* vol. 10, pp. 735–744, https://doi.org/10.1038/nri2850

Celussi, M., Cataletto, B., Umani, S.F. and Del Negro, P., 2009. Depth profiles of bacterioplankton assemblages and their activities in the Ross Sea. *Deep Sea Research Part I: Oceanographic Research Papers*, vol. 56, no. 12, pp. 2193-2205, https://doi.org/10.1016/j.dsr.2009.09.001

Chakraborty, S., Saha, A., Neelavar Ananthram, A. 2020. Comparison of DNA extraction methods for non-marine molluscs: is modified CTAB DNA extraction method more efficient than DNA extraction kits? *3 Biotech*, vol. 10, no. 69, https://doi.org/10.1007/s13205-020-2051-7

Chan, J., Geng, D., Pan, B., Zhang, Q., Xu, Q. 2021. Metagenomic insights into the structure and function of intestinal microbiota of the hadal amphipods. *Frontiers in Microbiology*, vol. 12, https://doi.org/10.3389/fmicb.2021.668989

Chan, J., Geng, D., Pan, B., Zhang, Q., Xu, Q. 2022. Gut microbial divergence between three hadal amphipod species from the isolated hadal trenches. *Microbial ecology*, vol. 84, no. 2, pp. 627-637, https://doi.org/10.1007/s00248-021-01851-3

Cheaib, B., Yang, P., Kazlauskaite, R., Lindsay, E., Heys, C., Dwyer, T., De Noia, M., Schaal, P., Sloan, W., Ijaz, U.Z., Llewellyn, M.S. 2021a. Genome erosion and evidence for an intracellular niche – exploring the biology of *Mycoplasmas* in Atlantic salmon. *Aquaculture*, vol. 541, https://doi.org/10.1016/j.aquaculture.2021.736772

Cheaib, Bachar, Seghouani, H., Llewellyn, M., Vandal-Lenghan, K., Mercier, P.-L., Derome, N. 2021b. The yellow perch (*Perca flavescens*) microbiome revealed resistance to colonisation mostly associated with neutralism driven by rare taxa under cadmium disturbance. *Animal Microbiome* vol. 3, https://doi.org/10.1186/s42523-020-00063-3

Cherel, Y. 2020. A review of Southern Ocean squids using nets and beaks. *Marine Biodiversity*, vol. 50, no. 6, p. 98, https://doi.org/10.1007/s12526-020-01113-4

Chiu, L., Guo, J.-L., Li, H.-W., Chang, H.-J., Yang, S.-H., Dufour, S., Chang, C.-F., Tseng, Y.-C., Wu, G.-C. 2025. Microbial diversity and pigment synthesis in the accessory nidamental gland: species-specific and color-associated patterns in bigfin reef squid (*Sepioteuthis lessoniana*). *Animal Microbiome,* vol. 7, no. 36, https://doi.org/10.1186/s42523-025-00402-2

Cho, J.C. & Giovannoni, S.J. 2004. Cultivation and growth characteristics of a diverse group of oligotrophic marine Gammaproteobacteria. *Applied and Environmental Microbiology*, vol. 70, no. 1, pp. 432-440, https://doi.org/10.1128/AEM.70.1.432-440.2004

Clarke, A., Clarke, M.R., Holmes, L.J., Waters, T.D. 1985. Calorific values and elemental analysis of eleven species of oceanic squids (Mollusca: Cephalopoda). *Journal of the Marine Biological Association of the United Kingdom*, vol. 65, pp. 983–986, https://doi.org/10.1017/S0025315400019457

Clarke, M. R. 1988. ‘Evolution of recent cephalopods - a brief review’, In: M.R. Clarke and E.R. Trueman (eds.), *The Mollusca, Paleontology and Neontology of Cephalopods*, Academic Press, San Diego, vol. 12, pp. 331–340.

Clarke, M.R., Denton, E.J., Gilpin-Brown, J.B. 1979. On the use of ammonium for buoyancy in squids. *Journal of the Marine Biological Association of the United Kingdom,* vol. 59, pp. 259–276, https://doi.org/10.1017/S0025315400042570

Corbari, L., Durand, L., Cambon-Bonavita, M.-A., Gaill, F., Compère, P. 2012. New digestive symbiosis in the hydrothermal vent amphipoda *Ventiella sulfuris*. *Comptes Rendus Biologies*, vol. 335, pp. 142–154, https://doi.org/10.1016/j.crvi.2011.12.005

Corinaldesi, C. 2015. New perspectives in benthic deep-sea microbial ecology. *Frontiers in Marine Science*, vol. 2, p. 17, https://doi.org/10.3389/fmars.2015.00017

Costas-Imbernón, D., Costas-Prado, C., Sequeiro, T., Touriñán, P., García-Fernández, P., Tur, R., Chavarrías, D., Saura, M., Rotllant, J. 2024. The skin microbiome as a new potential biomarker in the domestication and health status of *Octopus vulgaris*. *Frontiers in Marine Science,* vol. 11, https://doi.org/10.3389/fmars.2024.1435217

De la Cruz-Leyva, Ma.C., González-de la Cruz, J.U., Durán-Mendoza, T., López-Martínez, S., Corona-Cruz, A.I., Zamudio-Maya, M., Rojas-Herrera, R.A. 2019. Flora bacteriana asociada al *Octopus maya* comercial capturado en la Península de Yucatán, México. *Hidrobiológica,* vol. 29, no. 3, pp. 101-108, https://doi.org/10.24275/uam/izt/dcbs/hidro/2020v29n3/Delacruz

Daley, M.C., Urban-Rich, J., Moisander, P.H. 2016. Bacterial associations with the hydromedusa *Nemopsis bachei* and scyphomedusa *Aurelia aurita* from the North Atlantic Ocean. *Marine Biology Research*, vol. 12, pp. 1088–1100, https://doi.org/10.1080/17451000.2016.1228974

Davis, N.M., Proctor, D., Holmes, S.P., Relman, D.A., Callahan, B.J. 2017. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. _bioRxiv_, https://doi.org/10.1101/221499

de Castro, I., Ribeiro, S., Oliveira, V., Coelho, F.J.R.C., de Lurdes Dapkevicius, M., de Azevedo, E.B., Barcelos e Ramos, J. 2023. *Brachybacterium atlanticum* sp. nov., a novel marine bacterium isolated from the Atlantic Ocean. *International Journal of Systematic and Evolutionary Microbiology*, vol. 73, https://doi.org/10.1099/ijsem.0.005959

Destoumieux-Garzón, D., Canesi, L., Oyanedel, D., Travers, M.A., Charrière, G.M., Pruzzo, C., Vezzulli, L. 2020. *Vibrio*–bivalve interactions in health and disease. *Environmental Microbiology*, vol. 22, no. 10, pp. 4323-4341, https://doi.org/10.1111/1462-2920.15055

Devine, S.P., Pelletreau, K.N., Rumpho, M.E. 2012. 16S rDNA-based metagenomic analysis of bacterial diversity associated with two populations of the kleptoplastic sea slug *Elysia chlorotica* and its algal prey *Vaucheria litorea*. *The Biological Bulletin*, vol. 223, no. 1, pp. 138-154, https://doi.org/10.1086/BBLv223n1p138

Dick, G.J. 2019. The microbiomes of deep-sea hydrothermal vents: distributed globally, shaped locally. *Nature Reviews Microbiology,* vol. 17, pp. 271–283, https://doi.org/10.1038/s41579-019-0160-2

Distel, D.L., Altamia, M.A., Lin, Z., Shipway, J.R., Han, A., Forteza, I., Antemano, R., Limbaco, Ma.G.J.P., Tebo, A.G., Dechavez, R., Albano, J., Rosenberg, G., Concepcion, G.P., Schmidt, E.W., Haygood, M.G. 2017. Discovery of chemoautotrophic symbiosis in the giant shipworm *Kuphus polythalamia* (Bivalvia: Teredinidae) extends wooden-steps theory. *Proceedings of the National Academy of Sciences,* vol. 114, no. 18, pp. E3652–E3658, https://doi.org/10.1073/pnas.1620470114

Dobretsov, S., Dahms, H.U., Qian, P.Y. 2006. Inhibition of biofouling by marine microorganisms and their metabolites. *Biofouling*, vol. 22, no.1, pp. 43-54, https://doi.org/10.1080/08927010500504784

Douglas, A.E. 2019. Simple animal models for microbiome research. *Nature Review Microbiology,* vol. 17, pp. 764–775, https://doi.org/10.1038/s41579-019-0242-1

Duan, Y., Huang, J., Wang, Y., Zhang, J. 2020. Characterization of bacterial community in intestinal and rearing water of *Penaeus monodon* differing growth performances in outdoor and indoor ponds. *Aquaculture Research*, vol. 51, no. 10, pp. 4279-4289, https://doi.org/10.1111/are.14770

Dubilier, N., Bergin, C., Lott, C. 2008. Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nature Reviews Microbiology*, vol. 6, no. 10, pp. 725-740, https://doi.org/10.1038/nrmicro1992

Dugas, L.R., Fuller, M., Gilbert, J., Layden, B.T. 2016. The obese gut microbiome across the epidemiologic transition. *Emerging Themes in Epidemiology,* vol. 13, no. 2, https://doi.org/10.1186/s12982-015-0044-5

Dunlap, P.V., Ast, J.C., Kimura, S., Fukui, A., Yoshino, T., Endo, H. 2007. Phylogenetic analysis of host–symbiont specificity and codivergence in bioluminescent symbioses. *Cladistics*, vol. 23, no. 5, pp. 507-532, https://doi.org/10.1111/j.1096-0031.2007.00157.x

Dunn, M.R. 2009. Feeding habits of the ommastrephid squid *Nototodarus sloanii* on the Chatham Rise, New Zealand. *New Zealand Journal of Marine and Freshwater Research*, vol. 43, no. 5, pp. 1103-1113, https://doi.org/10.1080/00288330.2009.9626533

Egerton, S., Culloty, S., Whooley, J., Stanton, C., Ross, R.P. 2018. The gut microbiota of marine fish. *Frontiers in Microbiology*, vol. 9, pp. 873, https://doi.org/10.3389/fmicb.2018.00873

Ezenwa, V.O., Gerardo, N.M., Inouye, D.W., Medina, M., Xavier, J.B. 2012. Animal behavior and the microbiome. *Science*, vol. 338, pp. 198–199, https://doi.org/10.1126/science.1227412

Fan, Y. & Pedersen, O. 2021. Gut microbiota in human metabolic health and disease. *Nature Reviews Microbiology*, vol. 19, no. 1, pp. 55-71, https://doi.org/10.1038/s41579-020-0433-9

Fernández-Álvarez, F.Á., Taite, M., Vecchione, M., Villanueva, R., Allcock, A.L. 2022. A phylogenomic look into the systematics of oceanic squids (order Oegopsida). *Zoological Journal of the Linnean Society*, vol. 194, no. 4, pp. 1212-1235, https://doi.org/10.1093/zoolinnean/zlab069

Ferrera, I., Auladell, A., Balagué, V., Reñé, A., Garcés, E., Massana, R., Gasol, J.M. 2024. Seasonal and interannual variability of the free-living and particle-associated bacteria of a coastal microbiome. *Environmental Microbiology Reports*, vol. 16, pp. e13299, https://doi.org/10.1111/1758-2229.13299

Figuerola, B., Capdevila, P., Cerdà-Domènech, M., Garrabou, J., Mirasole, A., Bassols, P., del Campo, J., Teixidó, N. 2025. Interactive effects of ocean acidification and warming disrupt calcification and microbiome composition in bryozoans. *Communications Biology,* vol. 8, pp. 1135, https://doi.org/10.1038/s42003-025-08524-8

Click or tap here to enter text.Ford, L.A., Alexander, S.K., Cooper, K.M., Hanlon, R.T. 1986. Bacterial populations of normal and ulcerated mantle tissue of the squid, *Lolliguncula brevis*. *Journal of Invertebrate Pathology*, vol. 48, pp. 13–26. https://doi.org/10.1016/0022-2011(86)90138-2

Frickmann, H., Zautner, A.E., Moter, A., Kikhney, J., Hagen, R.M., Stender, H., Poppert, S. 2017. Fluorescence *in situ* hybridization (FISH) in the microbiological diagnostic routine laboratory: a review. *Critical Reviews in Microbiology*, vol. 43, pp. 263–293, https://doi.org/10.3109/1040841X.2016.1169990

Fujiwara, Y., Takai, K., Uematsu, K., Tsuchida, S., Hunt, J.C., Hashimoto, J. 2000. Phylogenetic characterization of endosymbionts in three hydrothermal vent mussels: influence on host distributions. *Marine Ecology Progress Series*, vol. 208, pp. 147-155, https://doi.org/10.3354/meps

Gao, X., Zhang, M., Xue, J., Huang, J., Zhuang, R., Zhou, X., Zhang, H., Fu, Q., Hao, Y. 2018. Body mass index differences in the gut microbiota are gender specific. *Frontiers in Microbiology*, vol. 9, p. 1250, https://doi.org/10.3389/fmicb.2018.01250

Gentès, S., Minet, A., Lopes, C., Tessier, E., Gassie, C., Guyoneaud, R., Swarzenski, P.W., Bustamante, P., Metian, M., Amouroux, D., Lacoue-Labarthe, T. 2023. *In vivo* mercury (de)methylation metabolism in cephalopods under different pCO2 scenarios. *Environmental Science Technology,* vol. 57, pp. 5761–5770, https://doi.org/10.1021/acs.est.2c08513

Gillan, D.C., Speksnijder, A.G., Zwart, G., De Ridder, C. 1998. Genetic diversity of the biofilm covering *Montacuta ferruginosa* (Mollusca, Bivalvia) as evaluated by denaturing gradient gel electrophoresis analysis and cloning of PCR-amplified gene fragments coding for 16S rRNA. *Applied and Environmental Microbiology*, vol. 64, no. 9, pp. 3464-3472, https://doi.org/10.1128/AEM.64.9.3464-3472.1998

Gobet, A., Mest, L., Perennou, M., Dittami, S.M., Caralp, C., Coulombet, C., Huchette, S., Roussel, S., Michel, G., Leblanc, C. 2018. Seasonal and algal diet-driven patterns of the digestive microbiota of the European abalone *Haliotis tuberculata*, a generalist marine herbivore. *Microbiome*, vol. 6, no. 1, p. 60, https://doi.org/10.1186/s40168-018-0430-7

González-Aravena, M., Perrois, G., Font, A., Cárdenas, C.A., Rondon, R. 2024. Microbiome profile of the Antarctic clam *Laternula elliptica*. *Brazilian Journal of Microbiology*, vol. 55, no. 1, pp. 487-497, https://doi.org/10.1007/s42770-023-01200-1

Guerrero-Ferreira, R.C, Gorman, C., Chavez, A.A., Willie, S., Nishiguchi, M.K. 2013. Characterization of the Bacterial Diversity in Indo-West Pacific Loliginid and Sepiolid Squid Light Organs. *Microbial Ecology,* vol. 65, pp. 214–226, https://doi.org/10.1007/s00248-012-0099-6

