The Microbiome of Colonial Ascidians

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Abstract

Colonial ascidians are prolific and successful marine invertebrates worldwide. Colonial ascidians harbour a diverse assemblage of microorganisms, some of which are known to produce secondary metabolites (bioactives) that contribute to the ascidians' ecological success. However, little is known about the microbial ecology of the putative symbionts associated with colonial ascidians and whether they consistently contribute to host competitiveness. We hypothesise that the colonial ascidian microbiome is host-specific and enables host ecological success in interacting with proximate competitor(s) through chemical competition facilitated by inducible microbially synthesised metabolites. We further theorise that, during invasion events, non-native colonial ascidians outcompete native ones using allelochemical capacities conferred by their microbiome and that non-native colonial ascidians, which are globally successful invaders, harbour unique microorganism assemblages.

To examine our hypotheses, it is necessary to characterise the relationship between ascidian ecological status (i.e., native, non-native, monospecific, or interacting) and the ascidian microbiome. We systematically sampled colonial ascidian specimens (both native and non-native species) engaged in competitive interactions as well as monospecific specimens. Microbiome analysis was conducted using 16S rRNA gene PCR amplicon sequencing to characterise baseline variability and identify microbial response patterns linked to the ecological status of host species.

Our findings revealed a high degree of species-specificity in the ascidian microbiome, and native and non-native species exhibited distinct microbial patterns linked with their ecological state. However, interacting ascidian species did not appear to harbour dominant unique microbial assemblages compared to their monospecific counterparts. We also conducted the first microbiome comparison for zooids and tunics of colonial ascidians and found that, contrary to common assumptions, zooids and tunics generally harbour similar microorganisms.

This study provides the first systematic characterisation of the colonial ascidian microbiome. We generated statistically informative baselines for the colonial ascidian microbiome and novel insight on its potential roles in mediating host ecology. Findings

from this study form a robust foundation for future chemical characterisation of potential bioactive compounds (using the same specimens) and shotgun metagenomic analyses of these unique microbial communities.

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Chapter 1

Introduction & Literature Review

1.1 Ascidians

1.1.1 Ascidian Morphology, Lifecycle, and Ecology

Ascidiacea, commonly known as ascidians, are sessile, soft-bodied, filter-feeding marine invertebrates (Shenkar & Swalla, 2011). Ascidiacea is a highly diverse class of the Chordata phylum with approximately 3,000 known species of ascidians inhabiting diverse benthic ecosystems in tropical, temperate, and polar marine environments, from shallow water to the deep sea (Erwin et al., 2014; Shenkar et al., 2022; Shenkar & Swalla, 2011). Ascidian diversity is also reflected in remarkable interspecific and intraspecific morphological differences, including colour, morphometry (size & shape), and other anatomical differences (Dalby, 1997; Lopez-Legentil & Turon, 2005). Ascidians can be either solitary or colonial, the latter consisting of social groups of individuals connected by their tunic base (Núñez-Pons et al., 2012). Solitary ascidians are separate individuals with a clear larger body structure and rough tunic (Chen et al., 2017; Lindquist et al., 1992). A relatively large number of studies has been conducted on solitary ascidians, perhaps due to their well-defined body structure which eases dissection and cell disaggregation (Chen et al., 2017).

Colonial ascidians are composed of many tiny morphologically and genetically identical individuals, called zooids, embedded in a gelatinous matrix sharing the external tunic (Chen et al., 2017; Núñez-Pons et al., 2012) (Figure 1.1). The zooids are the eukaryotic individuals of the colony; therefore, their cells are rich with mitochondria (Cooper, 2000; Goddard-Dwyer et al., 2020). Zooids are also the active filter feeder vessels of the colony (Martinez Garcia et al., 2009). The tunic is a multifunctional outer integumentary tissue, exhibiting diverse textures from gelatinous to leathery or cartilaginous (Hirose, 2009; Núñez-Pons et al., 2012). The tunic is composed mainly of acidic mucopolysaccharides associated with collagen and elastin-like proteins and is covered by a thin cuticle (Martínez-García et al., 2007). It harbours diverse cell types, including microbial symbionts in most cases and several eukaryotic cell lines. Adult zooids and larvae are surrounded by that protective tissue, which also forms the colony by keeping all zooids connected (Martínez-García et al., 2007).

The common ascidian life cycle includes two phases: a sessile adult stage and a free-living larval stage (Figure 1.2) (Núñez-Pons et al., 2012). Ascidian larvae exhibit all key features of the Chordata phylum: notochord, dorsal nerve cord, post-anal tail, and pharyngeal gills (Núñez-Pons et al., 2012). Adults are filter feeders, while larvae cannot feed through filtering since the digestive system only becomes functional after metamorphosis to the adult. In the filtration process, water enters through the incurrent siphon and passes through the mucous-covered pharynx, on which the food is captured, and then exits through the excurrent siphon (Hickman, C.P., 1988 cited by Martínez-García et al., 2007). Ascidians' natural dispersal is almost exclusively of gametes (sperm) and larvae, and is usually of short duration, resulting in dispersal distances of fewer than a few meters (Núñez-Pons et al., 2012). Colonial species produce fewer eggs in number, but they are larger in size. The eggs are rich in vitelum, forming lecithotrophic larvae that are brooded until released as tadpoles (Núñez-Pons et al., 2012). Colonial ascidians can also reproduce asexually by fragmentation, and colonies can fuse to form chimeras with zooids of different genotypes. This could enhance the colony's adaptive capacity and competitive abilities (Ben-Shlomo, 2017; Casso et al., 2019).

Ascidian defence mechanisms against predators in adults and larvae can be structural, behavioural, or chemical (López-Legentil et al., 2006). Colonial ascidians typically lack structural defence, such as the rough tunicate of some solitary species, which covers their body and protects them from predators. Consequently, colonial ascidians are more vulnerable to predation, yet they remain abundant across all the world's oceans (Tianero et al., 2015). Thus, colonial ascidians' main defence and offence mechanism is expected to be chemical and is attributed to the biosynthesis of potently toxic secondary metabolites (also referred to as bioactive compounds), many of which appear to be produced by their microbiome (Schmidt et al., 2005; Tarjuelo et al., 2002).

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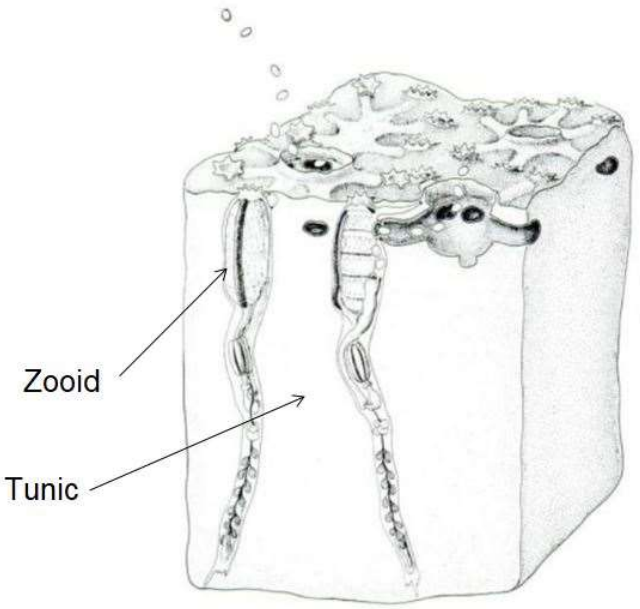


Figure 1.1: Schematic diagram adapted and modified from Moniot & Moniot (1991) showing the general structure of colonial ascidians: the tunic tissue and the zooids, i.e., the individuals of the colony.

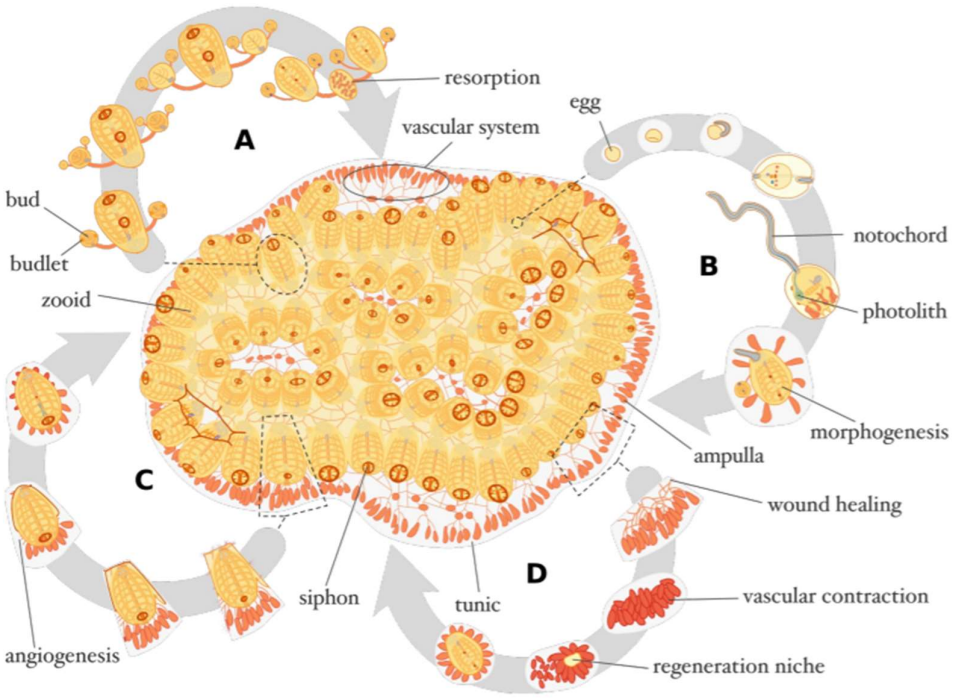


Figure 1.2: Adapted from Blanchoud et al. 2018. *Botrylloides leachii* schematic physiology. Top view of a stereotypical colony composed of 72 zooids. (A) Blastogenic cycle of an adult zooid with two daughter buds and four budlets. (B) Viviparous sexual reproduction

1.1.2 The Ascidian Microbiome

Ascidians and most other marine invertebrates are considered holobionts, a functional ecological unit combining the host and its associated microorganisms (Dror et al., 2019; Simon et al., 2019). The hologenome theory, which was first presented by Ilana Zilber-Rosenberg and Eugene Rosenberg (2008), considers the holobiont (the animal or plant with all its associated microorganisms) as a unit of selection in evolution (Rosenberg & Zilber-Rosenberg, 2018). The hologenome is defined as the sum of the genetic information of the host and its various taxa of microbiota (bacteria, archaea, fungi, microalgae, and viruses) (Zilber-Rosenberg & Rosenberg, 2008).

Very little currently is known about the microbiome of colonial ascidians (Casso et al., 2020; Erwin et al., 2014; Evans et al., 2021; Goddard-Dwyer et al., 2020). The feeding vessels of colonial ascidians (zooids) are assumed to contain planktonic microorganisms which they feed on (Chen et al., 2017). To exclude the influence of planktonic bacteria, in most studies, only the inner tunic of the ascidian was used for microbial analysis (Casso et al., 2019; Chen et al., 2017; Erwin et al., 2013, 2014; Goddard-Dwyer et al., 2020;

Martinez Garcia et al., 2009; Martínez-García et al., 2007). Furthermore, due to the diverse, complex, and fragile structure of many colonial ascidian species, dissection and tissue separation can be very challenging. Consequently, it has been common in studies of colonial ascidian microbiome to extract DNA from whole colonies or to remove and exclude the zooids (which also includes gut microbiome) from the analysis (Chen et al., 2017).

There have been some reports that the microbiome of solitary ascidians may be species-specific and tissue-specific (Chen et al., 2017; Utermann et al., 2020). Therefore, zooids of colonial ascidians could harbour an unexpected diversity of microorganisms different from the bacterioplankton and tunic microbiome, and they may make unique contributions to the biosynthesis of the secondary metabolites or other microbial-driven functionalities (Chen et al., 2017; Rosenberg & Zilber-Rosenberg, 2018). However, no zooid microbiota analysis of colonial ascidians has been conducted to date, and there is no empirical evidence supporting the exclusion of zooids from analyses of colonial ascidian microbiomes (Chen et al., 2017; Erwin et al., 2013, 2014; Goddard-Dwyer et al., 2020; Martinez Garcia et al., 2009).

Marine invertebrate microbiomes have been associated with a wide range of host-beneficial functions, such as synthesis of metabolic substances in the carbon, nitrogen (Martínez-García et al., 2007) and sulfur cycles (Hoffmann et al., 2005), and contributions to reproductive processes (Fitt et al., 1990) and structural rigidity (Dror et al., 2019; Wilkinson, 1978). Symbiotic bacteria may also support host growth by supplying vitamins and amino acids, assisting in digestion, and protecting the host from pathogens or predators by producing secondary metabolites for chemical defences (Dror et al., 2019; Schmidt et al., 2005). Ascidian microbiome is known to be involved in key metabolic abilities, such as nutrient fixation, heavy metal detoxification (Aires et al., 2016), and production of toxic chemicals (bioactives) that can increase competitive abilities (Amsellem et al., 2017; Schmidt & Donia, 2010) . Thus, in ecological invasion events, the associated microbiota of invasive colonial ascidians may allow their host to successfully adapt and establish into new and diverse environments, making them successful invaders worldwide (Casso et al., 2020; Dror et al., 2019; Evans et al., 2018).

1.2 Invasive Ascidians

1.2.1 Global Overview

In all natural ecosystems, terrestrial, marine, and other aquatic habitats, species invasion is considered a major threat to species biodiversity and the ecosystem's stability (Simberloff et al., 2013; Zhan et al., 2015). Marine invasions usually involve the introduction of a wide range of species into benthic ecosystems, probably due to the low number of physical barriers to inhibit those introductions (Dror et al., 2019; Rinkevich & Fidler, 2014). Therefore, marine habitat is a highly vulnerable ecosystem to species colonisation (Dror et al., 2019), with more than 80% of coastal habitats affected by invasive species (Molnar et al., 2008). Marine invasion has caused not only severe ecological damage and a global environmental concern but have also created an economical problem worldwide (Mačić et al., 2018; Simberloff et al., 2013).

Ascidians are among the most common fouling organisms in ports and harbours worldwide, as they are often abundant in urbanised and built areas, which allow them to settle and grow on artificial substrates such as wharf piles, seawalls, ship hulls, and aquaculture structures (Clark & Johnston, 2009; Dumont et al., 2011; Zhan et al., 2015). Ascidians have a negative economic effect on the aquaculture industry, reducing shellfish growth and causing displacement of shellfish, since they overgrow bivalves and foul gear, thereby adding weight and restricting water exchange and essential nutrients (Carman et al., 2010; G. Lambert, 2007). For example, massive fouling by the invasive ascidian *Ciona intestinalis* in Prince Edward Island (PEI, Canada) has been causing devastating losses to the local blue mussel farms (Utermann et al., 2020).

The negative ecological impacts of those introduction events are also significant. Invasive ascidians species often cannot coexist with the natives without dominating the habitat since invasive ascidians cause changes in the structure and composition of the benthic communities (Dijkstra et al., 2007; G. Lambert, 2001). Therefore, the ecosystem transforms from a native-dominated to an invasive-dominated community, which reduces the abundance and quantity of individuals among the existing native species (C. C. Lambert & Lambert, 1998). Ascidians are the ultimate competitors for space and resources due to their high reproduction abilities and rapid growth rate, allowing them to overgrow other sessile species and outcompete them (Zhan et al., 2015). Therefore, once invasion occurs, biodiversity decreases, shifting the food web and nutrient cycling

(Simberloff et al., 2013; Zhan et al., 2015). Thus, the ecosystems' natural balance can be affected, leading to an unreturnable tipping point with major environmental consequences (C. Heinze et al., 2021).

Globalisation has dramatically increased international marine shipping and the global aquaculture industry to supply the growing world population (Costello et al., 2020; Miralles et al., 2021). Big cargo ships serve as a great mobile surface for ascidians to settle on and attach to, carried within ballast water as larvae or attached to the ship hulls as juveniles and adults (Miralles et al., 2021). In addition, aquaculture often involves an artificial rearing of non-native species outside of their natural environment to cultivate them for nutritional requirements where they are not naturally found (Costello et al., 2020; Miralles et al., 2021). Consequently, unintentional release of non-native species is essentially unpreventable, making aquaculture a common pathway to exacerbate species introductions (Miralles et al., 2021). Therefore, international commerce using marine shipping and the growing aquaculture sector have led to an increased number of species introductions into new environments, and an increase in the frequency of human-induced species invasion (Molnar et al., 2008; Zhan et al., 2015).

1.2.2 Review of Species Invasions in New Zealand

New Zealand lies in the southern Pacific Ocean, 2,000 km east of Australia and with an Exclusive Economic Zone (EEZ) covering 2.2 million km^2 (Hewitt et al., 2004). Due to the lack of a terrestrial border with any adjacent landmasses, New Zealand relies on seaborne shipping for over 90% of its international commerce (Hewitt et al., 2004). In addition, New Zealand harbours unique fauna rich in endemic species (Kelly & Sullivan, 2010). For example, as reported by the Department of Conservation (2000), approximately 30% of the algae and over 95% of the sponge species are endemic to New Zealand, making them particularly vulnerable to the impacts of introduced species (Hewitt et al., 2004). Human colonisation of New Zealand during the last couple of centuries has also significantly impacted the country's ecology (Hayden et al., 2009). These geographical, biological, and anthropogenic factors make New Zealand particularly vulnerable to biological invasions both on land and in the oceans (Hayden et al., 2009; Hewitt et al., 2004).

Numerous invasive ascidians species have been documented to establish in New Zealand coastal environment (Smith et al., 2010). However, while terrestrial ecology and species invasion events are usually well described in the literature, marine ecological events are much less described or even known (Mačić et al., 2018). Moreover, the extent and ecological impact of marine invertebrates invasions have only recently been realised and are still poorly understood and quantified (Mačić et al., 2018; Simberloff et al., 2013). In New Zealand, coastal waters around Tauranga have a highly abundant biodiversity representation of non-native ascidians due to the Port of Tauranga (37°38'25.9"S 176°10'52.5"E), the largest shipping port of New Zealand (Miralles et al., 2021; Pyvis & Tull, 2017). The port plays a significant role in the international shipping industry, with a throughput of approximately 20 million tonnes of cargo a year (Pyvis & Tull, 2017). Vessels arriving at the port are primarily commercial vessels visiting from Australia (35%), the Northwest Pacific (24%), Northeast Pacific (15%), South Pacific (13%), East Asia (3%), and other New Zealand ports (Inglis et al., 2006). These international transport vectors increase the potential introduction events of invasive ascidians and contribute to the global spread of species beyond endemic origin (Miralles et al., 2021).

1.2.3 The Mechanism Beyond Invasive Species Success

With increasing rates of global invasions (Chan & Briski, 2017; Molnar et al., 2008), and the economic and ecologic damage they create, a full understanding of the mechanisms contributing to invasive species establishment, survival, and spread is critical to the development of successful management strategies (Miralles et al., 2021). Colonial ascidians are known to thrive and become abundant within coral reef ecosystems in which predation is high (Lindquist et al., 1992). The ability to avoid predation is attributed to a chemical defences and offence mechanism (Dror et al., 2019; Schmidt et al., 2005), such as acidity or the production of secondary metabolites (Lindquist et al., 1992). Thus, these secondary metabolites have been suggested as the primary means of predation avoidance and therefore a successful establishment within the invaded habitat (López-Legentil et al., 2006).

Many invasive ascidians persist and even thrive within highly polluted marine environments such as harbour systems (Evans et al., 2017). They exhibit a broad range of environmental tolerance to sewage, surface runoff, toxic heavy metals, organic and inorganic pollution, and resistance to wide fluctuations in temperature and salinity (C.

Lambert & Lambert, 2003; Pineda et al., 2012). Invasive ascidians are characterised by high growth and reproduction rates, leading to increases in local abundance and long-term survival (Evans et al., 2017; C. C. Lambert & Lambert, 1998). Once established, ascidians become strong ecological competitors for space using their epibiosis capabilities or by producing secondary metabolites to inhibit growth or reduce the larval recruitment of nearby competitors (Evans et al., 2017; López-Legentil et al., 2006). This results in a significant increase in the invasive ascidian abundance within the invaded habitat, and often at the expense of the native species (C. Heinze et al., 2021; Rpm et al., 1996).

Studies have reported that the associated microorganisms of many invasive species may play a role in their invasion and establishment abilities (Evans et al., 2018; Goddard-Dwyer et al., 2020). For example, the group of James Evan et al (2018) compared the prokaryotic symbiont communities of native and invasive colonial ascidian species across artificial (i.e., harbour system) and natural reef environments. They found that the microbiome of the native host *E. capsulatum* shifted across habitats, while the microbiome of the invasive host *D. bermudensis* remained stable. These findings suggested that there could be different ecological constraints affecting the stability and specificity of the associated microbiome of invasive ascidians (Evans et al., 2017, 2018)

1.3 Bioactives

1.3.1 Why Ascidians Produce Bioactives

In nature, colonial ascidians tend to grow on top of another, a phenomenon called epibiosis, due to inherent ecological competition over limited natural resources such as space, light, and nutrients (López-Legentil et al., 2006; Núñez-Pons et al., 2012). Colonial ascidians are extremely vulnerable to epibiosis, since they rely on their outer surfaces for vital life processes, such as gas and nutrient exchanges, waste expulsion of larvae and defence metabolite release (Wahl, 2008; Zhan et al., 2015). They are often subject to epibiosis since they are bound to the substrate on which they reside. During an ecological competition, overgrowing interacting colonies can lead to the mortality of the outcompeted colony (Wahl, 2008). To avoid epibiosis and predation, colonial ascidians use their allelochemical capacities (bioactive), induced by their symbiotic microbial communities (A. R. Davis et al., 1989; Núñez-Pons et al., 2012).

The same bioactives are also commonly produced during ecological invasions to help the invaders reduce predation pressure, settling of competitor species (weather ascidians or other benthic species) at a nearby environment, and fouling by epibionts (Goddard-Dwyer et al., 2020; Núñez-Pons et al., 2012). An invasive colonial species is likely to have a stronger bioactive reaction, which helps it defeat the endemic species and determines the extent of its ecological success (Dror et al., 2019). Thus, the microbiome of the invasive species can produce enhanced metabolic substances and gain a competitive advantage over the native species (Evans et al., 2017).

Moreover, colonial ascidians are softer and less protected than the rough tunics of solitary ascidians, therefore they may be under greater ecological pressures to synthesise different and possibly stronger chemical compounds (Koplovitz et al., 2009). Thus, successful epibionts species, such as the invasive *D. vexillum*, can adversely affect native sessile communities or even threaten their existence (Reinhardt et al., 2012; Stefaniak, 2009) due their overgrowth ability and perhaps stronger allelochemical response. Hence, bioactive biosynthesis in sessile communities, and specifically in colonial ascidians, may be a major factor under competitive ecological pressures, such as maintaining space, avoiding predation, and overcoming native species, (Green et al., 2002; Núñez-Pons et al., 2012).

Despite the greater probability to discover a stronger microbially driven bioactive compound for human purposes from the invasive colonial species due to the above mentioned, only recently invasive colonial ascidians became more common in microbiome studies as highly potential candidates for bioactive drug discovery source (Casso et al., 2020; Evans et al., 2018, 2021; Goddard-Dwyer et al., 2020).

1.3.2 Pharmaceutical and Other Applications of Bioactives

Many marine natural products (bioactives) are used for medical, pharmaceutical, and agricultural purposes (Fortman & Sherman, 2005). The first ascidian bioactive metabolite, geranyl hydroquinone, was isolated by Fenical in 1974 from *Aplidium sp.* (Núñez-Pons et al., 2012). In recent years, ascidians have yielded various novel compounds with remarkable bioactive abilities, including the first marine natural product to enter human clinical trials, didemnin B (Aplidin®), used for the treatment of specific cancers (Núñez-Pons et al., 2012; Palanisamy et al., 2017). Ascidians are the most

investigated group of marine organisms for identification of novel bioactives due to their high potential to apply in future biomedical applications (Palanisamy et al., 2017).

Natural products isolated from ascidians usually include polyketide, terpenoid, peptide, alkaloid, and other classes of natural products (Murray et al., 2020). Most of ascidian bioactive compounds contain nitrogen, and approximately 70% of nitrogenous metabolites are alkaloids (Proksch et al., 2002). These compounds often exhibit diverse biological activities such as cytotoxicity, antitumor, antimicrobial, immunosuppressive activities, inhibition of topoisomerases and cyclin kinases (Palanisamy et al., 2017). Therefore, ascidians compounds are widely used in the medical and pharmaceutical sectors (Proksch et al., 2003). However, a recent study showed that until recent years, only 5% of living ascidian species have been studied out of approximately 3,000 species, suggesting that many novel marine natural products are yet to be discovered (Palanisamy et al., 2017).

1.3.3 Putative Roles of the Ascidian Microbiome in Bioactive Synthesis

Molecular studies have shown that some of the abundant secondary metabolites from marine invertebrates originate in the associated bacteria and not in the animals themselves (Schmidt, 2015). Supporting evidence to the microbial origin of the bioactive compounds isolated from ascidians is that the single molecule dominating their biosynthesis resides exclusively in microbes (Dou & Dong, 2019).

Ascidians are associated with a wide diversity of bacteria (Chen et al., 2017; Evans et al., 2017; Utermann et al., 2020). Some bacterial phyla may play a specific physiological or ecological role that is crucial to the host's ability to survive (Chen et al., 2018). For example, Cyanobacteria may provide oxygen, nutrients, and defence for the ascidian host by means of carbon fixation, nitrogen recycling, and metabolite production (Chen et al., 2018). Another example is Actinobacteria which is known for their ability to produce bioactive compounds for the host defence and offence mechanisms (Chen et al., 2018). During species invasions in particular, microbial symbionts enhance the adaptive capacity and invasion success by means of heavy metal detoxification (Aires et al., 2016), production of toxic chemicals (Amsellem et al., 2017), and enhanced nutrient fixation (Arnaud-Haond et al., 2017; Casso et al., 2020; Evans et al., 2017).

1.4 Research Aims and Hypotheses

In this study, live and wild specimens of colonial ascidians were collected from locations around the Tauranga Harbour to examine their microbial communities across varied host ecological states. Specifically, this study aims to compare the associated microbiome between competing ascidian species (including the microbiome within interactive borders) and evaluate them against their ecological traits (native or invasive) and monospecific counterparts. This study is part of a larger project consisting of microbial and chemical analyses; chemical analyses will be conducted separately (not described in this thesis) using liquid chromatography–mass spectrometry (LC-MS) to detect and quantify bioactives.

Colonial ascidians were chosen as the subjects of this study since they are likely to have stronger allelochemical response than ascidian species with a rough tunic (i.e., solitary ascidians) to increase their probability of survival (Núñez-Pons et al., 2012). We examined the microbiome of species involved in an interaction because ecological competition has been theorised to trigger changes in microbial communities that lead to the biosynthesis of bioactive compounds (A. R. Davis et al., 1989; Lopez-Legentil & Turon, 2005; Núñez-Pons et al., 2012).

In addition, all colonial ascidian microbiome studies published so far have examined only the tunic and excluded the zooids, presumably due to the expected high abundance of bacterioplankton (Erwin et al., 2013, 2014; Goddard-Dwyer et al., 2020; Martinez Garcia et al., 2009). However, the hologenome theory (2008) supports the rationale that zooids' associated microbiome (excluding the bacterioplankton) should be separately characterised for their potential to produce bioactive compounds. This motivated us to conduct the first specific analysis of the zooid microbiome of colonial ascidians despite the greater experimental and procedural complexity (Chen et al., 2017).

Based on the assumptions that:

- bioactives determine the ecological success of colonial ascidians (A. R. Davis et al., 1989; Núñez-Pons et al., 2012); and
- bacteria in the tunic microbiome are involved in synthesising bioactives (Martinez Garcia et al., 2009; Martínez-García et al., 2007).