Guerrero-Ferreira, R.C. & Nishiguchi, M.K. 2007. Biodiversity among luminescent symbionts from squid of the genera *Uroteuthis*, *Loliolus* and *Euprymna* (Mollusca: Cephalopoda). *Cladistics*, vol. 23, no. 5, pp. 497-506, https://doi.org/10.1111/j.1096-0031.2007.00155.x

Guerrero-Ferreira, R.C., & Nishiguchi, M.K. 2010. Differential gene expression in bacterial symbionts from loliginid squids demonstrates variation between mutualistic and environmental niches. *Environmental Microbiology,* vol. 2, pp. 514–523, https://doi.org/10.1111/j.1758-2229.2009.00077.x

Hamilton, T.J., Paz-Yepes, J., Morrison, R.A., Palenik, B., Tresguerres, M. 2014. Exposure to bloom-like concentrations of two marine *Synechococcus* cyanobacteria (strains CC9311 and CC9902) differentially alters fish behaviour. *Conservation Physiology,* vol. 2, no. 1, cou020, https://doi.org/10.1093/conphys/cou020

Heys, C., Cheaib, B., Busetti, A., Kazlauskaite, R., Maier, L., Sloan, W.T., Ijaz, U.Z., Kaufmann, J., McGinnity, P., Llewellyn, M.S. 2020. Neutral Processes Dominate

Microbial Community Assembly in Atlantic Salmon, *Salmo salar*. *Applied and Environmental Microbiology*, vol. 86, e02283-19, https://doi.org/10.1128/AEM.02283-19

Holmström, C., Rittschof, D., Kjelleberg, S. 1992. Inhibition of settlement by larvae of *Balanus amphitrite* and *Ciona intestinalis* by a surface-colonizing marine bacterium. *Applied and Environmental Microbiology*, vol. 58, no. 7, pp. 2111-2115, https://doi.org/10.1128/aem.58.7.2111-2115.1992

Holt, C.C., Dhaliwal, S., Na, I., Mtawali, M., Boscaro, V., Keeling, P. 2023. Spatial compartmentalisation of bacteria in phoronid microbiomes. *Scientific Reports*, vol. 13, no. 18612, https://doi.org/10.1038/s41598-023-45652-9

Honda, K. & Littman, D.R. 2012. The microbiome in infectious disease and inflammation. *Annual Review of Immunology*, vol. 30, pp. 759-795, https://doi.org/10.1146/annurev-immunol-020711-074937

Hongxiang, X., Min, W., Xiaogu, W., Junyi, Y. and Chunsheng, W. 2008. Bacterial diversity in deep-sea sediment from northeastern Pacific Ocean. *Acta Ecologica Sinica*, vol. 28, no. 2, pp. 479-485, https://doi.org/10.1016/S1872-2032(08)60026-8

Hoving, H.J.T., Perez, J.A.A., Bolstad, K.S., Braid, H.E., Evans, A.B., Fuchs, D., Judkins, H., Kelly, J.T., Marian, J.E., Nakajima, R. and Piatkowski, U. 2014. The study of deep-sea cephalopods. *Advances in Marine Biology*, vol. 67, pp. 235-359, https://doi.org/10.1016/B978-0-12-800287-2.00003-2

Hu, M.Y., Yu, Q.Z., Lin, J.Q., Fang, S.G. 2022. Sexual Dimorphism of the Gut Microbiota in the Chinese Alligator and Its Convergence in the Wild Environment. *International Journal of Molecular Sciences,* vol. 23, no. 20, pp. 12140, https://doi.org/10.3390/ijms232012140

Hu, X., Su, H., Zhang, P., Chen, Z., Xu, Y., Xu, W., Li, J., Wen, G., Cao, Y. 2022b. Microbial community characteristics of the intestine and gills of medium-form populations of *Sthenoteuthis oualaniensis* in the South China Sea. *Frontiers in Marine Science,* vol. 9, https://doi.org/10.3389/fmars.2022.920536

Hu, X., Su, H., Zhang, P., Chen, Z., Xu, Y., Xu, W., Li, J., Wen, G., Cao, Y. 2022a. Microbial Community Structure and Metabolic Characteristics of Intestine and Gills of Dwarf-Form Populations of *Sthenoteuthis oualaniensis* in South China Sea. *Fishes*, vol. 7, no. 4, pp. 191, https://doi.org/10.3390/fishes7040191

Huang, Y., Callahan, S., Hadfield, M.G. 2012. Recruitment in the sea: bacterial genes required for inducing larval settlement in a polychaete worm. *Science Reports*. vol. 2, no. 228, https://doi.org/10.1038/srep00228

Huddedar, S.B., Shete, A.M., Tilekar, J.N., Gore, S.D., Dhavale, D.D., Chopade, B.A. 2002. Isolation, characterization, and plasmid pUPI126-mediated indole-3-acetic acid production in *Acinetobacter* strains from rhizosphere of wheat. *Applied biochemistry and biotechnology*, vol. 102, no. 1, pp. 21-39, https://doi.org/10.1385/ABAB:102-103:1-6:021

Hwang, D.F., Arakawa, O., Saito, T., Noguchi, T., Simidu, U., Tsukamoto, K., Shida, Y., Hashimoto, K. 1989. Tetrodotoxin-producing bacteria from the blue-ringed octopus *Octopus maculosus*. *Marine Biology*, vol. 100, pp. 327-332, https://doi.org/10.1007/BF00391147

Ibáñez, C.M. & Keyl, F. 2010. Cannibalism in cephalopods. *Reviews in Fish Biology and Fisheries*, vol. 20, no. 1, pp. 123-136, https://doi.org/10.1007/s11160-009-9129-y

Iehata, S., Valenzuela, F., Riquelme, C. 2015. Analysis of bacterial community and bacterial nutritional enzyme activity associated with the digestive tract of wild Chilean octopus (*Octopus mimus* Gould, 1852). *Aquaculture Research*, vol. 46, pp. 861–873, https://doi.org/10.1111/are.12240

Iehata, S., Valenzuela, F., Riquelme, C. 2016. Evaluation of relationship between Chilean octopus (*Octopus mimus* Gould, 1852) egg health condition and the egg bacterial community. *Aquaculture Research*, vol. 47, no.2, pp. 649-659, https://doi.org/10.1111/are.12525

Ilstrup, D.M. 1990. Statistical methods in microbiology. *Clinical Microbiology Reviews*, vol. 3, no.3, pp. 219-226, https://doi.org/10.1128/cmr.3.3.219

Isnansetyo, A., & Kamei, Y. 2009. Bioactive substances produced by marine isolates of *Pseudomonas*. *Fournal of Industrial Microbiology and Biotechnology,* vol. 36, no. 10, pp. 1239–1248, https://doi.org/10.1007/s10295-009-0611-2

Itoi, S., Okamura, T., Koyama, Y., Sugita, H. 2006. Chitinolytic bacteria in the intestinal tract of Japanese coastal fishes. *Canadian Journal of Microbiology*, vol. 52, no. 12, pp. 1158-1163, https://doi.org/10.1139/w06-082

Ivanova, E.P., Kiprianova, E.A., Mikhailov, V.V., Levanova, G.F., Garagulya, A.D., Gorshkova, N.M., Vysotskii, M.V., Nicolau, D.V., Yumoto, N., Taguchi, T., Yoshikawa, S. 1998. Phenotypic diversity of *Pseudoalteromonas citrea* from different marine habitats and emendation of the description*. International Journal of Systematic and Evolutionary Microbiology,* vol. 48, pp. 247–256, https://doi.org/10.1099/00207713-48-1-247

Jackson, G.D. 2004. Advances in defining the life histories of myopsid squid. *Marine and Freshwater Research*, vol. 55, no. 4, pp. 357-365, https://doi.org/10.1071/MF03152

Jackson, G.D., McKinnon, J.F., Lalas, C., Ardern, R., Buxton, N.G. 1998. Food spectrum of the deepwater squid *Moroteuthis ingens* (Cephalopoda: Onychoteuthidae) in New Zealand waters. *Polar Biology*, vol. 20, no. 1, pp.56-65, https://doi.org/10.1007/s003000050276

Jackson, G.D., Shaw, A.G.P., Lalas, C. 2000. Distribution and biomass of two squid species off southern New Zealand: *Nototodarus sloanii* and *Moroteuthis ingens*. *Polar Biology*, vol. 23, no. 10, pp.699-705, https://doi.org/10.1007/s003000000141

Jereb, P., & Roper, C.F.E. 2010. Cephalopods of the world - an annotated and illustrated catalogue of cephalopod species known to date. Vol 2. Myopsid and oegopsid squids. FAO, Rome.

Jereb, P., Vecchione, M. & Roper, C.F.E. 2010. 'Family Loliginidae'. In P. Jereb & C.F.E. Roper, eds. *Cephalopods of the world. An annotated and illustrated catalogue of species known to date. Volume 2. Myopsid and Oegopsid Squids.* FAO Species Catalogue for Fishery Purposes, vol. 2, no. 4, Rome, FAO. pp. 38–117.

Jin, Y., Wu, S., Zeng, Z., Fu, Z. 2017. Effects of environmental pollutants on gut microbiota. *Environmental Pollution*, vol. 222, pp.1-9, https://doi.org/10.1016/j.envpol.2016.11.045

Juni, E. 1992. The Genus Psychrobacter. In: Balows, A., Trüper, H.G., Dworkin, M., Harder, W., Schleifer, KH. (eds) The Prokaryotes. *Springer*, New York, NY. pp. 3241-3246, https://doi.org/10.1007/978-1-4757-2191-1_12

Kamp, D.L., Kerwin, A.H., McAnulty, S.J., Nyholm, S.V. 2025. Organ structure and bacterial microbiogeography in a reproductive organ of the Hawaiian bobtail squid reveal dimensions of a defensive symbiosis. *Applied and Environmental Microbiology*, vol. 91, no. 5, pp. e02163-24, https://doi.org/10.1128/aem.02163-24

Kang, W., Kim, P.S., Tak, E.J., Sung, H., Shin, N.-R., Hyun, D.-W., Whon, T.W., Kim, H.S., Lee, J.Y., Yun, J.-H., Jung, M.J., Bae, J.W. 2022. Host phylogeny, habitat, and diet are main drivers of the cephalopod and mollusk gut microbiome*. Animal Microbiome*, vol. 4, no. 30, https://doi.org/10.1186/s42523-022-00184-x

Kaufman, M.R., Ikeda, Y., Patton, C., van Dykhuizen, G., Epel, D. 1998. Bacterial Symbionts Colonize the Accessory Nidamental Gland of the Squid *Loligo opalescens* via Horizontal Transmission. *The Biological Bulletin*, vol. 194, pp. 36–43, https://doi.org/10.2307/1542511

Kaur, G., Kumar, N., Mual, P., Kumar, A., Kumar, R.M., Mayilraj, S. 2016. *Brachybacterium aquaticum* sp. nov., a novel actinobacterium isolated from seawater. *International Journal of Systematic and Evolutionary Microbiology*, vol. 66, pp. 4705–4710, https://doi.org/10.1099/ijsem.0.001414

Kellermann, M.Y., Schubotz, F., Elvert, M., Lipp, J.S., Birgel, D., Prieto-Mollar, X., Dubilier, N., Hinrichs, K.U. 2012. Symbiont–host relationships in chemosynthetic mussels: a comprehensive lipid biomarker study. *Organic Geochemistry*, vol. 43, pp. 112-124, https://doi.org/10.1016/j.orggeochem.2011.10.005

Kennedy, J., Flemer, B., Jackson, S.A., Morrissey, J.P., O’Gara, F., Dobson, A.D.W. 2014. Evidence of a putative deep sea specific microbiome in marine sponges. *PLoS One*, vol. 9, pp. e91092, https://doi.org/10.1371/journal.pone.0091092

Kerney, R., Kim, E., Hangarter, R.P., Heiss, A.A., Bishop, C.D., Hall, B.K. 2011. Intracellular invasion of green algae in a salamander host. *Proceedings of the National Academy of Sciences*, vol. 108, no. 16, pp. 6497-6502, https://doi.org/10.1073/pnas.1018259108

Kerwin, A.H., & Nyholm, S.V. 2017. Symbiotic bacteria associated with a bobtail squid reproductive system are detectable in the environment, and stable in the host and developing eggs. *Environmental Microbiology*, vol. 19, pp. 1463–1475, https://doi.org/10.1111/1462-2920.13665

Ketchum, R.N., Smith, E.G., Vaughan, G.O., McParland, D., Al-Mansoori, N., Burt, J.A., Reitzel, A.M. 2021. Unraveling the predictive role of temperature in the gut microbiota of the sea urchin *Echinometra* sp. *EZ* across spatial and temporal gradients. *Molecular Ecology*, vol. 30, pp. 3869–3881, https://doi.org/10.1111/mec.15990

Kim, Y.S., Son, H.J., Jeong, S.Y. 2015. Isolation of an algicide from a marine bacterium and its effects against the toxic dinoflagellate *Alexandrium catenella* and other harmful algal bloom species. *Journal of microbiology*, vol. 53, no. 8, pp. 511-517, https://doi.org/10.1007/s12275-015-5303-1

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, vol. 41, no. 1, pp. e1, https://doi.org/10.1093/nar/gks808

Koenig, K.M., Sun, P., Meyer, E., Gross, J.M. 2016. Eye development and photoreceptor differentiation in the cephalopod *Doryteuthis pealeii*. *Development*, vol. 143, no. 17, pp. 3168-3181,

Kormas, K.A., Meziti, A., Mente, E., Frentzos, A. 2014. Dietary differences are reflected on the gut prokaryotic community structure of wild and commercially reared sea bream (*Sparus aurata*). *MicrobiologyOpen*, vol. 3, no. 5, pp. 718-728, https://doi.org/10.1002/mbo3.202

Kremer, K.I., Hagen, W., Oesterwind, D., Duncan, S., Bode-Dalby, M., Dorschner, S., Dudeck, T., Sell, A.F. 2025. Trophic ecology of squids in the Benguela Upwelling System elucidated by combining stomach content, stable isotope and fatty acid analyses. *Marine Biology,* vol. 172, no. 32, https://doi.org/10.1007/s00227-024-04592-2

Kristjansson, J.K., & Alfredsson, G.A. 1991. ‘The Heterotrophic, Thermophilic Genera *Thermomicrobium*, *Rhodothermus*, *Saccharococcus*, *Acidothermus*, and *Scotothermus*’, in: *Thermophilic Bacteria*. CRC Press, pp. 14.