We hypothesis that:

- each colonial ascidian species harbours a species-specific microbiome;
- colonial ascidians interacting (i.e., competing chemically) with other colonial ascidian species have a different microbiome compared to their monospecific counterparts;
- the interactive border between two colonial ascidians has a unique microbiome compared to either of the interacting species within the specimen;
- invasive colonial ascidians harbour different microbiome to native ones; and colonial ascidians harbour tissue-specific (zooid and tunic) microbiomes.

Chapter 2

Methodology

2.1 Sampling Design

To test my research hypotheses, the following specimens were collected:

- Monospecific specimens: colonies composed of a single ascidian species (native or invasive) with no apparent epibiosis. These specimens were collected to provide baseline information on the ascidian microbiome, allowing us to evaluate species-specificity in specimens not undergoing ecological competition with another ascidian species.
- Interaction specimens: two colonies of different ascidian species (referred to hereafter as species one & species two) that meet at an interactive border (IB). The interaction specimens allowed us to compare the microbiomes of ascidian species in different ecological states. Specifically, these specimens enabled us to examine the influence of interaction with another species on the ascidian microbiome.
- Seawater samples - Seawater samples were collected to assess the microbial baseline (bacterioplankton) of each collection site.

An additional goal of this study was to conduct the first comparison of zooid and tunic microbiomes for colonial ascidians. Therefore, each ascidian specimen was dissected to obtain both tissue types (Table 2.2).

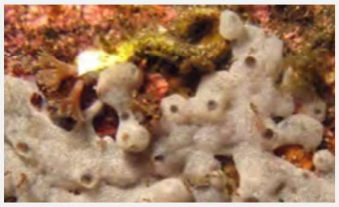
2.1.1 Sample Collection

During SCUBA and free dives, naturally grown competitive interactions between three native species (*Didemnum incanum* [DI]*, Lissoclinum notti* [LN]*, Aplidium phortax* [AP]) and three invasive species (*Didemnum vexillum* [DV]*, Botrylloides leachi/leachii* [BL]*, Aplidium kottae* [AK]) of ascidians (Table 2.1) were collected. Monospecific specimens of the same species were also collected (Table 3.1). Sample collection was conducted at three locations and on four dates (Figure 2.1, Table 3.1). On the 18th of February 2021 and the 15th of March 2021, specimens were collected from Tauranga Marine Bridge Marine Precinct (37°40'27"S, 176°10'18"E) (hereafter referred to as Bridge) and from Tauranga Bridge Marina, Pier A (37°40'09"S, 176°10'44"E) (hereafter referred to as Pier

A) respectively (Figure 2.1, Table 3.1). On the 25th of March and the 29th of April 2021, samples were collected from Salisbury wharf, Pilot Bay, Mount Maunganui (37°38'25"S, 176°10'52"E) (hereafter referred to as Mount) (Figure 2.1, Table 3.1).

All ascidian specimens were collected at least 2 m apart from each other to reduce the probability of pseudoreplication (Casso et al., 2020; Dror et al., 2019; Evans et al., 2017; Rinkevich & Fidler, 2014). All samples were collected from depths ranging from zero to 1.5 m using a cookie cutter, to ensure similar sizes and shapes for all specimens. During sampling, the specimens were collected by carefully detaching them from the concrete structures or floating docks and placing them in individual Ziploc plastic bags containing seawater. Ascidian samples were transported in oxygenated seawater to the Coastal Marine Field Station (37°40'15"S, 176°09'59"E) (approximately 1-6 km distance from collection sites) and identified by a morphological inspection. Seawater samples (~2 L) were collected from each location in sterile plastic jars and transported on ice to the Thermophile Research Unit at the University of Waikato Hillcrest campus.

Table 2.1: A table describing the experimental species models (initials in parenthesis) , divided into native and invasive species to NZ.

Native species to New-Zealand		Non-native species to New-Zealand	
Species name and initials	Image	**Species name and initials**	Image
Didemnum incanum (DI)		***Didemnum vexillum (DV)***	
Lissoclinum notti (LN)		***Botrylloides leachi/leachii (BL)***	
Aplidium phortax (AP)		***Aplidium kottae (AK)***	

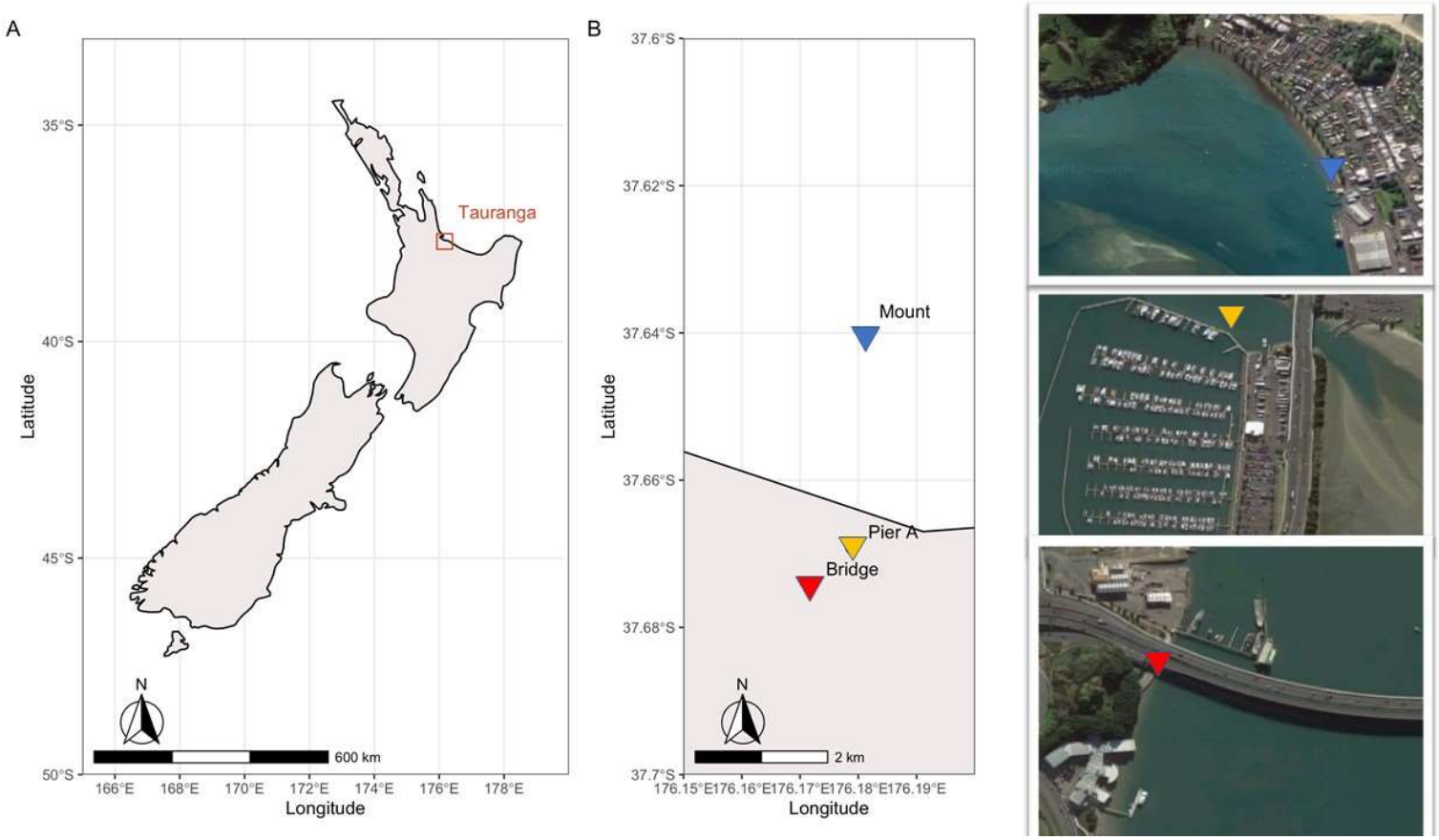


Figure 2.1: New Zealand map (A), and collection sites within Tauranga Harbour (B) created in R , and satellite images taken from google maps. The red triangle shows Tauranga Marine Bridge Marine Precinct (Bridge) (37°40'27"S, 176°10'18"E), the yellow triangle shows Tauranga Bridge Marina, Pier A (Pier A) (37°40'09"S, 176°10'44"E), & blue triangle shows Salisbury wharf, Pilot Bay, Mount Maunganui (Mount) (37°38'25"S, 176°10'52"E).

2.1.2 Initial Sample Processing

Upon arrival at the Coastal Marine Station, each interaction specimen was dissected into three fragments as shown in figure 2.2. One third of the specimen (the middle fragment) was used for microbial analysis, and the flanking fragments were preserved for chemical analysis since a larger biomass is required for liquid chromatography–mass spectrometry (LC–MS) analysis. Each fragment of an interaction specimen contained samples of species one, species two, and the IB in-between. To capture potential heterogeneities in monospecific specimens, they were dissected into eight segments: six from the outer ring and two from the centre (Figure 2.3). One half of the segments was for microbiology (M), and the other half was for chemistry (C).

Since ascidians have strong muscles in their body walls, it was essential to relax the specimens before freezing them to ease the separation of zooids and tunic during further dissection (Rosana M. Rocha, 2016). To relax the specimens, dissolved menthol crystals were used on the fragment designated for microbial analysis using the following procedure: 2 grams of crystals menthol was dissolved in 995 µL of 100% ethanol with 2 min of vortexing, then the menthol solution was mixed with approximately 0.5 L of seawater containing individual ascidian specimens. The jar containing the specimen and seawater (mixed with menthol) was sealed and cooled (4°C) for approximately one hour. The relaxed specimens were retrieved from the jar upside down (i.e., the siphon opening facing down) to remove excess menthol solution (Rosana M. Rocha, 2016). Samples were transported on ice to Thermophile Research Unit and stored in 15 ml Falcon tubes at -80°C.

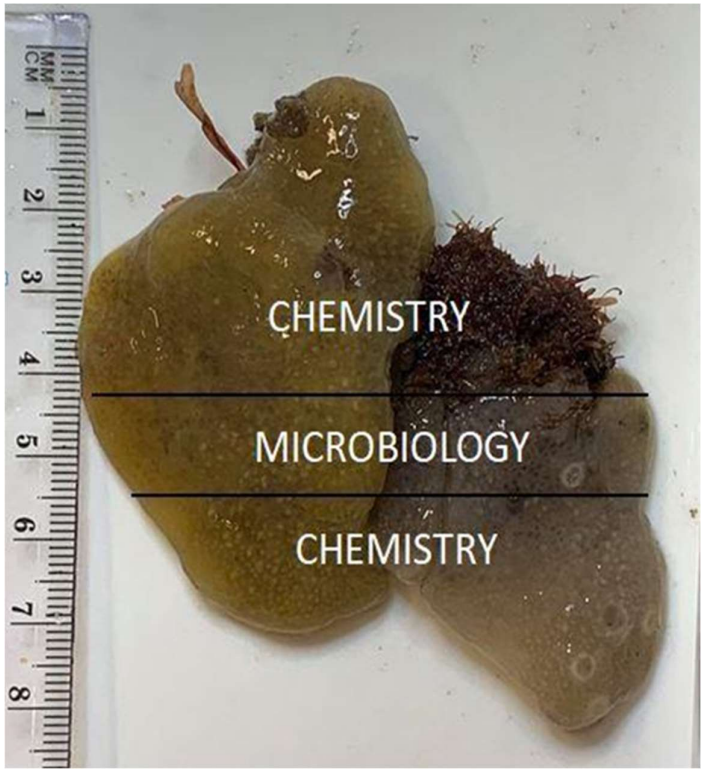


Figure 2.2:An interaction specimen and the dissection methodology to divide between the chemical and microbial analyses.

Figure 2.3: A monospecific specimen and the dissection methodology to equally divided between the chemical and microbial analyses (C- chemistry, M- microbiology)

2.2 Ascidian Sample Processing Prior to DNA Extraction

Each specimen was defrosted on ice and photographed before dissection (Table S1). Ascidian specimens were rinsed with filtered (0.2 µm, 47 mm, Whatman) artificial seawater (Red Sea Salt, 35 ppt) to remove loosely associated microorganisms. All exposed surfaces were sterilised with ethanol (70%) and bleach (5%). Dissection scalpels, Petri dishes, and all other associated equipment were bleached, sterilised with ethanol, and exposed to UV for 30 minutes prior to dissection. For each specimen a new sterile blade was used to avoid cross contamination.

2.2.1 Interaction Specimens

Using a dissection microscopy, interaction specimens were dissected into 3 samples: species one, species two, and IB to approximately 1 cm by 1 cm (Figure 2.4), and each of the samples was dissected into zooids and the tunic (approximately 40-80 milligrams). Therefore, each interaction specimen yielded six samples (Table 2.2).

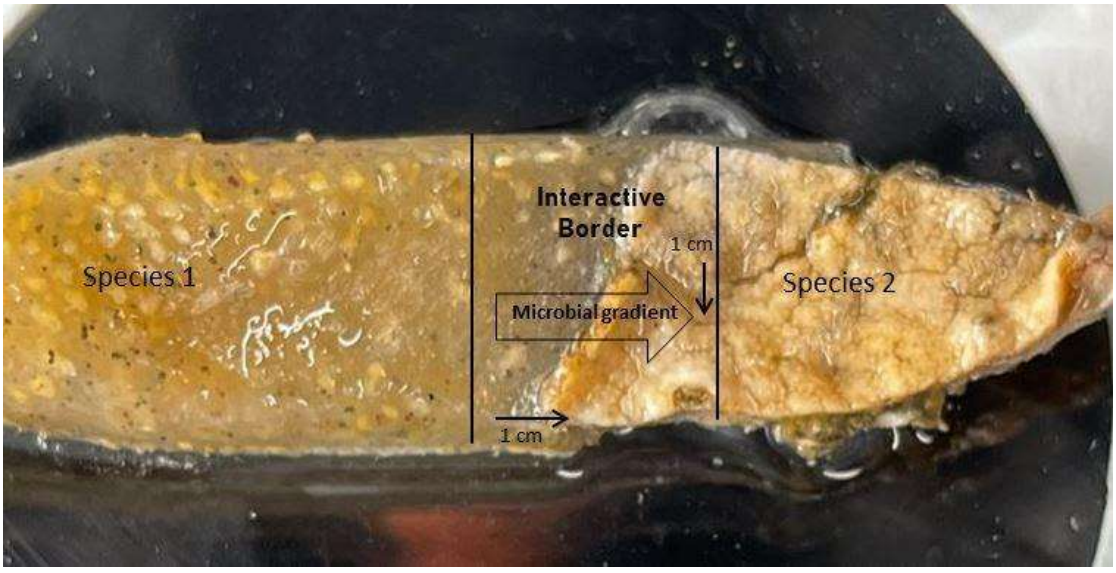


Figure 2.4: An interaction specimen including species 1, species 2, and the interactive border (IB) between them. The microbial gradient represents the sampling direction to contain the microbiome within the tissue of interest.

2.2.2 Monospecific Specimens

Each segment (from the outer ring and centre) (Figure 2.3) within a monospecific specimen was separated into zooids and tunic samples (approximately 40-80 milligrams). Therefore, each monospecific specimen yielded two samples (Table 2.2).

Table 2.2: A list of the derivative samples from each interaction and monospecific specimen.

Interaction specimen	Monospecific specimen
1) Species one zooids 2) Species one tunic 3) Species two zooids 4) Species two tunic 5) Interactive border (IB) zooids 6) Interactive border (IB) tunic	1) Species zooids (outer ring and centre) 2) Species tunic (outer ring and centre)

2.3 Seawater and Artificial Seawater Samples

Seawater samples (artificial and natural) were filtered with cellulose nitrate membrane filters (0.2 µm, 47 mm, Whatman) (Jeunen et al., 2019). Each filter was aseptically cut into small pieces prior to DNA extraction. Before processing samples and between each sample, the filtration apparatus was rinsed with bleach (5%), ethanol (70%), and MilliQ water (UV-sterilised for 30 minutes) and dried with sterile Kim Wipes.

2.4 DNA Extraction

DNA extraction was conducted using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol, with a final elution of 200 µl of AE buffer. To extract the microbiome of interest and limit eukaryotic DNA recovery (Figure 2.5), the following modifications were applied: lysis stage was conducted as an overnight incubation (approximately 12 hours lysis) in the thermomixer (56°C, 300 rpm). The extracted DNA samples were quantified using the Qubit broad range (BR) assay fluorometric quantification method following the dsDNA BR protocol (Thermo Fisher Scientific, Auckland). DNA was stored at -20°C until further analysis was conducted.

Prior to DNA extraction from the filters for seawater and artificial seawater samples, two bead-beating steps were conducted, and the remaining steps followed the manufacturer's protocol for the Qiagen DNeasy Blood and Tissue Kit with the following modifications according to Lamy et al. (2021): 600 µl of Buffer AL and 600 µl of 100% ethanol were used; 500 µl of lysate was pipetted to spin column and centrifuged at maximum speed until the entire volume of the lysate (1.8 mL) was pulled through the spin column; two 500-µl washes of Buffer AW1 and two 500-µl washes of Buffer AW2 were done; elutions were done in two 50-µl steps for a total of 100 µl extracted DNA. A procedural blank control was created during DNA extraction, containing only the reagents present in the DNeasy Blood and Tissue Kit.

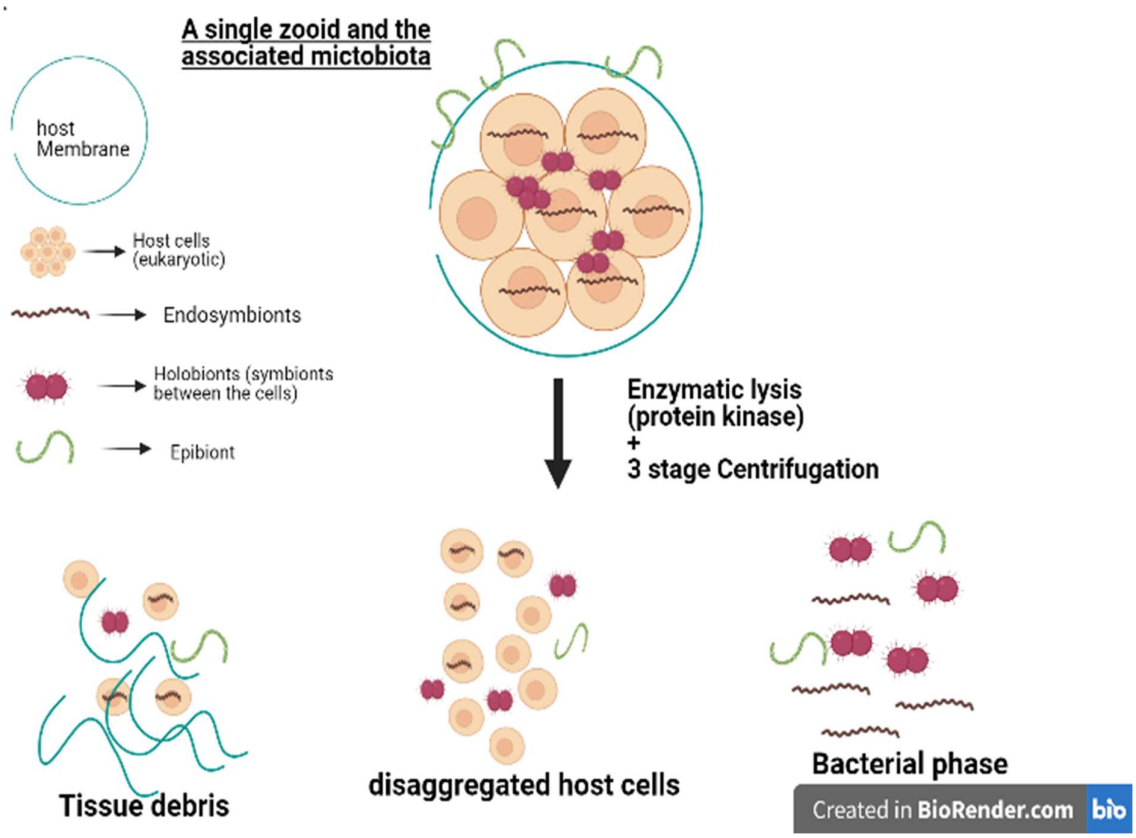


Figure 2.5: A diagram representing the lysis method of a single zooid and the associated microbiota of interest.

2.5 DNA Amplification of the 16S rRNA Gene

Polymerase chain reaction (PCR) was used to amplify the V4 and V5 hypervariable regions of the 16S rRNA gene from the DNA samples , using the fusion primers 515F-Y/926R (5'GTGYCAGCMGCCGCGGTAA [515F] 5'CCGYCAATTYMTTTRAGTTT [926R]). Barcoding of samples was conducted prior to DNA amplification to limit undetected cross contamination (fusion barcodes attached in supplementary material Table S2) (Comeau et al., 2017). 926R primers were initially used by Quince and colleagues (2011) and were chosen for the 16S rRNA amplification in this study, despite their tendency to amplify eukaryotic 18S rRNA. The reason is that they were proven to yield more accurate estimates of mock community composition and abundances and produce longer amplicons that can differentiate taxa, compared with 515F-C/806R (Apprill et al., 2015; Parada et al., 2016).

Extracted DNA samples were diluted to 5 ng/µL, and 1 µl of DNA was added to each PCR reaction (rxn) to achieve 5 ng of DNA in each rxn. Each reaction (20 µL) consisted

of 0.4 µL of bovine serum albumin (BSA) (0.8 mg/mL), 2.4 µL of dNTPs (2mM), 2.4 µL of 10X PCR buffer, 2.4 µL MgCl2 (50 mM), 0.4 µL of each primer (10 mM), 0.096 µL of Platinum Taq DNA Polymerase (Thermo Fisher Scientific, Auckland), 1 µL of template DNA (5 ng/ µL), and 10.504 µL molecular-grade DNA-free ultra-pure water (Thermo Fisher Scientific, Auckland) (Table 2.3). Samples with concentration lower than 5 ng/µL were not diluted and added to an adjustable master mix to achieve 5 ng of DNA per rxn (Table 2.4).

Table 2.3: Master mix recipe for samples diluted to 5 ng/µL

Concentration	Reagents	Volume µL	Final concentration
N/A	H_20	10.504	N/A
0.8mg/mL	BSA	0.4	0.016 mg/mL
2mM	dNTPs	2.4	0.24 mM
10x	PCR Buffer	2.4	1.2 mM
50mM	$MgCl_2$	2.4	6 mM
10mM	515Y FP	0.4	0.2 mM
10mM	926 RP	0.4	0.2 mM
5U/µL	Taq (Platinum)	0.096	<1 U
5ng/µL	DNA	1	
	Total	20µL	

Table 2.4: Master mix recipe for samples with < 5 ng/µL

Concentration	Reagents	Volume µL	Final concentration
N/A	H_20	10.504 - X	N/A
0.8mg/mL	BSA	0.4	0.016 mg/mL
2mM	dNTPs	2.4	0.24 mM
10x	PCR Buffer	2.4	1.2 mM
50mM	$MgCl_2$	2.4	6 mM
10mM	515Y FP	0.4	0.2 mM
10mM	926 RP	0.4	0.2 mM
5U/µL	Taq (Platinum)	0.096	<1 U
<5ng/µL	DNA	X	
	Total	20µL	

The following thermocycler conditions were applied: an initial 3-minute denaturation step at 94°C followed by 30 cycles of 45 seconds at 94°C, 60 seconds at 55°C, and 90 seconds at 72°C, with a final extension step at 72°C for 10 minutes. All reactions were run on an Applied Biosystems ProFlex PCR System (Thermo Fisher Scientific, Massachusetts, United States of America). PCR positive control (1 ng of E. Coli genomic DNA) and PCR no-templet control (NTC) were created during each batch of

PCR amplification. PCR NTC contained ultra-pure water instead of DNA template, and the reagents present in the prepared master mix (Table 2.3).

All PCR reactions were conducted in triplicates and pooled for a single sample post amplification. An agarose gel electrophoresis was used to confirm successful PCR amplification. For this step, 1µL of 2.5x loading dye (Thermo Fisher Scientific, Auckland) was combined with 5µL of the pooled PCR product and loaded into the wells of a 1.5% agarose gel. 10µL of a 1 Kb Plus DNA Ladder (Thermo Fisher Scientific, Auckland) was also loaded. Each gel ran for 37 minutes at 70 V and was visualised on an Alpha Innotech Imaging System (Alpha Innotech, California, USA).

2.6 Gel Extraction

To minimise non-target (i.e., eukaryotic 18S rRNA and mitochondrial 16S rRNA genes) reads in the sequencing library, non-target PCR amplicons were removed using gel extraction. Each sample that showed more than one amplicon band was electrophoretically resolved as follows: 2.5 µL of 10x loading dye (Thermo Fisher Scientific, Auckland) was combined with 60 µL of the pooled PCR product and loaded into the wells of a 1.5% agarose gel (240 ml 1x TAE buffer, 3.6 g agarose powder, 4 µL Syber Safe). 20 µL of a 1 Kb Plus DNA Ladder (Thermo Fisher Scientific, Auckland) and a positive control were also loaded. Each gel ran for 60 minutes at 70 V and the target PCR amplicon (bacterial 16S rRNA gene) was extracted using a sterile scalpel.

16S rRNA gene PCR amplicons were recovered using an E.Z.N.A. Gel Extraction Kit (catalogue no. D2500-01, Custom Science, Auckland) according to the manufacturer's protocol with the following modifications: extra 20% of binding buffer was combined with the gel band, the gel band was incubated for additional 8 minutes and at 300 RPM, samples were centrifuged at 4,000 RCF at step number 2 (later centrifuge steps remained as specified in the protocol), the elution buffer was heated (60ºC) before being added to the column (30 µL), and incubated on the column for additional 3 minutes.

2.7 DNA Sequencing and Data Analysis

2.7.1 Next-Generation Sequencing

Barcoded PCR amplicons were normalised using the SequelPrep Normalization Plate Kit (Thermo Fisher Scientific, Auckland) following the manufacturer's protocol. 5 µL of each normalised sample was pooled to form an amplicon sequencing library. 750 µL of the library was concentrated using isopropanol precipitation. Isopropanol precipitation was conducted by mixing 2 volumes of 100% isopropanol (1500 µL) and 75 µL of 3 M ammonium acetate with the library. The mixture was incubated at -20ºC overnight, spun down at 16,000 RCF for 30 minutes, and washed with 70% ethanol at -20ºC. The pellet was resuspended in double-filtered sterilised 1X TE buffer and sent at 4ºC to Auckland Genomics Facility (AGF) at the University of Auckland, New Zealand. At the AGF, the amplicon library was sequenced using an Illumina MiSeq in a 2 x 300 bp paired-end configuration using the MiSeq Reagent Kit V3.

2.7.2 Bioinformatic Analyses

Raw FASTQ files from the Illumina MiSeq were imported into R (v4.1). Amplicon Sequence Variant (ASV) inference and initial filtering were performed using the 'DADA2' package (v.1.22.0) (Callahan et al., 2016). The forward and reverse reads quality profiles were visually examined, and it was determined that truncLen trimming was necessary. Reads were then filtered to allow no unidentified nucleotides ("N") using the following filtering parameters: maxN=0, truncQ=2, rm.phix=TRUE, maxEE=2. The forward and reverse reads were then truncated and trimmed using the following function and parameters: filterAndTrim(fnFs, filtFs, fnRs, filtRs), truncLen=c(235,235), maxN=0, maxEE=c(2,2), truncQ=2, rm.phix=TRUE. Parametric error rates for the reads were visualised and evaluated, which showed that the estimated error rate had a good fit for the observed error rate. Sample inference was performed (i.e., denoised based on generated error model) using the filtered and trimmed sequence data. Denoised and trimmed forward and reverse reads were merged, and the ASVs table was constructed. Chimeras were removed using the "removeBimeraDenovo" function with the "consensus" method. Taxonomy was assigned using the Ribosomal Database Project (RDP) Naive Bayesian Classifier algorithm with kmer size 8 and 100 bootstrap replicates (Wang et al., 2007) and a DADA2-formatted reference database for the SILVA v138 database (Quast et al., 2012).