Kuang, T., He, A., Lin, Y., Huang, X., Liu, L., Zhou, L. 2020. Comparative analysis of microbial communities associated with the gill, gut, and habitat of two filter-feeding fish. *Aquaculture Reports*, vol. 18, p.100501, https://doi.org/10.1016/j.aqrep.2020.100501

Laptikhovsky, V.V. 1999. Fecundity and spawning in squid of families Enoploteuthidae and Ancistrocheiridae (Cephalopoda: Oegopsida). *Scientia Marina*, vol. 63, pp. 1–7, https://doi.org/10.3989/scimar.1999.63n11

Lasa, A. & Romalde, J.L. 2021. ‘Coevolution of Molluscs and Their Microbes’. In: Hurst, C.J., *Microbes: The Foundation Stone of the Biosphere*, Springer Nature, Cham, vol.8, pp. 513-526, https://doi.org/10.1007/978-3-030-63512-1_24

Lavy, O., Gophna, U., Gefen, E., Ayali, A. 2020. Dynamics of bacterial composition in the locust reproductive tract are affected by the density-dependent phase. *FEMS Microbiology Ecology,* vol. 96, no. 4, pp. fiaa044, https://doi.org/10.1093/femsec/fiaa044

Lawler, S.N., Kellogg, C.A., France, S.C., Clostio, R.W., Brooke, S.D., Ross, S.W. 2016. Coral-associated bacterial diversity is conserved across two deep-sea *Anthothela* species. *Frontiers in Microbiology*, vol. 7, p. 458, https://doi.org/10.3389/fmicb.2016.00458

Le Roux, F., Wegner, K.M., Polz, M.F. 2016. Oysters and *Vibrios* as a model for disease dynamics in wild animals. *Trends in microbiology*, vol. 24, no. 7, pp. 568-580, 10.1016/j.tim.2016.03.006

Lederberg, J. & McCray, A.T. 2001. ‘Ome SweetOmics--A genealogical treasury of words’. *The Scientist*, vol. 15, no. 7, pp. 8.

Lee, H., Choi, Y., Han, J.H., Lee, S.D., Park, S., Choi, J.H. 2024. Submarine volcanic microbiota record three volcano-induced tsunamis. *Communications Earth & Environment*, vol. 5, no. 1, p. 272, https://doi.org/10.1038/s43247-024-01443-2

Lesser, M.P., Fiore, C., Slattery, M., Zaneveld, J. 2016. Climate change stressors destabilize the microbiome of the Caribbean barrel sponge, *Xestospongia muta*. *Journal of Experimental Marine Biology and Ecology*, vol. 475, pp. 11–18, https://doi.org/10.1016/j.jembe.2015.11.004

Li, J., Zhang, Yang, Sun, J., Thompson, F., Zhang, Yanying. 2023. Editorial: Interaction between marine invertebrates and symbiotic microbes in a changing environment: Community structure and ecological functions. *Frontiers in Marine Science,* vol. 9, https://doi.org/10.3389/fmars.2022.1128906

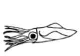
Li, Y.F., Xu, J.K., Chen, Y.W., Ding, W.Y., Shao, A.Q., Liang, X., Zhu, Y.T., Yang, J.L. 2019. Characterization of gut microbiome in the mussel *Mytilus galloprovincialis* in response to thermal stress. *Frontiers in Physiology*, vol. 10, p. 1086, https://doi.org/10.3389/fphys.2019.01086

Lin, N., Tao, Y., Gao, P., Xu, Y., Xing, P. 2021. Comparative Genomics Revealing Insights into Niche Separation of the Genus *Methylophilus*. *Microorganisms*, vol. 9, no. 8, pp. 1577, https://doi.org/10.3390/microorganisms9081577

Lischka, A., Pook, C.J., Pannell, J.L., Braid, H.E., Gaw, S., Bolstad, K.S. 2020. Distribution of trace elements in the tissues of arrow squid (*Nototodarus sloanii*) from the Chatham Rise, New Zealand: Human health implications. *Fisheries Research*, vol. 221, pp. 105383, https://doi.org/10.1016/j.fishres.2019.105383

Liu, H., Guo, X., Gooneratne, R., Lai, R., Zeng, C., Zhan, F., Wang, W. 2016. The gut microbiome and degradation enzyme activity of wild freshwater fishes influenced by their trophic levels. *Scientific Reports*, vol. 6, p. 24340, https://doi.org/10.1038/srep24340

Liu, H., Tan, K.S., Zhang, X., Zhang, H., Cheng, D., Ting, Y., Li, S., Ma, H., Zheng, H. 2020. Comparison of gut microbiota between golden and brown noble scallop *Chlamys*



nobilis and its association with carotenoids. *Frontiers in Microbiology*, vol. 11, p. 36, https://doi.org/10.3389/fmicb.2020.00036

Lo Giudice, A., & Rizzo, C. 2018. Bacteria Associated with Marine Benthic Invertebrates from Polar Environments: Unexplored Frontiers for Biodiscovery? *diversity,* vol. 10, no. 3, pp. 80, https://doi.org/10.3390/d10030080

Lovejoy, C., Bowman, J.P., Hallegraeff, G.M. 1998. Algicidal effects of a novel marine *Pseudoalteromonas* isolate (class Proteobacteria, gamma subdivision) on harmful algal bloom species of the genera *Chattonella*, *Gymnodinium*, and *Heterosigma*. *Applied and Environmental Microbiology*, vol. 64, no.8, pp.2806-2813, https://doi.org/10.1128/AEM.64.8.2806-2813.1998

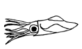
Lu, C.C. & Williams, R. 1994. Contribution to the biology of squid in the Prydz Bay region, Antarctica. *Antarctic Science*, vol. 6, no. 2, pp. 223-229, https://doi.org/10.1017/S0954102094000349

Lum-Kong, A. & Hastings, T.S. 1992. The accessory nidamental glands of *Loligo forbesi* (Cephalopoda: Loliginidae): characterization of symbiotic bacteria and preliminary experiments to investigate factors controlling sexual maturation. *Journal of Zoology*, vol. 228, no. 3, pp. 395-403, https://doi.org/10.1111/j.1469-7998.1992.tb04443.x

Lutz, H.L., Ramírez-Puebla, S.T., Abbo, L., Durand, A., Schlundt, C., Gottel, N.R., Sjaarda, A.K., Hanlon, R.T., Gilbert, J.A., Mark Welch, J.L. 2019. A simple microbiome in the European common cuttlefish, *Sepia officinalis*. *mSystems*, vol. 4, no.4, pp. e00177-19, https://doi.org/10.1128/msystems.00177-19

Ma, Y., Sun, F., Zhang, C., Bao, P., Cao, S., Zhang, M. 2014. Effects of *Pseudoalteromonas* sp. BC228 on digestive enzyme activity and immune response of juvenile sea cucumber (*Apostichopus japonicus*). *Journal of Ocean University of China*, vol. 13, pp. 1061–1066, https://doi.org/10.1007/s11802-014-2340-z

Mahadevan, P., & Middlebrooks, M.L. 2020. Bacterial diversity in the *clarki* ecotype of the photosynthetic sacoglossan, *Elysia crispata*. *MicrobiologyOpen*, vol. 9, pp. e1098, https://doi.org/10.1002/mbo3.1098



Mahadik, S., Kshirsagar, N., Tokshiya, S., Agnihotri, S., Pathade, G. 2025. Isolation, characterization and identification of bioluminescent bacteria from Indian squid (*Uroteuthis duvaucelii*). *Journal of Pure & Applied Microbiology*, vol. 19, no. 1, https://doi.org/10.22207/JPAM.19.1.47

Maoka, T. 2011. Carotenoids in marine animals. *Marine Drugs,* vol. 9, pp. 278–293, https://doi.org/10.3390/md9020278

Martino, C., Morton, J.T., Marotz, C.A., Thompson, L.R., Tripathi, A., Knight, R., Zengler, K. 2019. A novel sparse compositional technique reveals microbial perturbations. *mSystems*, vol. 4, no. 1, pp. 10-1128, https://doi.org/10.1128/msystems.00016-19

Maruyama, A., Honda, D., Yamamoto, H., Kitamura, K., Higashihara, T. 2000. Phylogenetic analysis of psychrophilic bacteria isolated from the Japan Trench, including a description of the deep-sea species *Psychrobacter pacificensis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, vol. 50, no. 2, pp. 835-846, https://doi.org/10.1099/00207713-50-2-835

Matsui, S., Seidou, M., Horiuchi, S., Uchiyama, I., Kito, Y. 1988. Adaptation of a deep-sea cephalopod to the photic environment. Evidence for three visual pigments. *The Journal of General Physiology*, vol. 92, no. 1, pp. 55-66, https://doi.org/10.1085/jgp.92.1.55

McAnulty, S.J., Kerwin, A.H., Koch, E., Nuttall, B., Suria, A.M., Collins, A.J., Schleicher, T.R., Rader, B.A., Nyholm, S.V. 2023. “Failure To Launch”: development of a reproductive organ linked to symbiotic bacteria. *mBio*, vol. 14, pp. e02131-22, https://doi.org/10.1128/mbio.02131-22

McFall-Ngai, M., Hadfield, M.G., Bosch, T.C.G., Carey, H.V., Domazet-Lošo, T., Douglas, A.E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S.F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A.H., Kremer, N., Mazmanian, S.K., Metcalf, J.L., Nealson, K., Pierce, N.E., Rawls, J.F., Reid, A., Ruby, E.G., Rumpho, M., Sanders, J.G., Tautz, D., Wernegreen, J.J. 2013. Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences,* vol. 110, pp. 3229–3236, https://doi.org/10.1073/pnas.1218525110

McMurdie, P.J. & Holmes, S. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*, vol. 8, no. 4, p.e61217, https://doi.org/10.1371/journal.pone.0061217

Meunier, L., Costa, R., Keller-Costa, T., Cannella, D., Dechamps, E., George, I.F. 2024. Selection of marine bacterial consortia efficient at degrading chitin leads to the discovery of new potential chitin degraders. *Microbiology Spectrum*, vol. 12, pp. e00886-24, https://doi.org/10.1128/spectrum.00886-24

Meziti, A., Ramette, A., Mente, E., Kormas, K.Ar. 2010. Temporal shifts of the Norway lobster (*Nephrops norvegicus*) gut bacterial communities. *FEMS Microbiology Ecology,* vol. 74, pp. 472–484, https://doi.org/10.1111/j.1574-6941.2010.00964.x

Mock, T. & Kirkham, A. 2012. What can we learn from genomics approaches in marine ecology? From sequences to eco-systems biology! *Marine Ecology*, vol. 33, no. 2, pp. 131-148, https://doi.org/10.1111/j.1439-0485.2011.00479.x

Moi, I.M., Roslan, N.N., Leow, A.T.C., Ali, M.S.M., Rahman, R.N.Z.R.A., Rahimpour, A., Sabri, S. 2017. The biology and the importance of *Photobacterium* species. *Applied Microbiology and Biotechnology*, vol. 101, no. 11, pp. 4371-4385, https://doi.org/10.1007/s00253-017-8300-y

Molloy, P.J., & Cowling, M.J. 1999. Buoyancy mechanisms of marine organisms: lessons from nature. *Underwater Technology*, vol. 24, pp. 41–49, https://doi.org/10.3723/175605499783259785

Moloney, C.L., St John, M.A., Denman, K.L., Karl, D.M., Köster, F.W., Sundby, S., Wilson, R.P. 2011. Weaving marine food webs from end to end under global change. *Journal of Marine Systems*, vol. 84, no. 3-4, pp. 106-116, https://doi.org/10.1016/j.jmarsys.2010.06.012

Montgomery, M.K. & McFall-Ngai, M. 1994. Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid *Euprymna scolopes*. *Development*, vol. 120, no. 7, pp. 1719-1729, https://doi.org/10.1242/dev.120.7.1719

Moreira, A.P.B., Duytschaever, G., Chimetto Tonon, L.A., Fróes, A.M., de Oliveira, L.S., Amado-Filho, G.M., Francini-Filho, R.B., De Vos, P., Swings, J., Thompson, C.C., Thompson, F.L. 2014. *Photobacterium sanctipauli* sp. nov. isolated from bleached *Madracis decactis* (Scleractinia) in the St Peter & St Paul Archipelago, Mid-Atlantic Ridge, Brazil. *PeerJ*, vol. 2, pp. e427, https://doi.org/10.7717/peerj.427

Moreno-Pino, M., Cristi, A., Gillooly, J.F., Trefault, N., 2020. Characterizing the microbiomes of Antarctic sponges: a functional metagenomic approach. *Scientific Report,* vol. 10, pp. 645, https://doi.org/10.1038/s41598-020-57464-2

MPI, Annual Review Report for Deepwater Fisheries 2022/2023. 2023. https://www.mpi.govt.nz/dmsdocument/68307-Annual-review-report-for-deepwater-fisheries-202223 [Accessed 7th September 2025].

Murray, A.E., Rack, F.R., Zook, R., Williams, M.J.M., Higham, M.L., Broe, M., Kaufmann, R.S., Daly, M. 2016. Microbiome Composition and Diversity of the Ice-Dwelling Sea Anemone, *Edwardsiella andrillae*. *Integrative & Comparative Biology,* vol. 56, no. 4, pp. 542-555, https://doi.org/10.1093/icb/icw095

Muyzer, G., & Smalla, K. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek,* vol. 73, pp. 127–141, https://doi.org/10.1023/A:1000669317571

Naguit, M.A.A., Plata, K.C., Abisado, R.G., Calugay, R.J. 2014. Evidence of bacterial bioluminescence in a Philippine squid and octopus hosts. *Aquaculture, Aquarium, Conservation & Legislation International Journal of the Bioflux Society,* vol. 7, no. 6, http://www.bioflux.com.ro/aacl

Naim, M.A., Morillo, J.A., Sørensen, S.J., Waleed, A.A.-S., Smidt, H., Sipkema, D. 2014. Host-specific microbial communities in three sympatric North Sea sponges. *FEMS Microbiology Ecology,* vol. 90, no. 2, pp. 390-403, https://doi.org/10.1111/1574-6941.12400

Nelson, T.M., Rogers, T.L., Carlini, A.R., Brown, M.V. 2012. Diet and phylogeny shape the gut microbiota of Antarctic seals: a comparison of wild and captive

animals. *Environmental Microbiology*, vol. 15, no. 4, pp. 1132-1145, https://doi.org/10.1111/1462-2920.12022

Ngangbam, A.K., Baten, A., Waters, D.L., Whalan, S., Benkendorff, K. 2015. characterization of bacterial communities associated with the tyrian purple producing gland in a marine gastropod. *PLoS One*, vol. 10, no. 10, p.e0140725, https://doi.org/10.1371/journal.pone.0140725

Ngugi, D.K., Miyake, S., Cahill, M., Vinu, M., Hackmann, T.J., Blom, J., Tietbohl, M.D., Berumen, M.L., Stingl, U. 2017. Genomic diversification of giant enteric symbionts reflects host dietary lifestyles. *Proceedings of the National Academy of Sciences*, vol. 114, no. 36, pp. E7592-E7601, https://doi.org/10.1073/pnas.170307011

Nishiguchi, M.K. & Nair, V.S. 2003. Evolution of symbiosis in the Vibrionaceae: a combined approach using molecules and physiology. *International Journal of Systematic and Evolutionary Microbiology*, vol. 53, no. 6, pp. 2019-2026, https://doi.org/10.1099/ijs.0.02792-0

Nishiguchi, M.K., Lopez, J.E., Boletzky, S.V. 2004. Enlightenment of old ideas from new investigations: more questions regarding the evolution of bacteriogenic light organs in squids. *Evolution & development*, vol. 6, no. 1, pp. 41-49, https://doi.org/10.1111/j.1525-142X.2004.04009.x