Analysis of the filtered sequence data was conducted in RStudio (v4.1.2 “Bird Hippie”). The taxonomic data and ASV data created using DADA2 were merged in R into a phyloseq object along with a metadata file created using Excel (composed of sample information, e.g., zooid/tunic, native/invasive etc.). This phyloseq object was statistically analysed using R packages, including phlyoseq (v1.38.0) (McMurdie & Holmes, 2013), ggplot2 (v3.3.5) (Wickham, 2016), vegan (v2.5.7) (Dixon, 2003), knitr (v1.37), tidyverse (v1.3.1) (Wickham et al., 2019), and decontam (v1.14.0) (N. M. Davis et al., 2018). To remove potential contaminant ASVs from the dataset, the ‘decontam’ package was used according to the prevalence-based method (N. M. Davis et al., 2018). This method uses PCR no-template controls [NTC] and procedural blank controls to calculate the prevalence of contaminants in samples (N. M. Davis et al., 2018). The threshold was set to 0.5 (higher sensitivity compared with the default threshold of 0.1). All ASVs that are more prevalent in the controls than in samples were identified as contaminants (N. M. Davis et al., 2018).

Microbiome data diversity were analysed using the ‘phyloseq’ R package. Species richness (alpha diversity) was calculated using observed richness and the Shannon index after all samples were rarefied to 1,000 reads. Normality of the data was evaluated using the quantile-quantile plot and the Shapiro-Wilk normality test, and non-gaussian distribution was observed from the datasets, suggesting non-normal distribution. Differences in the alpha diversity were evaluated using the Kruskal-Wallis Test (non-parametric ANOVA). If a significant difference was observed, pairwise analysis was conducted using the Wilcoxon test to compare between groups. Rarification was not performed prior to other analyses. Bacterial community composition was investigated using relative abundances of the most abundant phyla (abundance >1%). Principal Coordinate Analysis (PCoA) plots based on Euclidean dissimilarities of centred log ratio transformed data were used to examine the dissimilarity between samples (beta diversity analyses). PERMANOVA test using Adonis2 function in vegan was used to determine the statistical significance of observed differences in beta diversity or community composition. If a significant difference was observed, a permutation test for homogeneity of multivariate dispersions (i.e., group dispersions test) was conducted using the ‘betadisper’ function in vegan R package to confirm significance, and pairwise analysis was conducted by pairwise Adonis test to compare between groups.

Chapter 3

Results

3.1 General Data Description

3.1.1 Sample Description and Sequencing Data

Overall, 182 samples were included in this study. 164 samples were of ascidian microbiome and represented 3 replicates of 6 monospecific specimens and 7 interaction specimens of different species pairings (Table 3.1). The interaction specimens each yielded six samples (i.e., species one zooids, species one tunic, interactive border [IB] zooids, IB tunic, species two zooids, and species two tunic), totalling 126 samples (Tables 3.1 & 2.2). The monospecific specimens each yielded two samples (zooids and tunic), totalling 38 samples (four monospecific specimens of *Didemnum incanum* [DI] were collected, see Table 3.1). The interaction specimens included 2 invasive vs invasive and 5 native vs invasive species pairings (Figure 3.1 & Table 3.1), while no native vs native species pairings were observed at our field sites. The monospecific specimens included 3 native and 3 invasive species (Figure 3.1 & Table 3.1). Alongside the 164 samples, 1 procedural blank control and 13 PCR no-template controls (NTC) were also processed and analysed to check for potential contamination during DNA extraction and PCR. In addition, 4 seawater samples were included, one from each collection site (Table 3.1) and one of the unfiltered artificial seawater (ASW) used for rinsing during specimen dissection. After the sequencing data underwent de-noising and quality filtering using the DADA2 pipeline, 33,747 amplicon sequence variants (ASVs) were identified across the 182 samples in total (including ascidian specimens, seawater samples, the procedural blank, and PCR NTC).

Table 3.1: The collected interaction and monospecific specimens and number of replicates (n) by the collection date. The blue color represents native species (Didemnum incanum [DI], Lissoclinum notti [LN], Aplidium phortax [AP]), and red represents invasive species (Didemnum vexillum [DV], Botrylloides leachi/leachii [BL], Aplidium kottae [AK])

Collection date	18/02/21	15/03/21	25/03/21	29/04/21
Location	Bridge	Pier A	Mount	Mount
Interaction specimens	DV-DI (n=2)	DV-LN (n=3) DV-BL (n=3)	DV-AK (n=3) DV-AP (n=3) AK-AP (n=3) AK-DI (n=3) DV-DI (n=1)	N/A
Monospecific specimen	N/A	DV (n=3) BL (n=3) LN (n=3)	DI (n=4)	AK (n=3) AP (n=3)

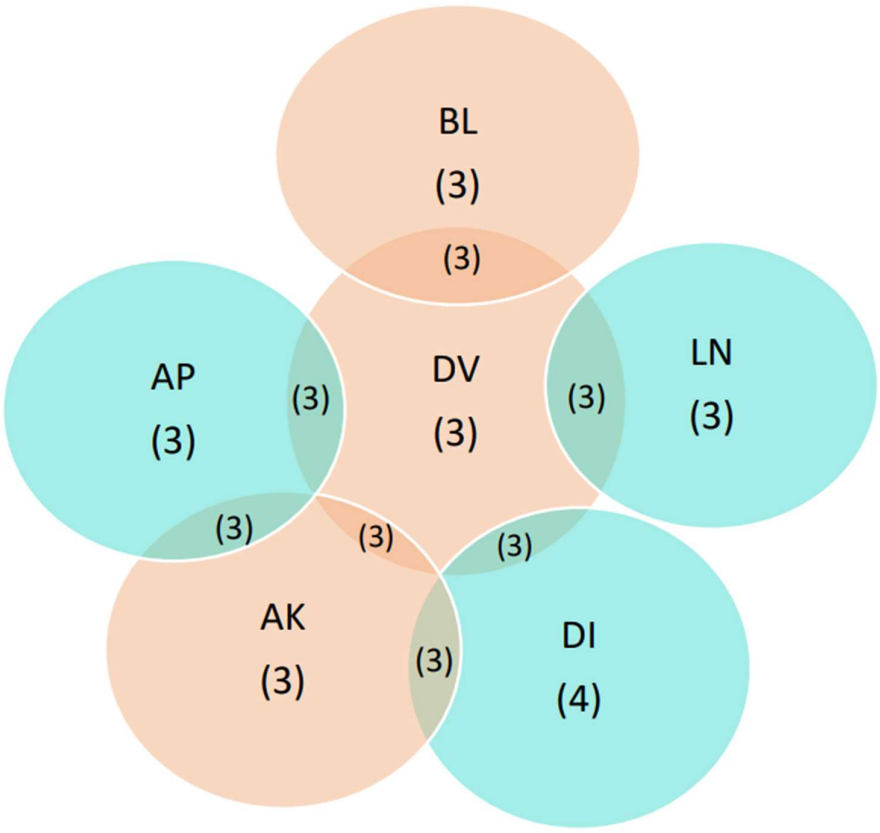


Figure 3.1: A Venn diagram showing the collected monospecific and interaction specimens and quantities (in parentheses). The overlaps between two species represent interactions and the number of replicates. Red circles are invasive species, and blue circles are native species.

3.2 Quality Control and Decontamination of the Sequencing Data

Low number of reads (Figure 3.2A) and limited diversity for contaminants (Figure 3.2B) were identified in the PCR NTC, and in the procedural blank control (S-164). The most abundant phylum in the controls was *Proteobacteria* (Figure 3.2B). Sample S-163 was unfiltered ASW used for rinsing during dissection. Although the ASW used to rinse specimens during dissection was always filtered (see section 2.2), we sequenced DNA extracted from unfiltered ASW to maximise the sensitivity of contaminant detection.

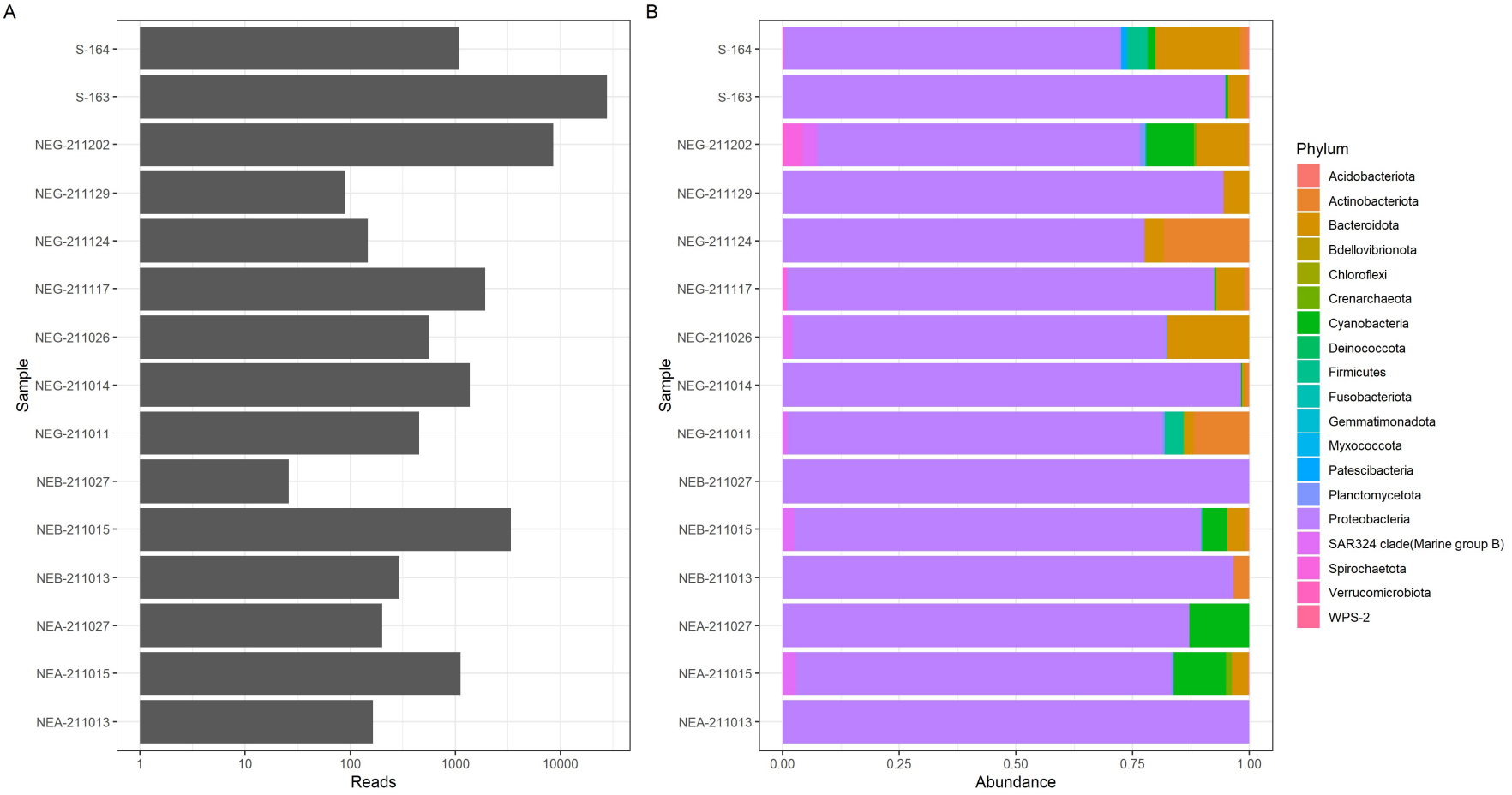


Figure 3.2: Reads number (A) and phylum-level composition (B) for control samples, including the procedural blank control (S-164, n=1), unfiltered ASW (S-163, n=1) , and PCR NTC (NEG-YYMMDD, n=13).

A plot of read numbers for all samples showed a clear separation between samples and controls (Figure 3.3). The only control with more than 10,000 reads was the unfiltered ASW (S-163). 'decontam' identified 119 out of 33,747 ASVs (0.4 %) as contaminants prevalent in both samples and controls (Figure S1). Those 119 ASVs were removed from the data set, leaving a total of 33,628 ASVs. Following this step, the 15 control samples (unfiltered ASW, PCR NTC, and procedural blank control) were removed from the dataset, leaving 33,403 ASVs across 167 samples in the dataset.

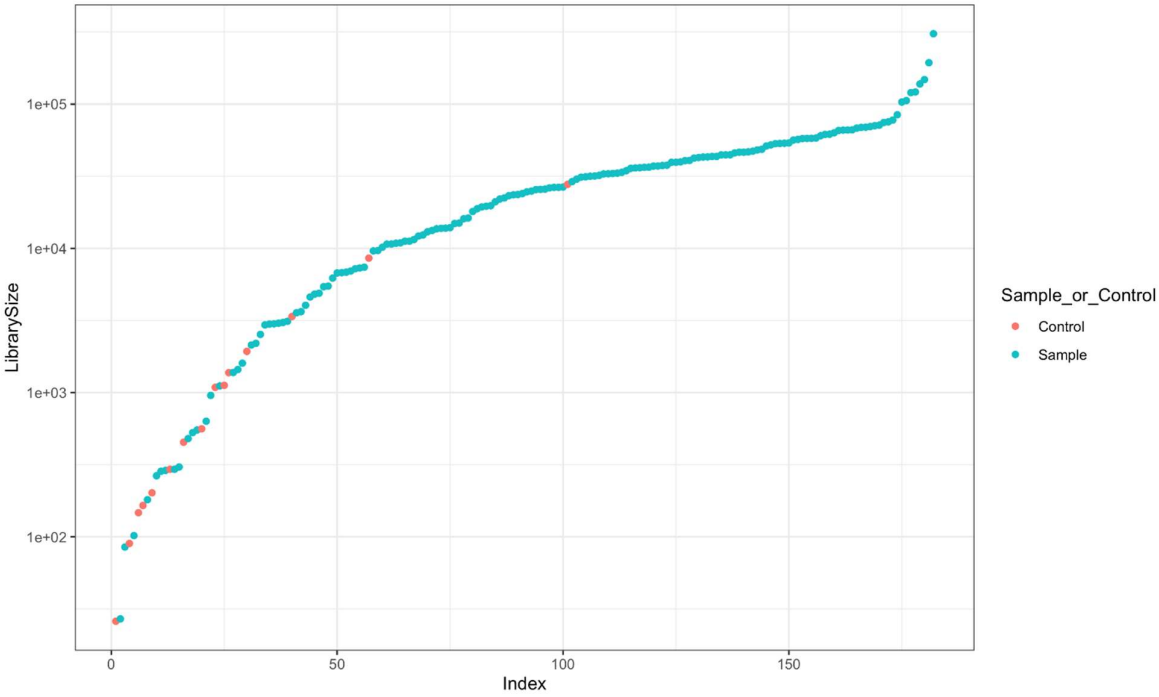


Figure 3.3: Decontam plot differentiating samples and controls by library size

Further filtering of this dataset was performed to remove non-target reads such as mitochondria (1,930 ASVs), chloroplasts (2,520 ASVs), eukaryotes and ASVs not assigned at the domain level (34 ASVs), leaving a total of 28,875 (86.5%) ASVs across 167 samples. After trimming for non-target reads, 15 samples had fewer than 1000 reads (Table 3.2). Those samples did not exhibit trends in regard to sample type (zooids or tunic, Figure 3.4A) and were removed from the dataset, leaving a total of 28,795 ASVs across 152 samples. ASVs that did not appear more than twice across all samples were also removed, resulting in a total of 26,705 ASVs across 152 samples (retaining

approximately 92% ASVs and 93% reads present in the dataset). After quality control and filters, the remaining samples had a median of 26,855 (min = 1,250, max = 303,785) reads (Figure 3.4 A), and a median of 457 (min = 35, max = 1,989) ASVs per sample (Figure S2). ASVs contained a median of 31 (min = 3, max = 24,187) reads (Figure 3.4 B). There was no obvious correlation between the number of reads and recovered DNA concentration and/or whether the sample underwent the gel extraction procedure (Figure S3).

Table 3.2: Samples removed due to low read count (<1,000 reads). The specimen identifier comprises of collection date, species, the species it interacts with (M if monospecific), replicate ID, zooid or tunic (Z/T)

Sample	Identifier	Species in sample	Interacting /Monospecific /Interactive border	Zooid/ Tunic	Replicate ID	Read count
S-087	2503AP_AK_3Z	AP	I	Z	3	21
S-135	1503BL_S_2Z	BL	M	Z	2	50
S-078	2503AKAP_I_1T	AKAP	IB	T	1	82
S-145	2904AP_S_1Z	AP	M	Z	1	82
S-023	1503DVBL_I_1Z	DVBL	IB	Z	1	141
S-146	2904AP_S_1T	AP	M	T	1	171
S-150	2904AP_S_3T	AP	M	T	3	225
S-088	2503AP_AK_3T	AP	I	T	3	265
S-102	1503DVLN_I_2T	DVLN	IB	T	2	292
S-139	2904AK_S_1Z	AK	M	Z	1	464
S-109	1802DV_DI_1Z	DV	I	Z	1	480
S-143	2904AK_S_3Z	AK	M	Z	3	528
S-106	1503LN_DV_3T	LN	I	T	3	633
S-041	2503DVAP_I_1Z	DVAP	IB	Z	1	756
S-076	2503AP_AK_1T	AP	I	T	1	853

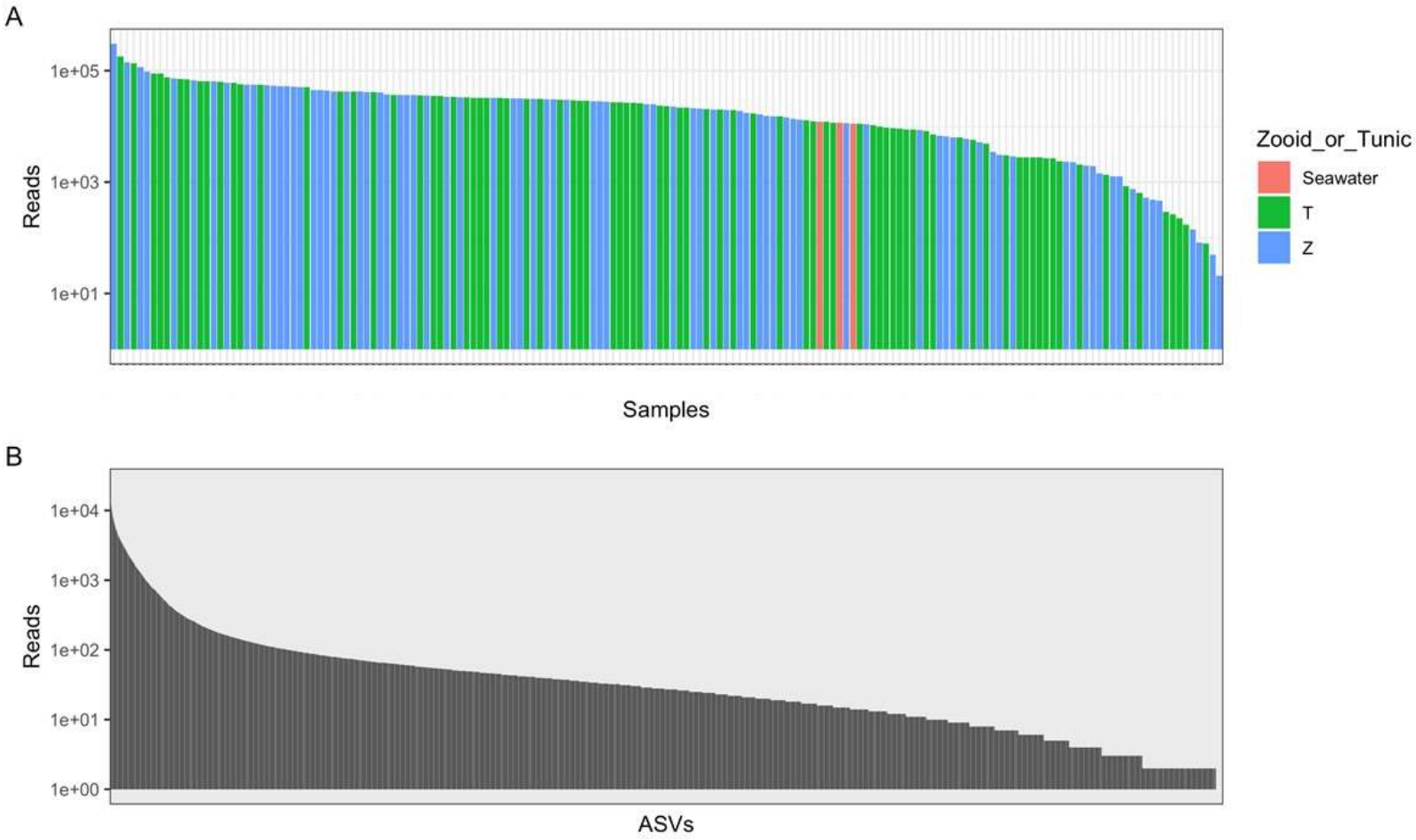


Figure 3.4: Numbers of reads per sample (A) and per ASV (B) after quality control and filters

3.3 Comparing Zooid, Tunic, and Seawater Microbiomes

3.3.1 Community Structure

Microbial community structure was analysed using all tunic and zooid samples as well as seawater samples (Figure 3.5). The PCoA plot showed no obvious difference in microbial community structure of the zooids compared to the tunic. In addition, zooid and tunic samples harbour significantly different microbiomes from the seawater samples, which appear to cluster. PERMANOVA test supported those findings, with almost no significant differences between zooids and tunic samples (p-value = 0.042), and both zooids and tunic exhibit a significant difference from the seawater samples (zooids vs seawater: p-value = 0.01, tunic vs seawater: p-value = 0.029). To examine the distribution of non-microbial signals (i.e., mitochondria and chloroplast), we carried out the same analyses with those sequences included, and the results showed similar trends (PERMANOVA, p-value = 0.045) (Figure S4).

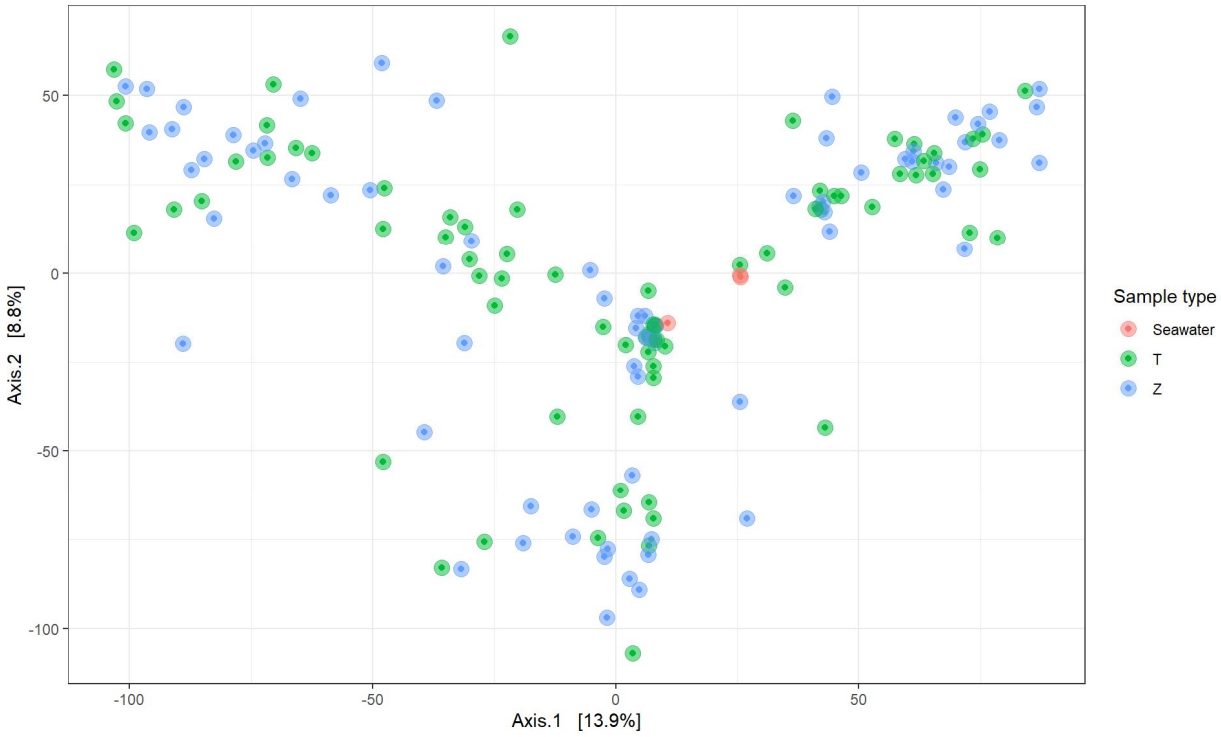


Figure 3.5: PCoA plot showing microbial community structure across tunic, zooid, and seawater samples.

3.3.2 Overlapping ASVs Between Zooids, Tunic, and Seawater Samples

A Venn diagram was generated to visualise shared and unique ASVs for each sample type (Figure 3.6). Tunic samples collectively have 12,490 unique ASVs, and zooid samples have 8,936. 4,541 ASVs were shared between these sample types. Seawater samples have 580 unique ASVs, and only 15 and 37 shared ASVs with zooids and tunic, respectively.

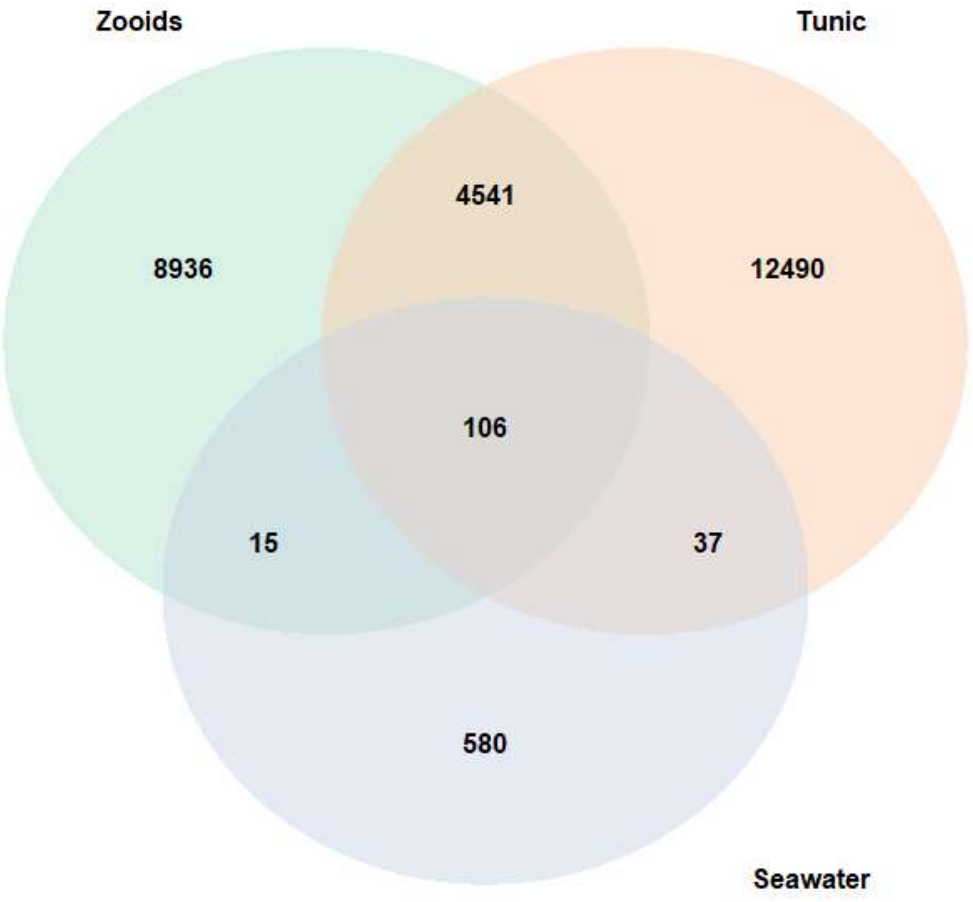


Figure 3.6: Venn diagram presenting the unique and shared ASVs across zooid, tunic, and seawater samples.