Nogi, Y., & Kato, C. 1999. Taxonomic studies of extremely barophilic bacteria isolated from the Mariana Trench and description of *Moritella yayanosii* sp. nov., a new barophilic bacterial isolate. *Extremophiles*, vol. 3, pp. 71–77, https://doi.org/10.1007/s007920050101

Nyholm, S.V. & McFall-Ngai, M.J. 2021. A lasting symbiosis: how the Hawaiian bobtail squid finds and keeps its bioluminescent bacterial partner. *Nature Reviews Microbiology*, vol. 19, no. 10, pp. 666-679, https://doi.org/10.1038/s41579-021-00567-y

O’Brien, P.A., Webster, N.S., Miller, D.J., Bourne, D.G. 2019. Host-microbe coevolution: applying evidence from model systems to complex marine invertebrate holobionts. *mBio*, vol. 10, https://doi.org/10.1128/mbio.02241-18

O’Connor, R.M., Fung, J.M., Sharp, K.H., Benner, J.S., McClung, C., Cushing, S., Lamkin, E.R., Fomenkov, A.I., Henrissat, B., Londer, Y.Y., Scholz, M.B. 2014. Gill bacteria enable a novel digestive strategy in a wood-feeding mollusk. *Proceedings of the National Academy of Sciences*, vol. 111, no. 47, pp. E5096-E5104, https://doi.org/10.1073/pnas.1413110111

Offret, C., Rochard, V., Laguerre, H., Mounier, J., Huchette, S., Brillet, B., Le Chevalier, P., Fleury, Y. 2019. Protective efficacy of a *Pseudoalteromonas* strain in European abalone, *Haliotis tuberculata*, infected with *Vibrio harveyi* ORM4. *Probiotics and Antimicrobial Proteins*, vol. 11, no. 1, pp. 239-247, https://doi.org/10.1007/s12602-018-9389-8

Ohwada, K., Tabor, P.S., Colwell, R.R. 1980. Species composition and barotolerance of gut microflora of deep-sea benthic macrofauna collected at various depths in the Atlantic Ocean. *Applied and Environmental Microbiology*, vol. 40, no. 4, pp. 746-755, https://doi.org/10.1128/aem.40.4.746-755.1980

Oksanen, J., Simpson, G., Blanchet, F., Kindt, R., Legendre, P., Minchin, P., O'Hara, R., Solymos, P., Stevens, M., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Borman, T., Carvalho, G., Chirico, M., De Caceres, M., Durand, S., Evangelista, H., FitzJohn, R., Friendly, M., Furneaux, B., Hannigan, G., Hill, M., Lahti, L., Martino, C., McGlinn, D., Ouellette, M., Ribeiro Cunha, E., Smith, T., Stier, A., Ter Braak, C., Weedon, J. 2025. _vegan: Community Ecology Package_. https://doi.org/10.32614/CRAN.package.vegan, R package version 2.7-1, https://CRAN.R-project.org/package=vegan

Orsod, M., Joseph, M., Huyop, F. 2012. Characterization of exopolysaccharides produced by *Bacillus cereus* and *Brachybacterium* sp. isolated from Asian sea bass (*Lates calcarifer*). *Malaysian Journal of Microbiology*, vol. 8, no. 3, pp. 170-174.

Ortiz-Burgos, S. 2016. ‘Shannon-weaver diversity index’. In: *Encyclopedia of estuaries*, Springer, Dordrecht, pp. 572-573.

Osman, E.O., & Weinnig, A.M. 2022. Microbiomes and Obligate Symbiosis of Deep-Sea Animals. *Annual Review of Animal Biosciences*, vol. 10, pp. 151–176, https://doi.org/10.1146/annurev-animal-081621-112021

Ottman, N., Smidt, H., De Vos, W.M., Belzer, C. 2012. The function of our microbiota: who is out there and what do they do? *Frontiers in Cellular and Infection Microbiology*, vol. 2, p. 104, https://doi.org/10.3389/fcimb.2012.00104

Paulson, J.N., Stine. O.C., Bravo, H.C., Pop, M. 2013. "Differential abundance analysis for microbial marker-gene surveys." *Nature Methods*, doi:10.1038/nmeth.2658, http://www.nature.com/nmeth/journal/vaop/ncurrent/abs/nmeth.2658.html

Peng, L.H., Liang, X., Xu, J.K., Dobretsov, S., Yang, J.L. 2020. Monospecific Biofilms of *Pseudoalteromonas* Promote Larval Settlement and Metamorphosis of *Mytilus coruscus*. *Scientific Reports,* vol. 10, pp. 2577, https://doi.org/10.1038/s41598-020-59506-1

Pertiwi, M.P., Praseptin, P.K.H., Werdani, I.D., Listyorini, D., Prabaningtyas, S. 2018. Identification and phylogenetic study of bioluminescent bacteria from squid (*Loligo duvaucelii*) based on 16S rRNA gene. *AIP Conference Proceedings*, vol. 2002, no. 1, pp. 020038, https://doi.org/10.1063/1.5050134

Petersen, J.M. & Osvatic, J. 2018. Microbiomes in natura: importance of invertebrates in understanding the natural variety of animal-microbe interactions. *mSystems*, vol. 3, no. 2, pp. e00179-17, https://doi.org/10.1128/msystems.00179-17

Pethybridge, H., Virtue, P., Casper, R., Yoshida, T., Green, C.P., Jackson, G., Nichols, P.D. 2012. Seasonal variations in diet of arrow squid (*Nototodarus gouldi*): stomach content and signature fatty acid analysis. *Journal of the Marine Biological Association of the United Kingdom*, vol. 92, no. 1, pp. 187-196, doi:10.1017/S0025315411000841

Pethybridge, H.R., Nichols, P.D., Virtue, P., Jackson, G.D. 2013. The foraging ecology of an oceanic squid, *Todarodes filippovae*: The use of signature lipid profiling to monitor ecosystem change. *Deep Sea Research Part II: Topical Studies in Oceanography*, vol. 95, pp. 119–128, https://doi.org/10.1016/j.dsr2.2012.07.025

Pichon, D., Gaia, V., Norman, M.D., Boucher-Rodoni, R. 2005. Phylogenetic diversity of epibiotic bacteria in the accessory nidamental glands of squids (Cephalopoda: Loliginidae and Idiosepiidae). *Marine Biology,* vol. 147, pp. 1323–1332, https://doi.org/10.1007/s00227-005-0014-5

Pimentel, Z.T., Dufault-Thompson, K., Russo, K.T., Scro, A.K., Smolowitz, R.M., Gomez-Chiarri, M., Zhang, Y. 2021. Microbiome analysis reveals diversity and function of mollicutes associated with the Eastern Oyster, *Crassostrea virginica*. *Msphere*, vol. 6, no. 3, pp. 10-1128, https://doi.org/10.1128/msphere.00227-21

Portela, E., Simões, N., Rosas, C., Mascaró, M. 2014. Can preference for crabs in juvenile *Octopus maya* be modified through early experience with alternative prey? *Behaviour*, vol. 151, no. 11, pp. 1597-1616, https://doi.org/10.1163/1568539X-00003206

Prakash, O., Shouche, Y., Jangid, K., Kostka, J.E. 2013. Microbial cultivation and the role of microbial resource centers in the omics era. *Applied Microbiology and Biotechnology,* vol. 97, pp. 51–62, https://doi.org/10.1007/s00253-012-4533-y

Pratt, A.M., & Anderson, F.E. 2025. Evolution of ammoniacal buoyancy within oegopsid squids and its relationship with depth. *Journal of Molluscan Studies*, vol. 91, no. 3, eyaf013, https://doi.org/10.1093/mollus/eyaf013

Priest, T., Oldenburg, E., Popa, O., Dede, B., Metfies, K., von Appen, W.-J., Torres-Valdés, S., Bienhold, C., Fuchs, B.M., Amann, R., Boetius, A., Wietz, M. 2025. Seasonal recurrence and modular assembly of an Arctic pelagic marine microbiome. *Nature Communications,* vol. 16, no. 1326, https://doi.org/10.1038/s41467-025-56203-3

Pringgenies, D., & Jørgensen, J.M. 1994. Morphology of the Luminous Organ of the Squid *Loligo duvauceli*d'Orbigny, 1839. *Acta Zoologica*, vol. 75, pp. 305–309, https://doi.org/10.1111/j.1463-6395.1994.tb00967.x

Pucciarelli, S., Chiappori, F., Devaraj, R.R., Yang, G., Yu, T., Ballarini, P., Miceli, C. 2014. Identification and analysis of two sequences encoding ice-binding proteins obtained from a putative bacterial symbiont of the psychrophilic Antarctic ciliate *Euplotes* *focardii*. *Antarctic Science*, vol. 26, no. 5, pp. 491-501, doi:10.1017/S0954102014000017

R Core Team. 2021. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Radjasa, O.K. & Sabdono, A. 2006. Phylogenetic diversity of secondary metabolite producing-bacteria associated with sponges from Bandengan waters, Jepara. *Journal of Coastal Development*, vol. 10, no. 1, pp. 47-54.

Ramírez, A.S., Vega-Orellana, O.M., Viver, T., Poveda, J.B., Rosales, R.S., Poveda, C.G., Spergser, J., Szostak, M.P., Caballero, M.J., Ressel, L., Bradbury, J.M., Mar Tavío, M., Karthikeyan, S., Amann, R., Konstantinidis, K.T., Rossello-Mora, R. 2019. First description of two moderately halophilic and psychrotolerant *Mycoplasma* species isolated from cephalopods and proposal of *Mycoplasma marinum* sp. nov. and *Mycoplasma todarodis* sp. nov. *Systematic and Applied Microbiology*, vol. 42, pp. 457–467, https://doi.org/10.1016/j.syapm.2019.04.003

Rasmussen, J.A., Kiilerich, P., Madhun, A.S., Waagbø, R., Lock, E.J.R., Madsen, L., Gilbert, M.T.P., Kristiansen, K., Limborg, M.T. 2023. Co-diversification of an intestinal Mycoplasma and its salmonid host. *The ISME Journal*, vol. 17, no.5, pp. 682-692, https://doi.org/10.1038/s41396-023-01379-z

Rasmussen, J.A., Villumsen, K.R., Duchêne, D.A., Puetz, L.C., Delmont, T.O., Sveier, H., Jørgensen, L. von G., Præbel, K., Martin, M.D., Bojesen, A.M., Gilbert, M.T.P., Kristiansen, K., Limborg, M.T. 2021. Genome-resolved metagenomics suggests a mutualistic relationship between *Mycoplasma* and salmonid hosts. *Communications Biology,* vol. 4, pp. 1–10, https://doi.org/10.1038/s42003-021-02105-1

Rasmussen, J.A., Villumsen, K.R., Ernst, M., Hansen, M., Forberg, T., Gopalakrishnan, S., Gilbert, M.T.P., Bojesen, A.M., Kristiansen, K., Limborg, M.T. 2022. A multi-omics approach unravels metagenomic and metabolic alterations of a probiotic and synbiotic additive in rainbow trout (*Oncorhynchus mykiss*). *Microbiome*, vol. 10, no. 1, p. 21, https://doi.org/10.1186/s40168-021-01221-8

Rawls, J.F., Samuel, B.S., Gordon, J.I. 2004. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Academy of Sciences*, vol. 101, no. 13, pp. 4596-4601, https://doi.org/10.1073/pnas.0400706101

Remeslo, A., Yukhov, V., Bolstad, K., Laptikhovsky, V. 2019. Distribution and biology of the colossal squid, *Mesonychoteuthis hamiltoni*: New data from depredation in toothfish fisheries and sperm whale stomach contents. *Deep Sea Research Part I: Oceanographic Research Papers*, vol. 147, pp. 121–127, https://doi.org/10.1016/j.dsr.2019.04.008

Remeslo, A.V., Yakushev, M.R., Laptikhovsky, V. 2015. Alien vs. Predator: interactions between the colossal squid (*Mesonychoteuthis hamiltoni*) and the Antarctic toothfish (*Dissostichus mawsoni*). *Journal of Natural History*, vol. 49, pp. 2483–2491, https://doi.org/10.1080/00222933.2015.1040477

Riesenfeld, C.S., Murray, A.E., Baker, B.J. 2008. Characterization of the microbial community and polyketide biosynthetic potential in the palmerolide-producing tunicate *Synoicum adareanum*. *Journal of Natural Products*, vol. 71, no. 11, pp. 1812-1818, https://doi.org/10.1021/np800287n

Rintoul, S.R. 2000. ‘Southern Ocean currents and climate’. In: *Papers and proceedings of the Royal Society of Tasmania*, vol. 133, no. 3, pp. 41-50, https://doi.org/10.26749/rstpp.133.3.41

Rivaro, P., & Ianni, C. 2025. Biogeochemistry of the Ross Sea and its ecosystem implication. *Deep Sea Research Part II: Topical Studies in Oceanography*, vol. 219, pp. 105449, https://doi.org/10.1016/j.dsr2.2024.105449

Roberts, J., Xavier, J.C., Agnew, D.J. 2011. The diet of toothfish species *Dissostichus eleginoides* and *Dissostichus mawsoni* with overlapping distributions. *Journal of Fish Biology*, vol. 79, no. 1, pp. 138-154, https://doi.org/10.1111/j.1095-8649.2011.03005.x

Rodríguez-Barreto, D., Sanz-González, J.C., Martín, M.V., Arrieta, J.M., Almansa, E. 2024. Sex-specific bacterial microbiome variation in *Octopus vulgaris* skin. *Frontiers in Microbiology,* vol. 14, https://doi.org/10.3389/fmicb.2023.1233661

Rodríguez-Marconi, S., De la Iglesia, R., Díez, B., Fonseca, C.A., Hajdu, E., Trefault, N. 2015. Characterization of bacterial, archaeal and eukaryote symbionts from Antarctic sponges reveals a high diversity at a three-domain level and a particular signature for this ecosystem. *PloS one*, vol. 10, no. 9, p. e0138837, https://doi.org/10.1371/journal.pone.0138837

Romalde, J.L., Diéguez, A.L., Lasa, A., Balboa, S. 2014. New *Vibrio* species associated to molluscan microbiota: a review. *Frontiers in Microbiology*, vol. 4, p. 413, https://doi.org/10.3389/fmicb.2013.00413

Romanenko, L.A., Schumann, P., Rohde, M., Lysenko, A.M., Mikhailov, V.V., Stackebrandt, E. 2002. *Psychrobacter submarinus* sp. nov. and *Psychrobacter marincola* sp. nov., psychrophilic halophiles from marine environments. *International Journal of Systematic and Evolutionary Microbiology*, vol. 52, no. 4, pp. 1291-1297, https://doi.org/10.1099/00207713-52-4-1291

Romanenko, L.A., Uchino, M., Tanaka, N., Frolova, G.M., Slinkina, N.N., Mikhailov, V.V. 2008. Occurrence and antagonistic potential of *Stenotrophomonas* strains isolated from deep-sea invertebrates. *Archives of Microbiology,* vol. 189, pp. 337–344, https://doi.org/10.1007/s00203-007-0324-8

Roper, C.F.E., Nigmatullin C., Jereb P. 2010. ‘Family Ommastrephidae’. *In* P. Jereb & C.F.E. Roper, eds. *Cephalopods of the world. An annotated and illustrated catalogue of species known to date. Volume 2. Myopsid and Oegopsid Squids.* FAO Species Catalogue for Fishery Purposes. vol. 2, no. 4, Rome, FAO. pp. 269–347.

Rosa, R. & Seibel, B.A. 2010. Slow pace of life of the Antarctic colossal squid. *Journal of the Marine Biological Association of the United Kingdom*, vol. 90, no. 7, pp. 1375-1378, https://doi.org/10.1017/S0025315409991494

Rosa, R., Lopes, V.M., Guerreiro, M., Bolstad, K., Xavier, J.C. 2017. Biology and ecology of the world’s largest invertebrate, the colossal squid (*Mesonychoteuthis hamiltoni*): a short review. *Polar Biology,* vol. 40, pp. 1871–1883. https://doi.org/10.1007/s00300-017-2104-5

Rosenberg, E., Koren, O., Reshef, L., Efrony, R., Zilber-Rosenberg, I. 2007. The role of microorganisms in coral health, disease and evolution. *Nature Reviews Microbiology*, vol. 5, no. 5, pp. 355-362, https://doi.org/10.1038/nrmicro1635

Roura, Á., Doyle, S.R., Nande, M. and Strugnell, J.M. 2017. You are what you eat: a genomic analysis of the gut microbiome of captive and wild *Octopus vulgaris* paralarvae and their zooplankton prey. *Frontiers in Physiology*, vol. 8, p. 362, https://doi.org/10.3389/fphys.2017.00362

Rusanova, P., Nikouli, E., Casini, M., Bono, G., Mente, E., Meziti, A., Kormas, K. 2025. Sex-related gut microbiota in three geographically separated Norway lobster (*Nephrops norvegicus*) populations. *The Microbe*, vol. 7, pp. 100396, https://doi.org/10.1016/j.microb.2025.100396

Sahu, R.L. & Minj, R.A. 2025. ‘Life in the Abyss: Exploring Microbial Diversity and Adaptation in the Deep Sea’. In: *Polar Microbes and Climate Change: A Molecular Understanding for Sustainable Future*. Cham: Springer Nature Switzerland, pp. 139-150, https://doi.org/10.1007/978-3-032-07096-8_4

Scanes, E., Parker, L.M., Seymour, J.R., Siboni, N., King, W.L., Wegner, K.M., Dove, M.C., O'Connor, W.A., Ross, P.M. 2021. Microbiome response differs among selected lines of Sydney rock oysters to ocean warming and acidification. *FEMS Microbiology Ecology*, vol. 97, no. 8, p. fiab099, https://doi.org/10.1093/femsec/fiab099

Scheuring, I., Rasmussen, J.A., Bozzi, D., Limborg, M.T. 2022. A strategic model of a host–microbe–microbe system reveals the importance of a joint host–microbe immune response to combat stress-induced gut dysbiosis. *Frontiers in Microbiology*, vol. 13, p. 912806, https://doi.org/10.3389/fmicb.2022.912806

Schramm, A., Davidson, S.K., Dodsworth, J.A., Drake, H.L., Stahl, D.A., Dubilier, N. 2003. *Acidovorax*-like symbionts in the nephridia of earthworms. *Environmental Microbiology*, vol. 5, no. 9, pp. 804-809, https://doi.org/10.1046/j.1462-2920.2003.00474.x

Seibel, B.A., Goffredi, S.K., Thuesen, E.V., Childress, J.J., Robison, B.H. 2004. Ammonium content and buoyancy in midwater cephalopods. *Journal of Experimental*

Marine Biology and Ecology, vol. 313, pp. 375–387, https://doi.org/10.1016/j.jembe.2004.08.015

Seibel, B.A., Hochberg, F.G., Carlini, D.B. 2000. Life history of *Gonatus onyx* (Cephalopoda: Teuthoidea): deep-sea spawning and post-spawning egg care. *Marine Biology*, vol. 137, no. 3, pp. 519-526, https://doi.org/10.1007/s002270000359

Seiblitz, I.G.L., Capel, K.C.C., Oliveira, R.R., Zilberberg, C., Cordeiro, C.A.M.M., Francini, C.L.B., Zanotti, A.A., Ciotti, A.M., Kitahara, M.V. 2025. Composition and rhythmic variations in the microbiome of Southwestern Atlantic corals. *Environmental Microbiome*, vol. 20, no. 71. https://doi.org/10.1186/s40793-025-00725-2

Seymour, J.R. 2014. A sea of microbes: the diversity and activity of marine microorganisms. *Microbiology Australia*, vol. 35, no.4, pp. 183-187, https://doi.org/10.1071/MA14060

Shete, A.M., Mujumdar, S.S., Bhuyan-Pawar, S., Chopade, B.A. 2015. Isolation, biotyping, biochemical and physiological characterization of marine *Acinetobacter* isolated from west coast of India. *International Journal of Current Microbiology and Applied Sciences*, no. 2, pp. 277-301.

Shropshire, J.D. & Bordenstein, S.R. 2016. Speciation by symbiosis: the microbiome and behavior. *mBio*, vol. 7, no. 2, pp. e01785-15, https://doi.org/10.1128/mbio.01785-15

Simidu, U., Taga, N., Colwell R. R., Schwarz J. R. 1980. Heterotrophic bacterial flora of the seawater from Nansei Shoto (Ryukyo Retto) area. *Bulletin of the Japanese Society of Scientific Fisheries,* vol. 46, no. 4, pp. 505-510, https://doi.org/10.2331/suisan.46.505

Skovhus, T.L., Ramsing, N.B., Holmström, C., Kjelleberg, S., Dahllöf, I. 2004. Real-time quantitative PCR for assessment of abundance of *Pseudoalteromonas* species in marine samples. *Applied and Environmental Microbiology*, vol. 70, pp. 2373–2382, https://doi.org/10.1128/AEM.70.4.2373-2382.2004

Sogin, M.L., Morrison, H.G., Huber, J.A., Welch, D.M., Huse, S.M., Neal, P.R., Arrieta, J.M., Herndl, G.J. 2006. Microbial diversity in the deep sea and the underexplored “rare

biosphere". *Proceedings of the National Academy of Sciences*, vol. 103, no. 32, pp. 12115-12120, https://doi.org/10.1073/pnas.0605127103

Song, J., Choi, A., Im, M., Joung, Y., Yoshizawa, S., Cho, J.-C., Kogure, K. 2015. *Aurantivirga profunda* gen. nov., sp. nov., isolated from deep-seawater, a novel member of the family Flavobacteriaceae. *International Journal of Systematics and Evolutionary Microbiology*, vol. 65, pp. 4850–4856, https://doi.org/10.1099/ijsem.0.000662

Sonnega, S. & Sheriff, M.J. 2024. Harnessing the gut microbiome: a potential biomarker for wild animal welfare. *Frontiers in Veterinary Science*, vol. 11, p. 1474028, https://doi.org/10.3389/fvets.2024.1474028

South, A., Michael, S., Massicotte, P. 2024. _rnaturalearthdata: World Vector Map Data from Natural Earth Used in 'rnaturalearth'_. https://doi.org/10.32614/CRAN.package.rnaturalearthdata, R package version 1.0.0, https://CRAN.R-project.org/package=rnaturalearthdata

Star, B., Haverkamp, T.H., Jentoft, S., Jakobsen, K.S. 2013. Next generation sequencing shows high variation of the intestinal microbial species composition in Atlantic cod caught at a single location. *BMC Microbiology,* vol. 13, pp. 248, https://doi.org/10.1186/1471-2180-13-248

Stoffel, M.A., Acevedo-Whitehouse, K., Morales-Durán, N., Grosser, S., Chakarov, N., Krüger, O., Nichols, H.J., Elorriaga-Verplancken, F.R., Hoffman, J.I. 2020. Early sexual dimorphism in the developing gut microbiome of northern elephant seals. *Molecular Ecology*, vol. 29, no. 11, pp. 2109-2122, https://doi.org/10.1111/mec.15385

Suzuki, S., Kaneko, R., Kodama, T., Hashihama, F., Suwa, S., Tanita, I., Furuya, K. and Hamasaki, K. 2017. Comparison of community structures between particle-associated and free-living prokaryotes in tropical and subtropical Pacific Ocean surface waters. *Journall Oceanography,* vol. 73, pp. 383–395, https://doi.org/10.1007/s10872-016-0410-0

Szafranski, K.M., Gaudron, S.M., Duperron, S. 2014. Direct evidence for maternal inheritance of bacterial symbionts in small deep-sea clams (Bivalvia: Vesicomyidae).

Naturwissenschaften, vol. 101, pp. 373–383, https://doi.org/10.1007/s00114-014-1165-3

Tak, E.J., Kim, P.S., Hyun, D.W., Kim, H.S., Lee, J.Y., Kang, W., Sung, H., Shin, N.R., Kim, M.S., Whon, T.W., Bae, J.W. 2018. Phenotypic and Genomic Properties of *Brachybacterium vulturis* sp. nov. and *Brachybacterium avium* sp. nov. *Frontiers in Microbiology*, vol. 9, p.1809, https://doi.org/10.3389/fmicb.2018.01809

Tarnecki, A.M., Burgos, F.A., Ray, C.L., Arias, C.R. 2017. Fish intestinal microbiome: diversity and symbiosis unravelled by metagenomics. *Journal of Applied Microbiology*, vol. 123, pp. 2–17, https://doi.org/10.1111/jam.13415

Taschuk, R. & Griebel, P.J. 2012. Commensal microbiome effects on mucosal immune system development in the ruminant gastrointestinal tract. *Animal Health Research Reviews*, vol. 13, no. 1, pp. 129-141, doi:10.1017/S1466252312000096

Taylor, M.W., Radax, R., Steger, D., Wagner, M. 2007. Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiology and Molecular Biology Reviews*, vol. 71, no. 2, pp. 295-347, https://doi.org/10.1128/mmbr.00040-06

Thurber, A.R., Jones, W.J., Schnabel, K. 2011. Dancing for Food in the Deep Sea: Bacterial Farming by a New Species of Yeti Crab. *PLoS One,* vol. 6, pp. e26243, https://doi.org/10.1371/journal.pone.0026243

Torres, J.P., Tianero, M.D., Robes, J.M.D., Kwan, J.C., Biggs, J.S., Concepcion, G.P., Olivera, B.M., Haygood, M.G., Schmidt, E.W. 2017. *Stenotrophomonas*-Like Bacteria Are Widespread Symbionts in Cone Snail Venom Ducts. *Applied and Environmental Microbiology*, vol. 83, pp. e01418-17, https://doi.org/10.1128/AEM.01418-17

Tseng, P.-W., Li, H.-W., Chen, C., Tseng, Y.C., Chang, C.F., Wu, G.C. 2023. Transcriptomic profile of symbiotic accessory nidamental gland during female maturation in bigfin reef squid. *Frontiers in Marine Science*, vol. 9, https://doi.org/10.3389/fmars.2022.1026742

Urbanczyk, H., Ast, J.C., Dunlap, P.V. 2011. Phylogeny, genomics, and symbiosis of *Photobacterium*. *FEMS Microbiology Reviews*, vol. 35, no.2, pp. 324-342, https://doi.org/10.1111/j.1574-6976.2010.00250.x

van de Water, J.A., Voolstra, C.R., Rottier, C., Cocito, S., Peirano, A., Allemand, D. and Ferrier-Pagès, C. 2018. Seasonal stability in the microbiomes of temperate gorgonians and the red coral *Corallium rubrum* across the Mediterranean Sea. *Microbial Ecology*, vol. 75, no. 1, pp. 274-288, https://doi.org/10.1007/s00248-017-1006-y

Vermeij, G.J. 2016. Gigantism and Its Implications for the History of Life. *PLoS One,* vol. 11, pp. e0146092, https://doi.org/10.1371/journal.pone.0146092

Vijayan, N., McAnulty, S., Kerwin, A., Jolly, J., Rokhsar, D., Ikeda, Y., Spady, B., Reveillac, E., Gestal, C., Nishiguchi, M., Nyholm, S. 2022. Characterization of symbiotic bacteria found in a female reproductive organ of multiple cephalopod species. *Cephalopod International Advisory Council Conference book of abstracts:* vol. *92,* http://hdl.handle.net/10261/307174

Vijayan, N., McAnulty, S.J., Sanchez, G., Jolly, J., Ikeda, Y., Nishiguchi, M.K., Réveillac, E., Gestal, C., Spady, B.L., Li, D.H., Burford, B.P., Kerwin, A.H., Nyholm, S.V. 2024. Evolutionary history influences the microbiomes of a female symbiotic reproductive organ in cephalopods*.* *Applied and Environmental Microbiology*, vol. 90, pp. e00990-23, https://doi.org/10.1128/aem.00990-23

Villanueva, R. 1992. Interannual growth differences in the oceanic squid Todarodes angolensis Adam in the northern Benguela upwelling system, based on statolith growth increment analysis. *Journal of Experimental Marine Biology and Ecology*, vol. 159, no. 2, pp. 157-177, https://doi.org/10.1016/0022-0981(92)90034-8

Viver, T., Orellana, L.H., Hatt, J.K., Urdiain, M., Díaz, S., Richter, M., Antón, J., Avian, M., Amann, R., Konstantinidis, K.T., Rosselló-Móra, R. 2017. The low diverse gastric microbiome of the jellyfish *Cotylorhiza tuberculata* is dominated by four novel taxa. *Environmental Microbiology*, vol. 19, pp. 3039–3058, https://doi.org/10.1111/1462-2920.13763

Voight, J.R., Pörtner, H.O., O’Dor, R.K. 1995. A review of ammonia-mediated buoyancy in squids (cephalopoda: Teuthoidea). *Marine and Freshwater Behaviour and Physiology*, vol. 25, pp. 193–203, https://doi.org/10.1080/10236249409378917

Voss, N.A. 1980. A Generic Revision of the Cranchiidae (Cephalopoda; Oegopsida). *Bulletin of Marine Science,* vol. 30, no. 2, pp. 365–412.