3.3.3 Community Taxonomic Composition

Bar plots were used to visualise phylum-level compositions for zooids and tunic from all specimens (Figure 3.7). No compositional difference was detected between the two tissue types, regardless of species and sample type. Both tissue types (zooids and tunic) are generally dominated by *Proteobacteria* (>50 % relative abundance in most samples). All seawater samples exhibit similar taxonomic composition for each collection site (Figure S6) and demonstrated a high relative abundance of *Bacteroidota* (Figure 3.7, S6).

There is no obvious and consistent difference between zooid and tunic samples, either by community structure (Figure 3.5) or taxonomic composition (Figure 3.7), and both zooid and tunic samples were significantly distinct from seawater samples (Figure 3.5) and shared a large microbial core (Figure 3.6). We therefore limited downstream data analyses to only the tunic samples in keeping with earlier publications (Chen et al., 2017), leaving a total of 17,174 ASVs across 75 samples.

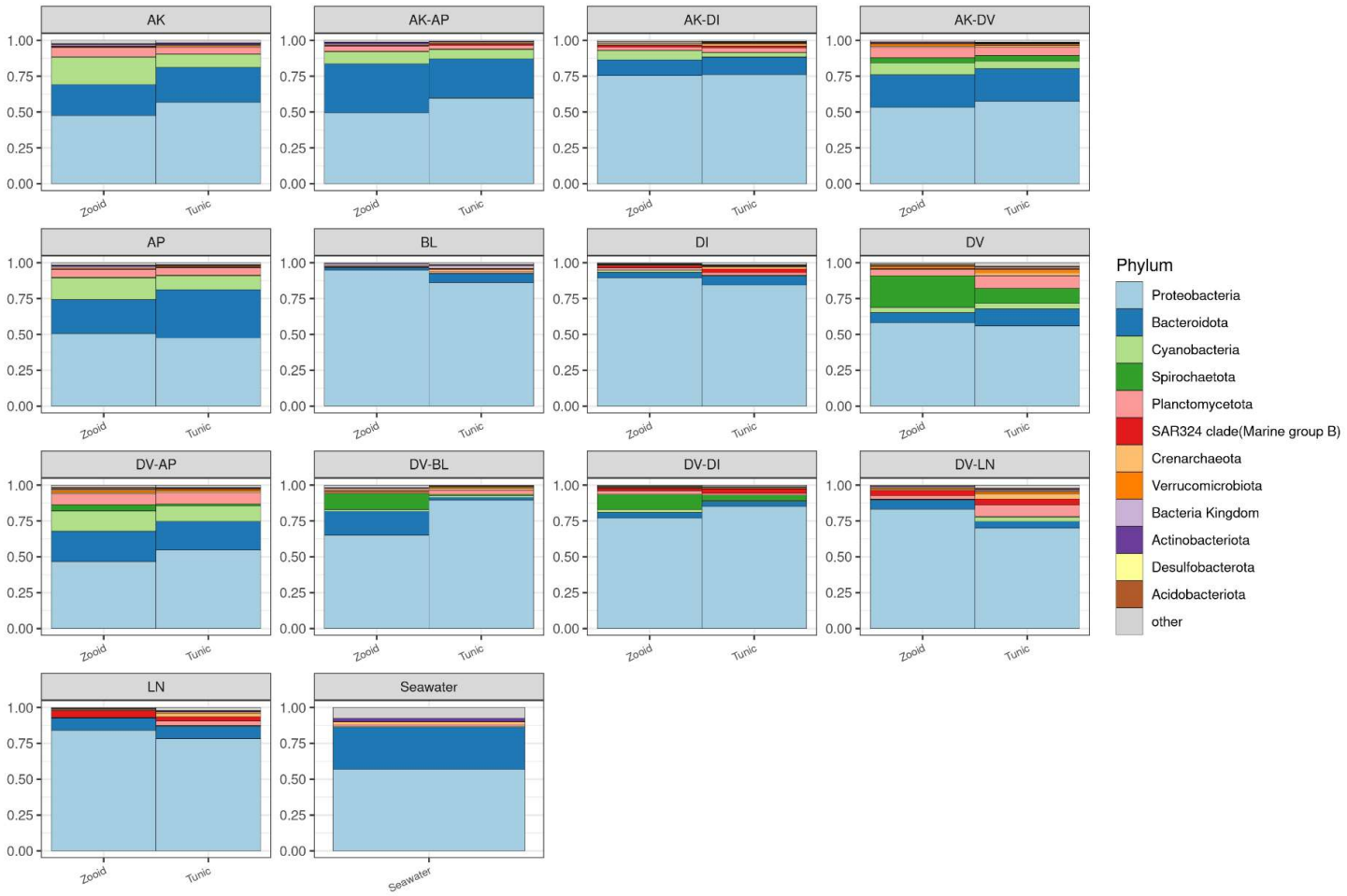
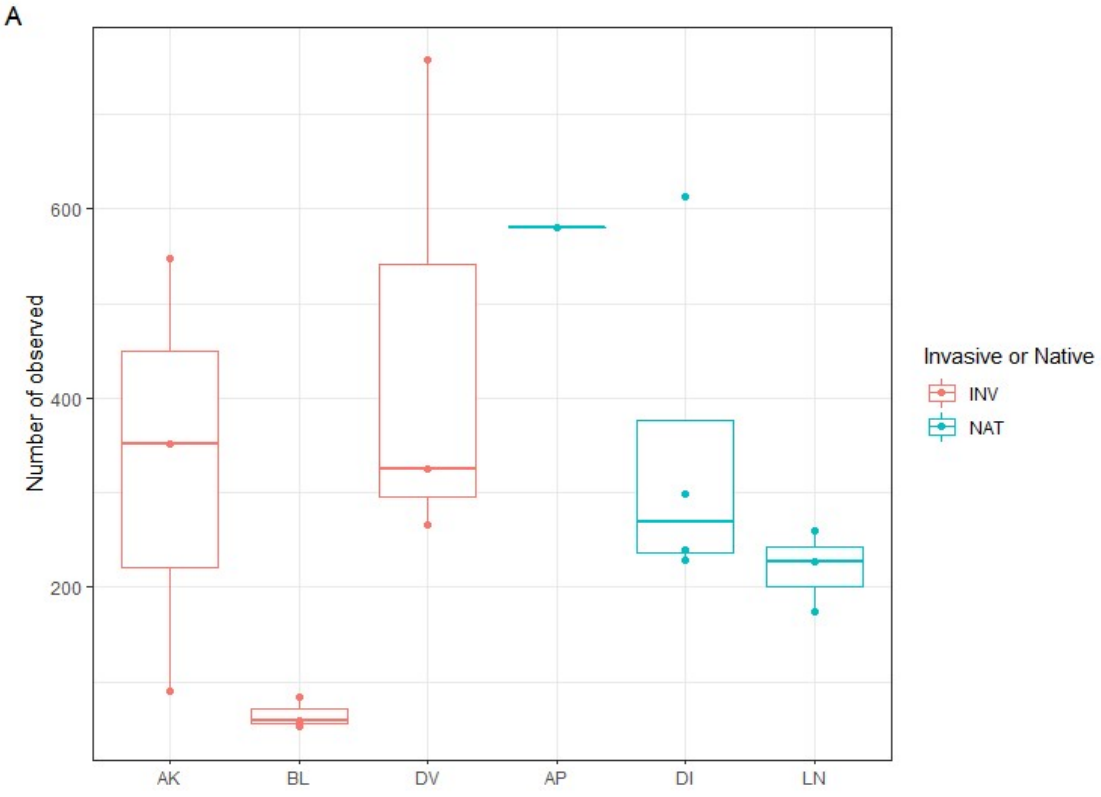


Figure 3.7: Averaged phylum-level composition of seawater, zooid, and tunic samples. Single-species bars (e.g., AK) contain both monospecific and interacting samples, and two-species bars (e.g., AK-AP) contain IB sample

3.4 Do Monospecific Specimens of Ascidians Contain Similar Organisms?

3.4.1 Community Richness

After rarifiaction (to 1,000 reads), alpha diversity of monospecific specimens was compared using observed richness and the Shannon index (Figure 3.8) with the exception of AP due to very low numbers of reads. The BL microbiome had potentially lower alpha diversity than all other species, which had similar levels of diversity (between 200 to 400 ASVs). Statistical tests showed no significant differences in observed richness and Shannon indices across all species (Kruskal-Wallis, p-values = 0.068 & 0.067, respectively) or between species, , including BL (Wilcoxon, p-values > 0.1).



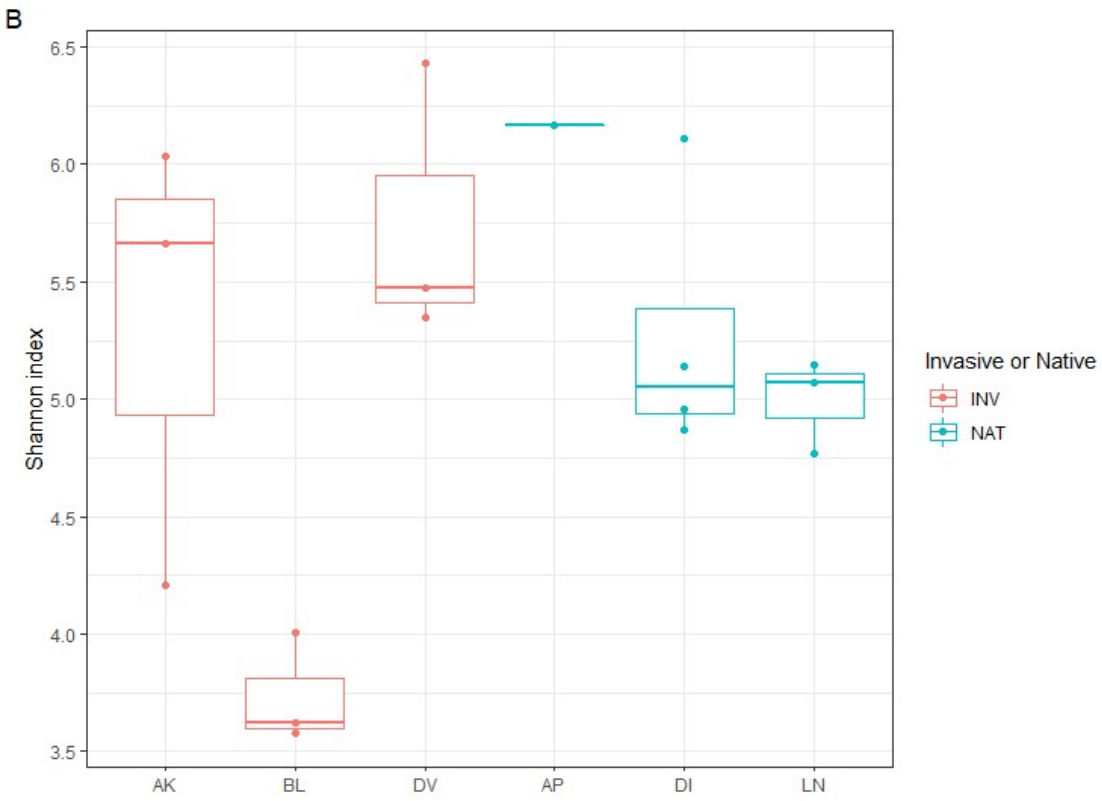


Figure 3.8: Alpha diversity of each examined species, by their monospecific form, using observed richness (A) and Shannon (B) index, calculated using rarefied data. The blue colour represents the native species while the red represents the invasive species.

3.4.2 Community Structure

A PCoA plot was used to visualise the dissimilarity among monospecific samples and showed that samples from the same species tend to cluster closely together (Figure 3.9). PERMANOVA test showed a highly significant difference among all samples (p-value = 0.002), and group dispersions test was not significant ("betadisper", p-value = 0.164); therefore, there is a significant difference between microbiomes of different species. When comparing between species pairs ("pairwise.adonis"), significant differences were observed between DV & DI (PERMANOVA, p-value = 0.034), BL & DI (PERMANOVA, p-value = 0.03), and AK & DI (PERMANOVA, p-value = 0.026).