Wang, W., Shao, Z., Liu, Y., Wang, G. 2009. Removal of multi-heavy metals using biogenic manganese oxides generated by a deep-sea sedimentary bacterium–*Brachybacterium* sp. strain Mn32. *Microbiology*, vol. 155, no. 6, pp. 1989-1996, https://doi.org/10.1099/mic.0.024141-0

Wang, Y., Huang, J.M., Wang, S.L., Gao, Z.M., Zhang, A.Q., Danchin, A., He, L.S. 2016. Genomic characterization of symbiotic *Mycoplasmas* from the stomach of deep-sea isopod *Bathynomus* sp. *Environmental Microbiology*, vol. 18, no. 8, pp. 2646-2659, https://doi.org/10.1111/1462-2920.13411

Wang, Y., Wang, S., Xu, Z., Ni, S., Xu, M., Kao, S.J. 2025. Phosphonate-driven oxic CH4 production by *Variovorax xiamenensis* sp. nov.: insights from marine and freshwater microbial adaptations to P-limitation. *ISME Communications*, vol. 5, no. 1, p. ycaf100, https://doi.org/10.1093/ismeco/ycaf100

Webber, D.M., Aitken, J.P., O'Dor, R.K. 2000. Costs of locomotion and vertic dynamics of cephalopods and fish. *Physiological and Biochemical Zoology*, vol. 73, no. 6, pp. 651-662, https://doi.org/10.1086/318100

Webster, N.S. & Bourne, D. 2007. Bacterial community structure associated with the Antarctic soft coral, *Alcyonium antarcticum*. *FEMS Microbiology Ecology*, vol. 59, no. 1, pp. 81-94, https://doi.org/10.1111/j.1574-6941.2006.00195.x

Wenzel, M.A., Douglas, A., sPiertney, S.B. 2018. Microbiome composition within a sympatric species complex of intertidal isopods (Jaera albifrons). *PLoS One*, vol. 13, no. 8, p.e0202212, https://doi.org/10.1371/journal.pone.0202212

Wessels, W., Sprungala, S., Watson, S.-A., Miller, D.J., Bourne, D.G. 2017. The microbiome of the octocoral *Lobophytum pauciflorum*: minor differences between

sexes and resilience to short-term stress. *FEMS Microbiology Ecology,* vol. 93,https://doi.org/10.1093/femsec/fix013

Wickham, H. 2016. ‘Data Analysis’, In: Wickham, H. (Ed.), *Ggplot2: Elegant Graphics for Data Analysis, Use R!* Springer International Publishing, Cham, pp. 189–201, https://doi.org/10.1007/978-3-319-24277-4_9

Wood, A.P., & Kelly, D.P. 1989. Methylotrophic and autotrophic bacteria isolated from lucinid and thyasirid bivalves containing symbiotic bacteria in their gills. *Journal of the Marine Biological Association of the United Kingdom*, vol. 69, pp. 165–179, https://doi.org/10.1017/S0025315400049171

Wu, G., Zhang, X., Wei, L., Wu, G., Kumar, A., Mao, T., Liu, Z. 2015. A cold-adapted, solvent and salt tolerant esterase from marine bacterium *Psychrobacter pacificensis*. *International Journal of Biological Macromolecules*, vol. 81, pp. 180-187, https://doi.org/10.1016/j.ijbiomac.2015.07.045

Wu, W.J., Liu, Q.Q., Chen, G.J., Du, Z.J. 2015. *Roseimarinus sediminis* gen. nov., sp. nov., a facultatively anaerobic bacterium isolated from coastal sediment. *International Journal of Systematic and Evolutionary Microbiology*, vol. 65, pp. 2260–2264, https://doi.org/10.1099/ijs.0.000250

Xavier, J.C., Golikov, A.V., Queirós, J.P., Perales-Raya, C., Rosas-Luis, R., Abreu, J., Bello, G., Bustamante, P., Capaz, J.C., Dimkovikj, V.H., González, A.F. 2022. The significance of cephalopod beaks as a research tool: An update. *Frontiers in Physiology*, vol. 13, p. 1038064, https://doi.org/10.3389/fphys.2022.1038064

Xu, Y., Nogi, Y., Kato, C., Liang, Z., Rüger, H.J., De Kegel, D., Glansdorff, N. 2003. *Moritella profunda* sp. nov. and *Moritella abyssi* sp. nov., two psychropiezophilic organisms isolated from deep Atlantic sediments. *International Journal of Systematic and Evolutionary Microbiology*, vol. 53, pp. 533–538, https://doi.org/10.1099/ijs.0.02228-0

Yabe, S., Sakai, Y., Yokota, A. 2016. *Thermosporothrix narukonensis* sp. nov., belonging to the class Ktedonobacteria, isolated from fallen leaves on geothermal soil, and emended description of the genus *Thermosporothrix*. *International Journal of*

Systematic and Evolutionary Microbiology, vol. 66, pp. 2152–2157, https://doi.org/10.1099/ijsem.0.001004

Yang, S.H., Chen, C., Hsieh, Y.E., Yang, S.Y., Li, H.W., Ching, T.Y., Wang, C.H., Chang, C.F., Tang, S.L., Wu, G.C. 2021. Bacterial Dynamics in the Accessory Nidamental Gland of *Sepioteuthis lessoniana* throughout Maturation. *Microbes and Environments*, vol. 36, https://doi.org/10.1264/jsme2.ME21030

Yaser, N., Foong, M., Mohd, A., Izni, I. 2014. Isolation and identification of bioluminescent bacteria in squid and water of Malaysia. *Journal of Advances in Agricultural and Environmental*, vol. 1, no. 2, pp. 225-228, http://dx.doi.org/10.15242/IJAAEE.C0215136

Yilmaz, P., Parfrey, L.W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., Schweer, T., Peplies, J., Ludwig, W., Glöckner, F.O. 2014. The SILVA and "all-species living tree project (LTP)" taxonomic frameworks. *Nucleic Acids Research*, vol. 42, no. D1, pp. D643-D648, https://doi.org/10.1093/nar/gkt1209

Yu, X., Yu, K., Liao, Z., Chen, B., Deng, C., Yu, J., Yao, Q., Qin, Z., Liang, J. 2021. Seasonal fluctuations in symbiotic bacteria and their role in environmental adaptation of the scleractinian coral *Acropora pruinosa* in high-latitude coral reef area of the South China Sea. *Science of the Total Environment*, vol. 792, p. 148438, https://doi.org/10.1016/j.scitotenv.2021.148438

Yukhov V.L. 2012. Antarctic deep-water squid (ADS) Mesonychoteuthis hamiltoni, Robson, 1925. Distribution and borders of the geographic range. Main results of complex research in the Azov and Black Seas Basin and the World Ocean. *Kerch: YugNIRO,* vol. 50, pp. 241–248. Russian.

Zari, A., CH, R., & Mohanraju, R. 2020. Non-species specific composition of bioluminescent bacteria in non- bioluminescent squid *Sepioteuthis lessoniana* (Lesson, 1830). *Indian Journal of Geo-Marine Sciences*, vol. 49, no. 6, pp. 975–981.

Zeng, Z., Zhu, S., Zhou, F., Chen, J., Shen, X., Liu, W., Zhang, X., Yao, G. 2025. Acute Effects of Ammonia Nitrogen or Nitrite Nitrogen Exposure on *Micropterus salmoides*

and the Post-Exposure Recovery. *Aquaculture, Fish and Fisheries*, vol. 5, no. 3, pp. e70085, https://doi.org/10.1002/aff2.70085

Zhang, M., Sun, Y., Liu, Y., Qiao, F., Chen, L., Liu, W.T., Du, Z., Li, E. 2016. Response of gut microbiota to salinity change in two euryhaline aquatic animals with reverse salinity preference. *Aquaculture*, vol. 454, pp. 72–80, https://doi.org/10.1016/j.aquaculture.2015.12.014

Zhang, N., Song, C., Wang, M., Liu, Y., Hui, M., Cui, Z. 2017. Diversity and characterization of bacteria associated with the deep-sea hydrothermal vent crab *Austinograea* sp. comparing with those of two shallow-water crabs by 16S ribosomal DNA analysis. *PLoS One*, vol. 12, no. 11, p.e0187842, https://doi.org/10.1371/journal.pone.0187842

Zhu, Z., Sun, Y., Zhu, F., Liu, Z., Pan, R., Teng, L., Guo, S. 2020. Seasonal variation and sexual dimorphism of the microbiota in wild blue sheep (*Pseudois nayaur*). *Frontiers in Microbiology*, vol. 11, p. 1260, https://doi.org/10.3389/fmicb.2020.01260

Appendices

Appendix A

Figure 1: PhD candidate next to giant squid for scale

Appendix B *Chapter One Supplementary Materials*

Citation	Squid Species	Body Site	Reported Core Taxa	Methods	Year	Location	Collection	Number	Extra Methods	Publicly available data
Alias *et al.,* 2017	*Loligo* sp.	ink sac	*Vibrio spp*	DNA extraction using Bioteke protocol and LuxAB PCR was conducted (The Lux-AB gene with 611R reverse primer and 66F forward primer (Gabriela et al., 2009))	2017	Malaysia	Wild caught	3	culturing in luminescent agar/broth	n/a

Barbieri *et al.,* 2001	*Doryteuthis pealeii*	accessory nidamental glands and egg capsules	*Roseobacter, Shewanella, Pseudoalteromonas, Vibrio.* Alpha-proteobacteria	DNA extraction with Gene Releaser kit or standard phenol DNA extraction with bead-beating, PCR with universal prokaryotic primers and *Roseobacter* specific primer, LICOR automated sequencer. Sequencing was preformed using an ABI 377 DNA sequencer	2001	mature from Marine Resources Centre, Immature from National Marine Fisheries Service	Wild caught	females (5 mature, 3 immature)	FISH and SEM	accession numbers AF022392–AF022413 and AF034927–AF034938
Biggs and Epel, 1991	*Doryteuthis opalescens*	egg capsule	n/a	light microscopy	1991	Monterey Bay	wild caught and then laboratory reproduction	150	n/a	n/a

Bloodgood, 1977	*Doryteuthis pealeii*	accessory nidamental glands	*n/a*	Released tubule contents was inoculated into a sterile medium	1977	n/a	n/a	n/a	pigment was isolated from freshly collected gland bacteria and a spectrum from 300-800 nm was onstained with a spectrophotometer	n/a
Chiu et al., 2025	*Sepioteuthis lessoniana, Uroteuthis edulis*	accessory nidamental glands	Alphaproteobacteria(Hyphomicrobiaceae, Fodinicurvataceae), Flavobacteriaceae. Only goes down to family	DNA and RNA extraction, 16S rRNA gene sequencing	2025	Heping Island, Keelung, Taiwan	wild caught	6 uroteuthis, 8 sepioteuthis	n/a	BioProject ID PRJNA1182936.

Ford *et al.*, 1986	*Lolliguncula brevis*	Normal and ulcerated mantle tissue	*Aeromonas, Pseudomonas, Vibrio*, and *Bacillus*	cotton swabs streaked on different agars (such as Marine Agar 2216), homogenisation of tissue sample was then diluted and spread-plated	1986	1-6m depth near shore Galveston ship channel	Laboratory-maintained and wild caught	11 Lab, 30 wild	n/a	n/a
Guerrero-Ferreira and Nishiguchi, 2010	*Uroteuthis chinensis*	light organs	*Vibrio*	cDNA libraries were constructed	2013	Australia	Wild caught	n/a	Cultured on seawater tryptone agar	n/a

Guerrero-Ferreira *et al.,* 2013	*Uroteuthis etheriogei, Uroteuthis noctiluca, U. chinensis, U. duvaucelii*	light organs	*Vibrio*	DNA isolation with DNAesy Isolation Kit, PCR with universal primers (16SF (5’-GCAAGCCTGATGCAGCCATG-3’) and 16SR (5’-ATCGTTTACGGCGTGGACTA-3’)). Sequencing reactions were executed by the dideoxy chain termination method using the Big Dye™ Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA).	2013	Indo-West Pacific	Wild caught	10	tenfold dilutions of homogenate plated on seawater tryptone agar	n/a

Hu *et al.*, 2022	*Sthenoteuthis oualaniensis* mid-form	intestine and gills	*Mycoplasma, Vibrio, Photobacterium, Lactobacillus*, Bacteroidales S24-7, BD1-7 clade, *Mesorhizobium*	Dna extraction with MOBio PowerFecal DNA isolation kit and PCR (515F and 806R, V4 regions). Illumina HiSeq	2022	offshore, stations N1 (11.00°N, 114.00°E) and N2 (9.00°N, 114.00°E)	Wild caught	24	n/a	accession number was PRJNA822533
Hu *et al.*, 2022	*Sthenoteuthis oualaniensis* dwarf-form	intestine and gills	*Mycoplasma, Vibrio, Photobacterium, Lactobacillus*, Bacteroidales S24-7, BD1-7 clade, *Mesorhizobium*	Dna extraction with MOBio PowerFecal DNA isolation kit and PCR (515F and 806R, V4 regions). Illumina HiSeq	2022	stations N1 (11.00° N, 114.00° E) and N2 (9.00° N, 114.00° E) in the Nansha area of China.	Wild caught	24	n/a	accession number is PRJNA856085
Kang *et al.*, 2022	*Loliolus beka, Uroteuthis edulis, Todarodes pacificus*	Stomach, Stomach caecum, Gut	*Photobacterium, Aliivibrio, Psychrilyobacter, Mycoplasma,* and *Arcobacter*	DNA extraction with Qiagen mini stool kit and V3-V4 PCR (341F and 805R), MiSeq Illumina	2022	offshore waters surrounding the Republic of Korea	Wild caught	5 for each squid taxa	COI genes used for taxonoic identification	accession number PRJEB27490

Kaufman *et al.,* 1998	*Doryteuthis opalescens*	accessory nidamental glands	*n/a*	Transmission electron microscopy	1998	Monterey Bay	Wild caught and laboratory reared	15 juvenile specimens observed from 0 to 129 days	n/a	n/a
Lum-Kong and Hastings, 1992	*Loligo forbesii*	accessory nidamental glands, egg casings	ANG: Pseudomonas, Vibrio	culturing on TSA +2% NaCl	1992	Plymouth	wild caught	10 maturing and 5 mature females, 3 egg casinings	n/a	n/a
Mahadik et al., 2025	*Uroteuthis duvaucelii*	eye part, tentacles, arms, body surface and abdomen and ink sac	*Shewanella seohaensis and Shewanella hafniensis* (isolated and identified)	sea water agar and 16S rRNA gene sequencing	2025	Ganesh Peth and Camp area of Pune district, Maharashtra	Fish Market	n/n	done in two seasons	n/a

Naguit *et al.,* 2014	*Uroteuthis* sp.	inside head and inside the vitreous sac of the eyes	*Photobacterium* sp.	DNA extraction from bioluminescent bacteria using Wang *et al.,* 1994 method. PCR with 27F and 1492R primers and sequenced at AITBiotech labs in Singapore	2014	Seafood market Manila, Philippines	Wild caught	3	Swabs streaked on liminescence agar	n/a
Pertiwi *et al.,* 2018	*Uroteuthis duvaucelii*	ink sac	*Photobacterium* sp.	DNA isolation with Roche High Pure Plasmid kit and PCR with 27F and 1492R with sequencing at First BASE Laboratories, Malaysia	2018	Malang region	Wild caught	1 (?)	Culturing in NA medium	n/a

Pichon *et al.,* 2005	Loliginidae	accessory nidamental glands and egg capsules	*Egg capsules: Vibrio, Shewanella, Agrobacterium, Roseobacter, Rhodobium.* ANG: *Vibrio, Shewanella, Pseudoalteromonas, Rhodobiu-Stappia, Roseobacter-Agrobacterium-Silicibacter*	DNA extraction with DNeasy Tissue Kit and PCR with universal prokaryote primers (27F and 1385R) and cloned	2005	Taiwan, English Cannel, Australia, Thailand	Wild caught	3 *photololigo ducaucelii*, 4 *P. chinensis*, 1 *Loligo edulis*, 2 *L. forbesii,* 3 *L. vulgaris,* 1 *Loliolus uyii,* 1 *L. beka, 3 Sepioteuthis lessoniana*	FISH	full table found in paper
Pringgenies and Jørgensen 1994	*Uroteuthis duvaucelii*	light organs	*n/a*	n/a	1994	Indonesia and Thailand	Wild caught	n/a	n/a	n/a

Ramírez *et al.,* 2019	*Todarodes sagittatus*	intestine and gills	*Mycoplasma*	Swabs inoculated in SP4-II broth medium and cultured, DNA extraction using a Realpure Genomic DNA extraction kit. PCRMacrogen Europe preformed the sequencing with *Mycoplasma* appropriate primers	2019	south coast of El Hierro island	Wild caught	2	Genome sequencing	n/a
Tseng *et al.,* 2023	*Sepioteuthis lessoniana*	accessory nidamental glands	n/a	Transcriptomic profile	2023	Taiwan	Wild caught	3 juvenile, 1 immature, 4 maturing, 5 mature	De novo transcriptome assembly	accession number is PRJNA814067

Vijayan *et al.,* 2024	*Uroteuthis, Doryteuthis, Sepioteuthis*	accessory nidamental glands	*Rhodobacteraceae in loliginids, Hyphmicrobiaceae* in *S. lessoniana*, *Bacteroidia* in *Doryeuthis pealeii*, Actinomycetota in *S. officinalis* and *S. esculenta*	DNA extraction with Qiagen DNEasy Blood and Tissue kit and V4 region PCR was conducted (primers 515F and 806R), MiSeq used for sequencing	2024	India, USA, Japan (respectively)	Wild caught	5 *Uroteuthis*, 4,4 *Doryteuthis*, 6 *Sepioteuthis*	n/a	n/a
Yang *et al.,* 2021	*Sepioteuthis lessoniana*	accessory nidamental glands	*Mycoplasma, Lactobacillus, Bacteroidia, Flavobacteriales*	DNA extraction using phenol-chloroform and PCR with 361F and 806R and Illumina MiSeq was used to sequence	2021	northeast coast of Taiwan	Wild caught	total of 36 squid, female squid from 3 separate yearly batches	haplotype-specific PCR to identify the lineage of squids used in the study	n/a
Yaser *et al.,* 2014	n/a	inner body, eyes, under the skin and ink	*Photobacterium leiognathi*	DNA extraction with Dneasy Blood and Tissue Kit, PCR with 27F and 1492R and BGI sequencing	2014	Malaysia	Wild caught	2	swabs from squid with cotton buds and streaked on luminescence agar	n/a

Zari *et al.*, 2020	*Sepioteuthis lessoniana*	surface, caecal fluid, gut, and ink gland	*Vibrio campbellii, V. vulnificus B2, V. alginolyticus, V. harveyi,* and *Photobacterium damselae*	Cultured on luminescent agar	2020	Catham Jetty	Wild caught	n/a	bacterial identification carried out with standard biochemical identification keys and online software used	n/a

Appendix C *Chapter Two Supplementary Materials*

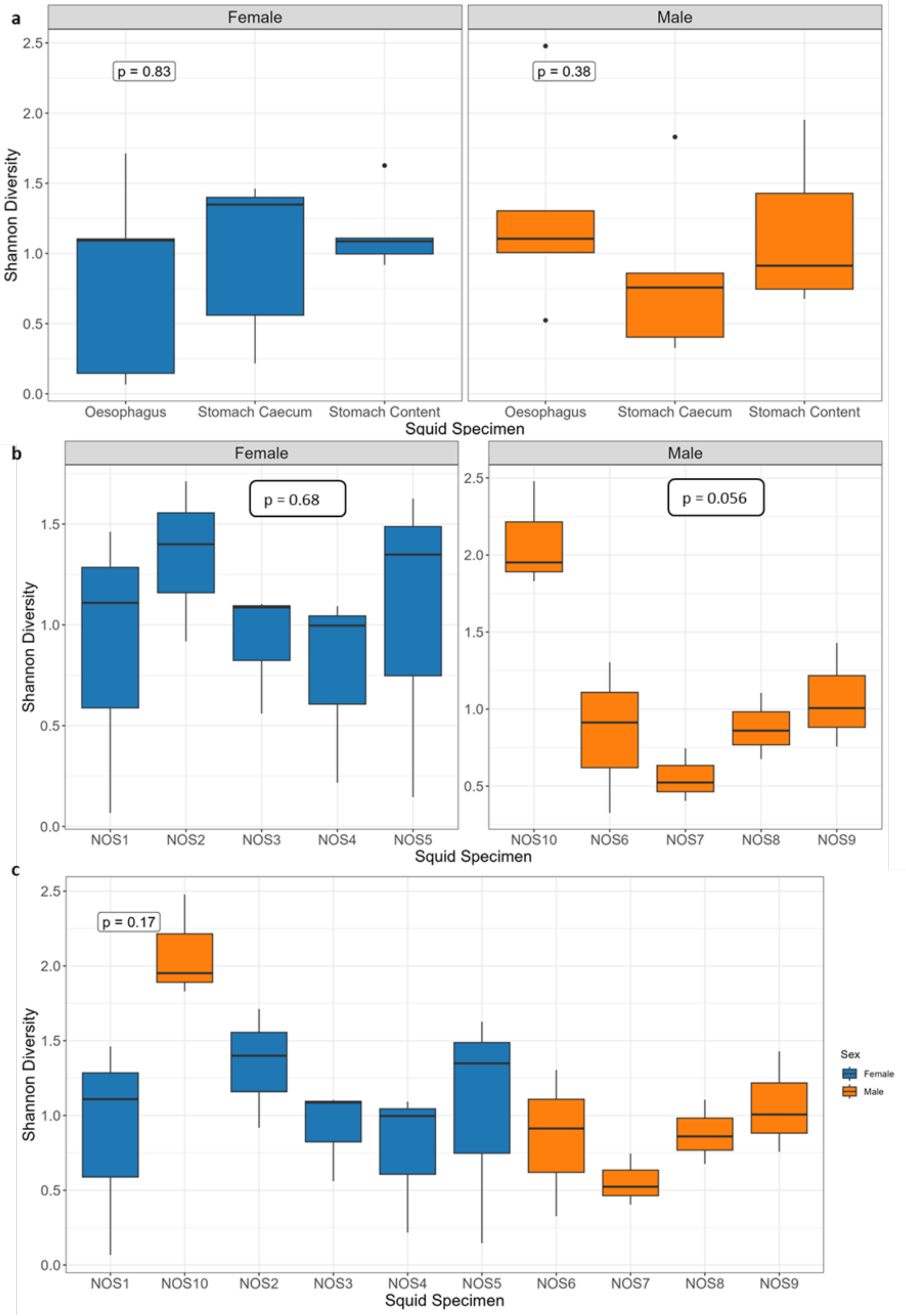


Figure 2: Shannon diversity index with rarefied data for the microbiota of the digestive tracts of female (blue) and male (orange) N. sloanii*; significance within a group was tested with the Kruskal–Wallis test. (a) Among body sites within a sex, (b) among individuals within a sex, and (c) among all individuals within* N. sloanii*. Boxes represent the interquartile range (IQR, 25–75%) of the data. Median values are indicated by the bar within each box and whiskers show the values within 1.5 times the IQR.*

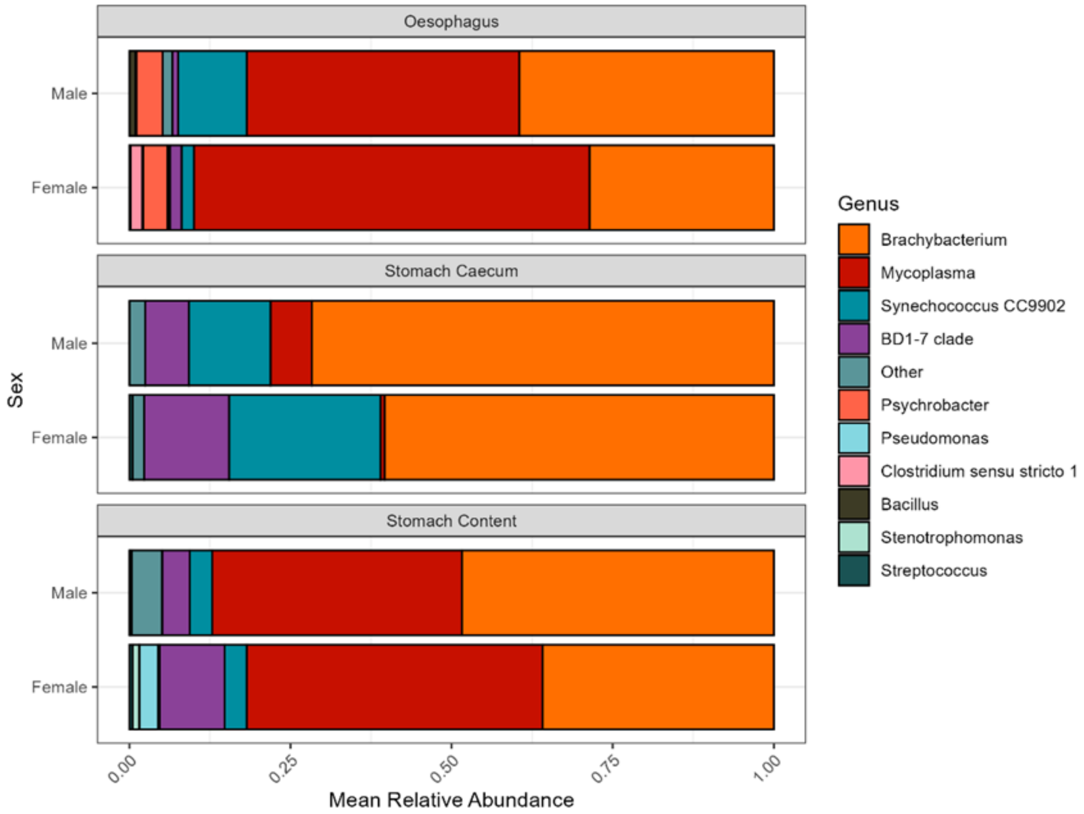


Figure 3: Top ten most abundant bacterial genera within the female and male digestive tract microbiota, composed of oesophagus, stomach caecum, and stomach content, of N. sloanii*, with the other genera grouped into "other".*

Table 1: Relative abundance (%) of microbial taxa observed along the digestive tract of N. sloanii (N = 15 for each male and female squids).

Genus	**Female (%)**	**Male (%)**
BD1-7 clade	8.38	4
Bacillus	0.02	0.33
Brachybacterium	41.61	53.19
Clostridium sensu stricto 1	0.61	0.04
Mycoplasma	35.99	29.14
Other	0.77	2.9
Pseudomonas	1.01	0.02
Psychrobacter	1.26	1.45
Stenotrophomonas	0.38	0
Streptococcus	0.35	0
Synechococcus CC9902	9.61	8.92

Appendix D *Chapter Three Supplementary Materials*

Table 2: Samples removed during the data processing due to insufficient reads. Highlighted in grey were removed during the filtering process. Highlighted in yellow were removed during rarefaction.

	Brain	Eye In	Digestive Tract	Siphon	Beak In	Beak Out	Reproductive Organs
N. sloanii	2	3	2	3			2
N. gouldi	2		3	3			
T. angolensis			3	1	1	1	
T. filippovae			4				

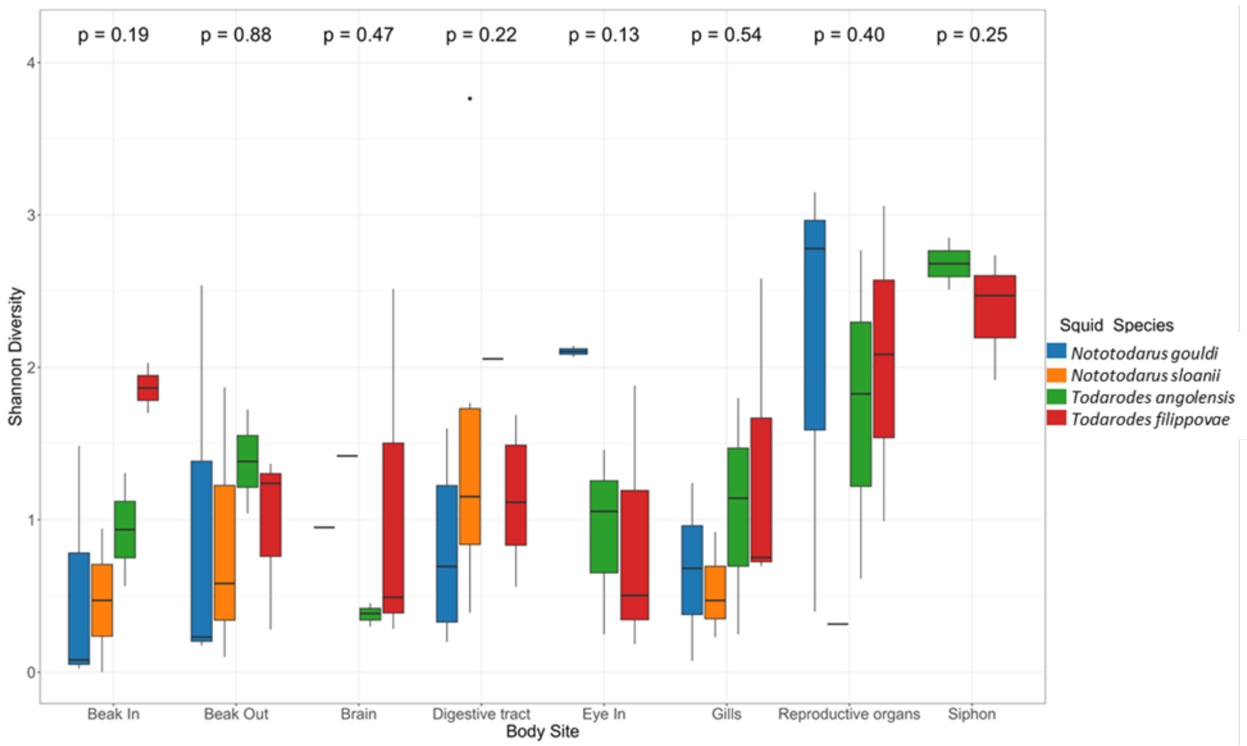


Figure 4: Shannon's diversity index among the four species tested in chapter three for the same body sites. The significance within a group of body sites was assessed using the Kruskal–Wallis test. Boxes represent the interquartile range (IQR, 25–75%) of the data, with median values indicated by the bar within each box, and whiskers show values within 1.5 times the IQR.

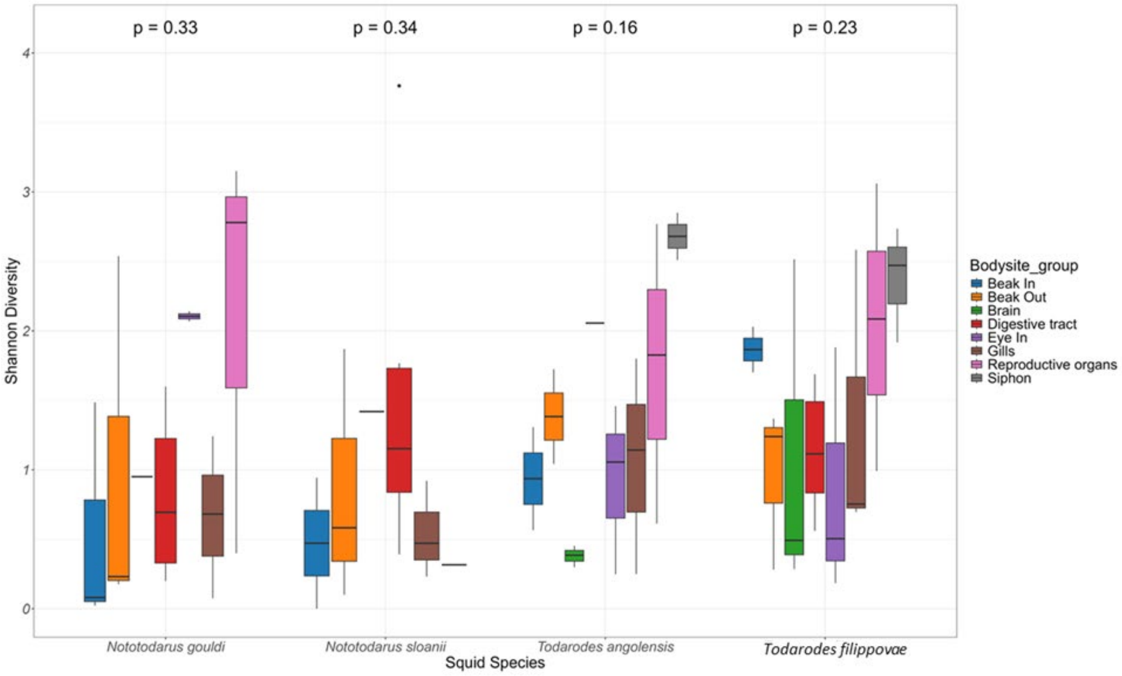


Figure 5: Shannon's diversity index among body sites within a single species of squid, for the four squid species tested in chapter three. The significance within a group of body sites was assessed using the Kruskal–Wallis test. Boxes represent the interquartile range (IQR, 25–75%) of the data, with median values indicated by the bar within each box, and whiskers show values within 1.5 times the IQR.

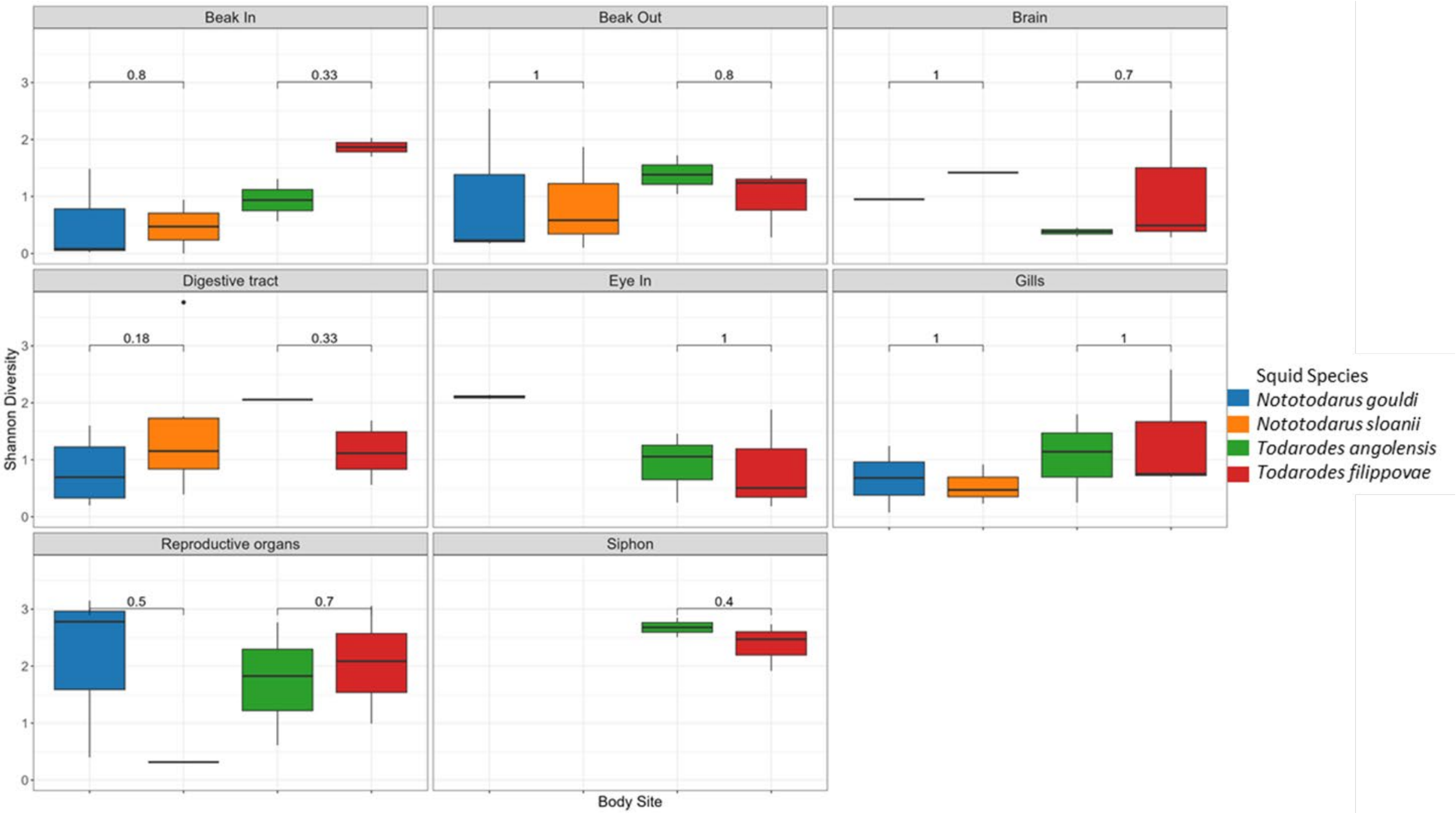


Figure 6: Shannon's diversity index for the eight different body sites among squid species within a genus, for a single species of squid. The significance within a group of body sites was assessed using the Kruskal–Wallis test. Boxes represent the interquartile range (IQR, 25–75%) of the data, with median values indicated by the bar within each box, and whiskers show values within 1.5 times the IQR.

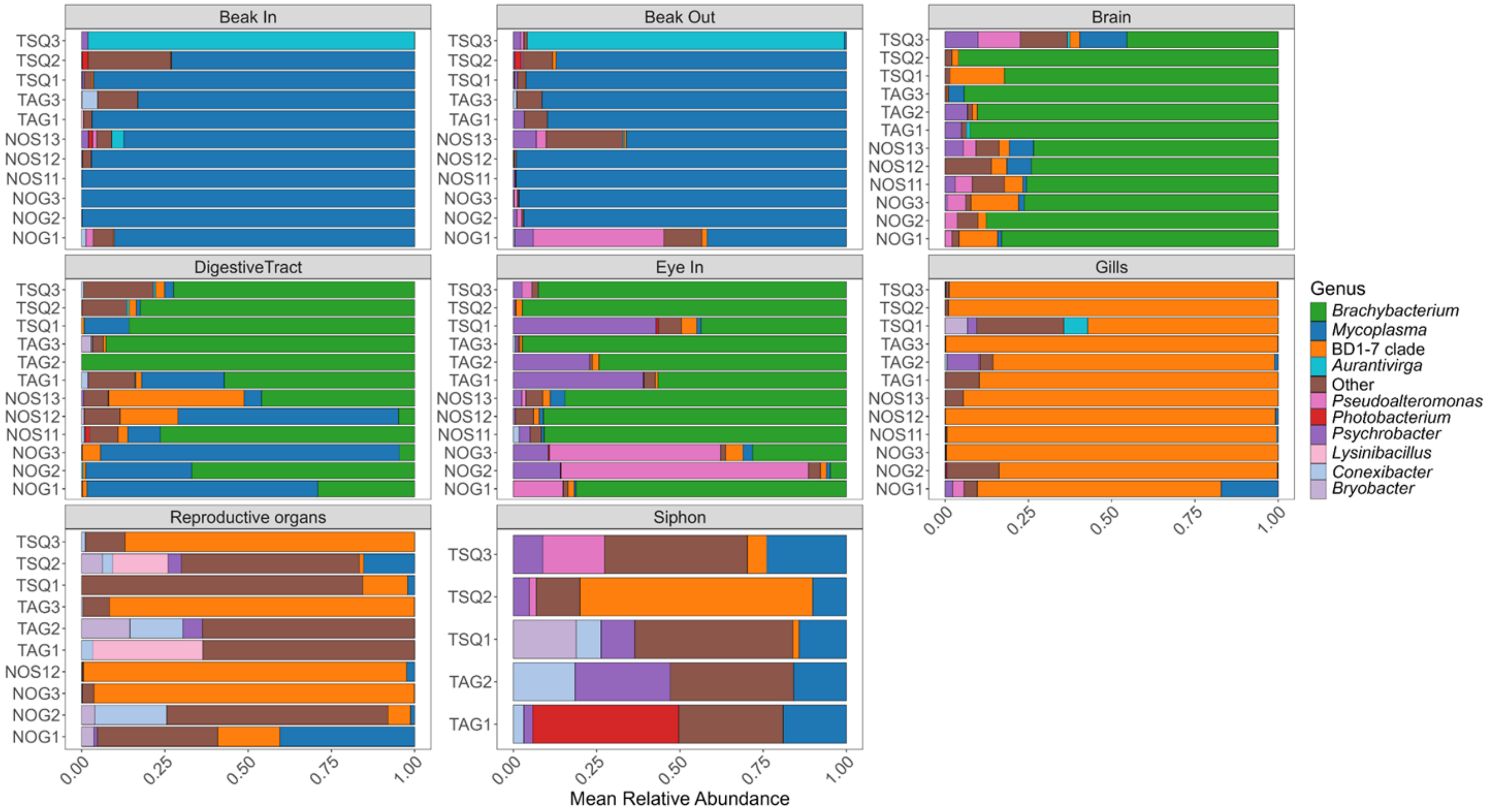


Figure 7: The top ten most abundant bacterial genera within the eight body sites of individual specimens of N. sloanii, N. gouldi, T. angolensis*, and T. filippovae, with the other genera grouped into "other".* Todarodes angolensis *inner and outer beaks, and the siphon had one individual specimen that did not pass the filtering stage. The same was true for N. sloanii reproductive organs. All siphon samples from the three* N. sloanii *and* N. gouldi *individuals did not pass the filtering stage.*

Appendix E *Chapter Four Supplementary Materials*

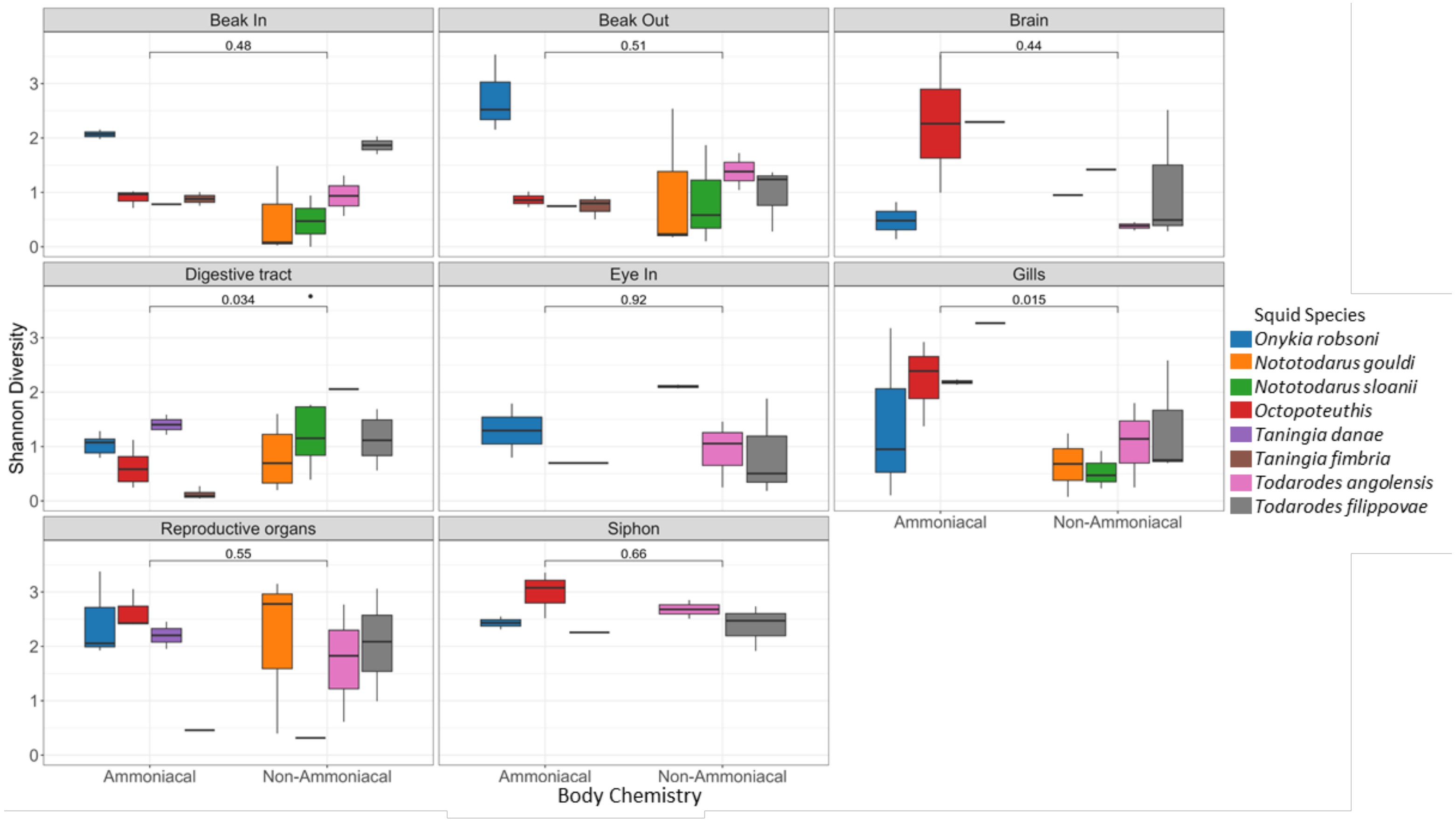


Figure 9: Shannon's diversity index among body sites between ammoniacal and non-ammoniacal squids. The significance within a group of body sites was assessed using the Kruskal–Wallis test. The digestive tract and gills are observed to be statistically significant between groups. Boxes represent the interquartile range (IQR, 25–75%) of the data, with median values indicated by the bar within each box, and whiskers show values within 1.5 times the IQR.