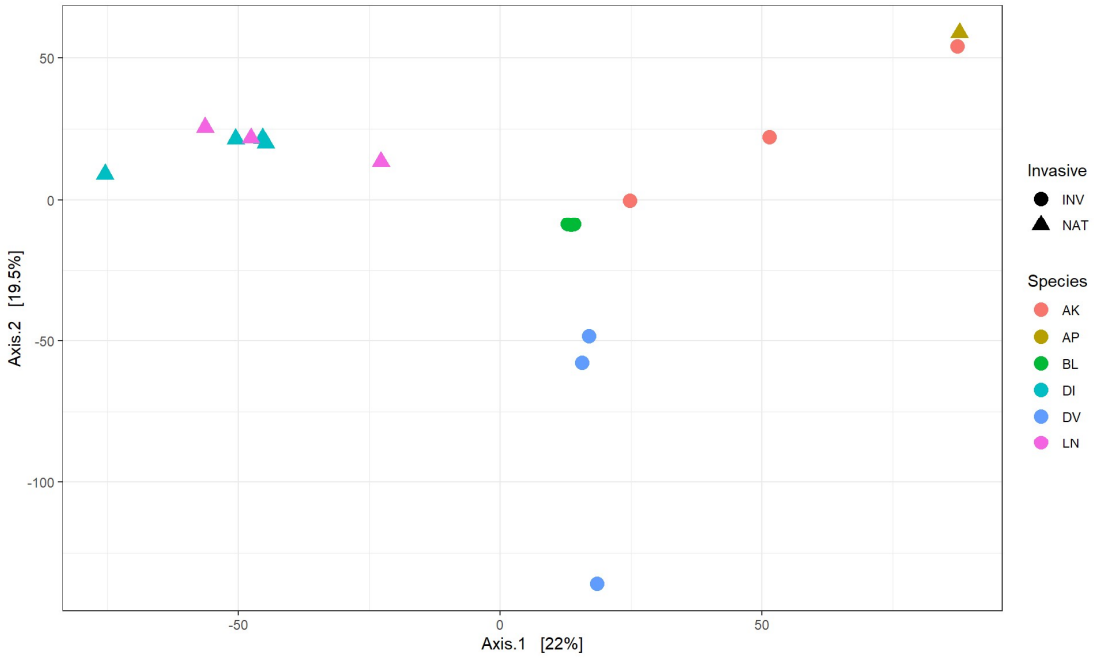


Figure 3.9: PCoA plot showing the dissimilarity between the microbiomes of monospecific specimens.

Table 3.3: P-values calculated in the PERMANOVA Adonis pairwise test, showing the differences between monospecific specimens.

Species	P-value
AK vs DV	0.1
AK vs BL	0.1
DV vs BL	0.1
AK vs AP	0.25
AK vs DI	**0.035**
AK vs LN	0.1
DV vs AP	0.25
DV vs DI	**0.033**
DV vs LN	0.1
BL vs AP	0.25
BL vs DI	**0.002**
BL vs LN	0.1
AP vs DI	0.2
AP vs LN	0.25
DI vs LN	0.535

3.4.3 Community Composition

A comparison of phylum-level composition for monospecific specimen microbiomes supported broad species-specific patterns (Figure 3.10). With the exception of BL, most monospecific microbiomes appeared to be dominated by *Proteobacteria*. The sole AP sample showed a similar microbial composition to the AK species with high abundance of *Bacteroidota* and *Cyanobacteria* phyla.

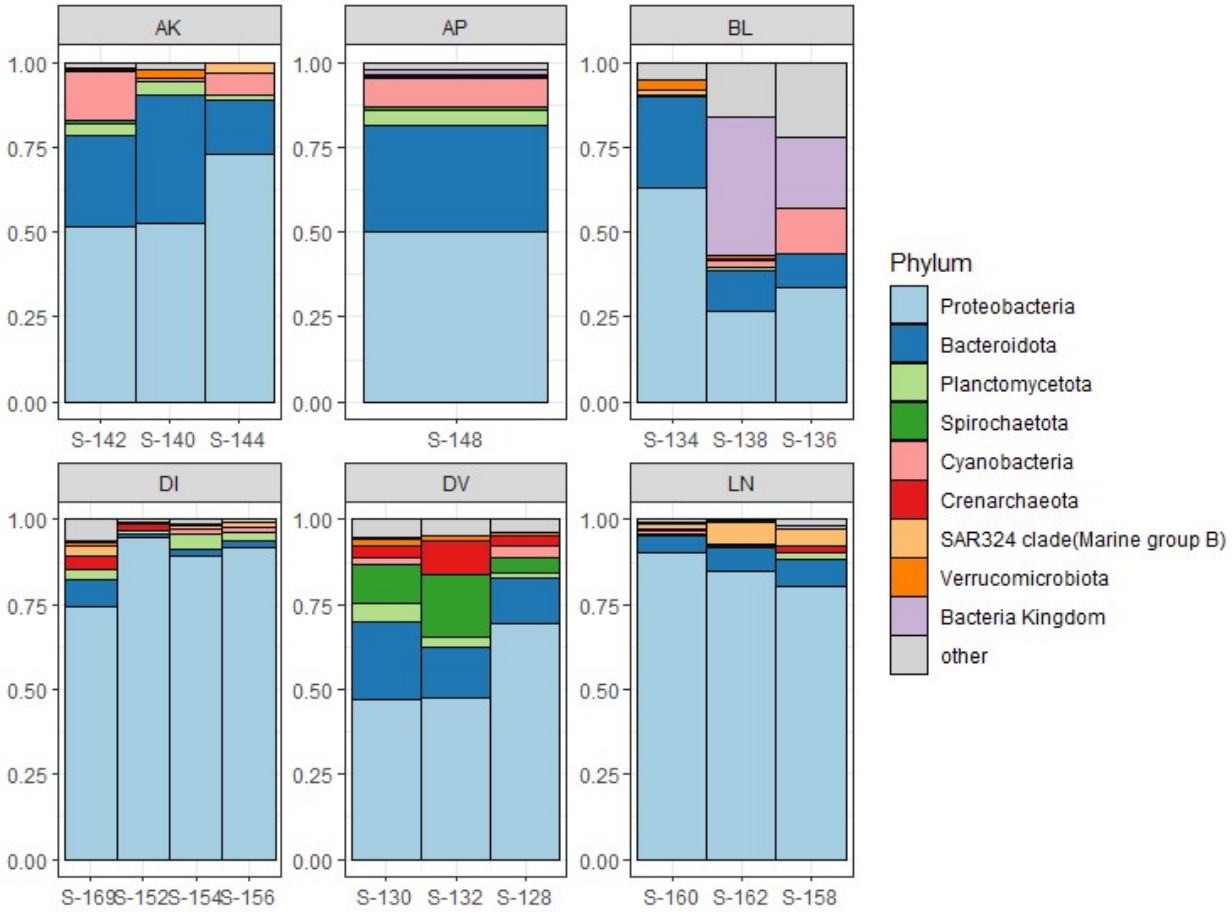
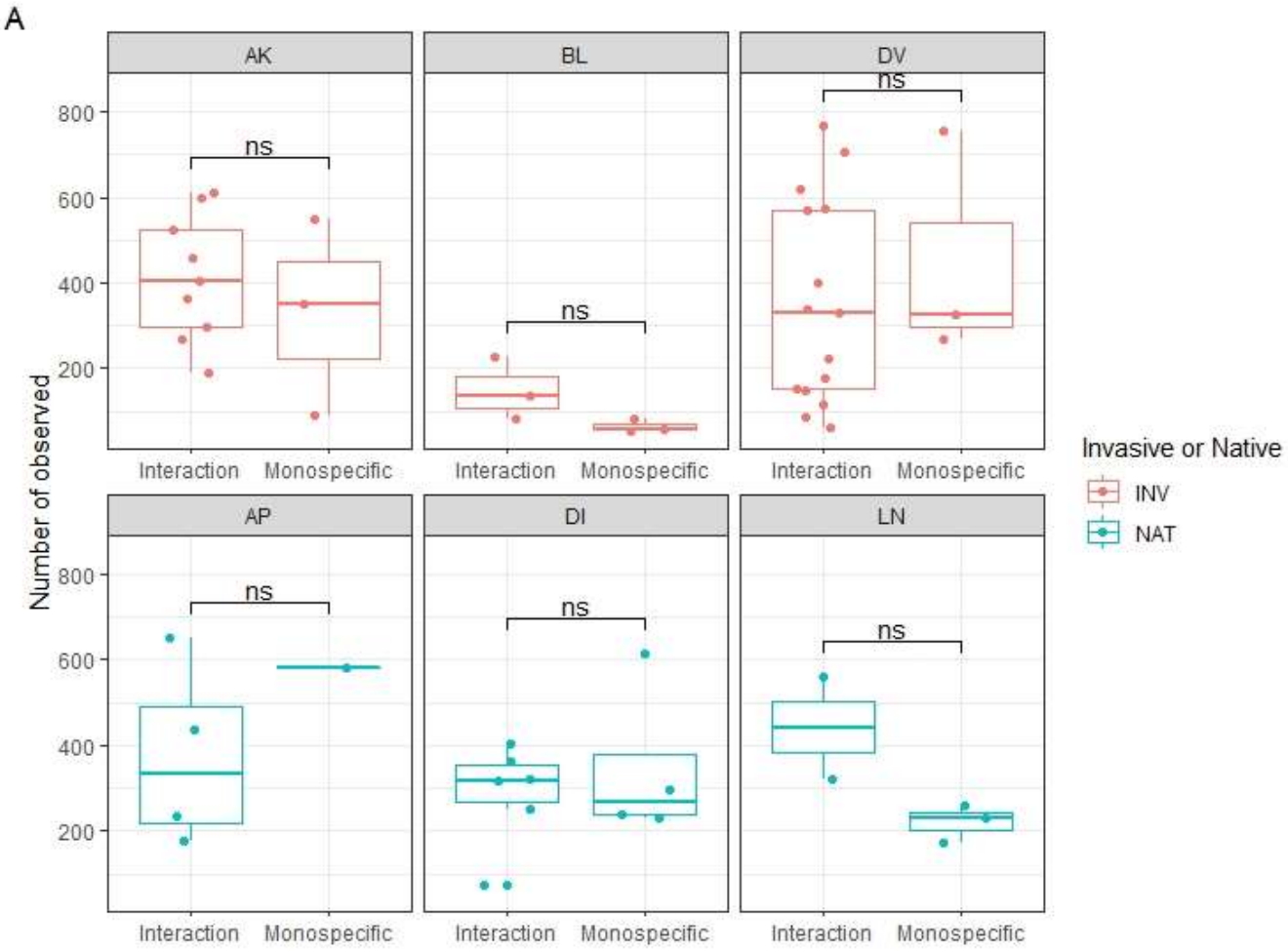


Figure 3.10: Microbial composition across all replicates of the monospecific samples of each species.

3.5 Do Interacting Ascidians Harbour a Different Microbiome to Their Monospecific Counterparts?

3.5.1 Community Richness

The alpha diversity for interacting and monospecific samples for all species (excluding IB samples) was examined using observed richness (Figure 3.11A) and Shannon index (figure 3.11B) (after rarifiaction to 1,000 reads). Both indices showed no significant difference between the monospecific and interacting samples for all species (Wilcoxon, p value > 0.2) (Figure 3.11).



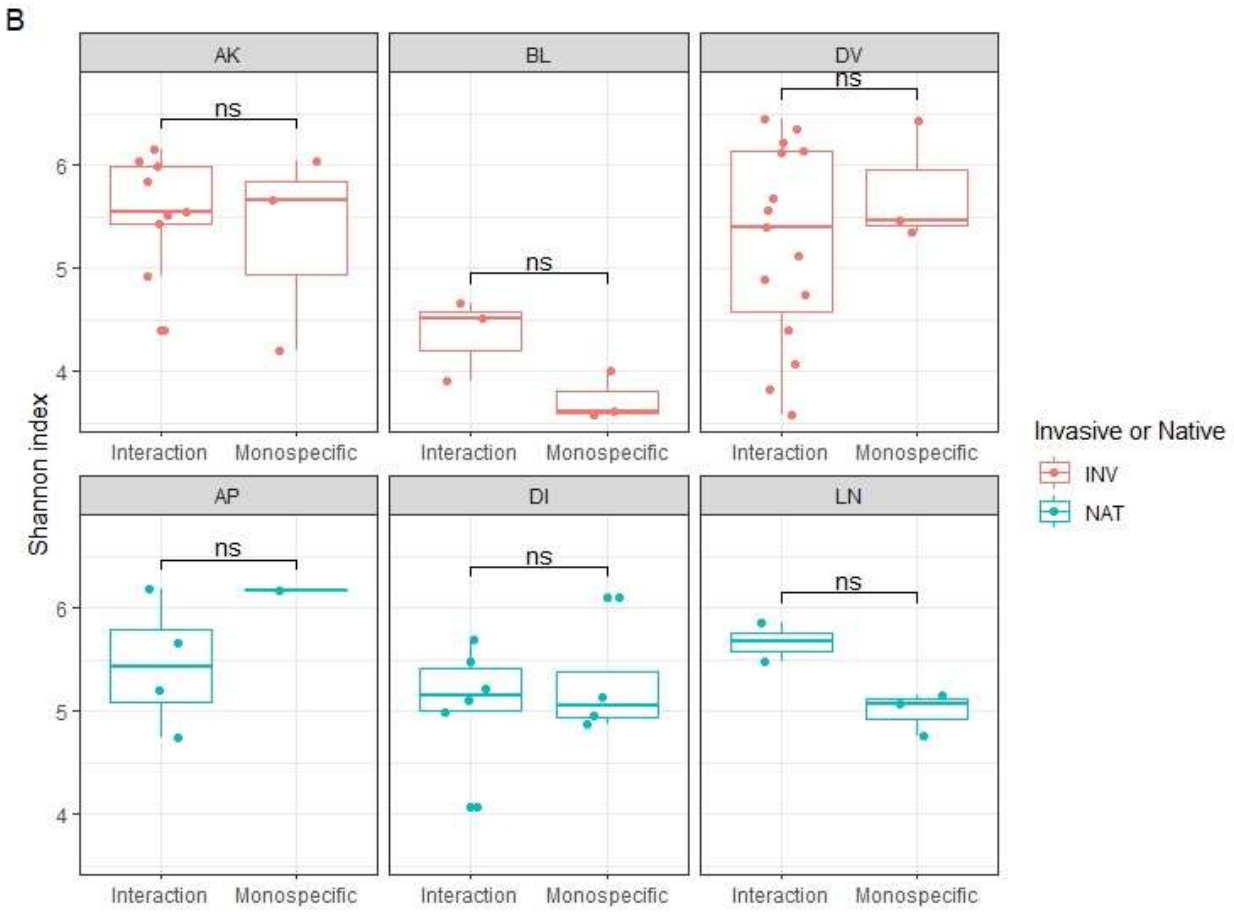


Figure 3.11: Alpha diversity per species, by their monospecific and interacting form (no IB samples were included), using observed richness (A) and Shannon (B) index.

3.5.2 Community Structure

The PCoA plot showed no obvious difference in microbial community structure between monospecific and interacting samples of the same species (Figure 3.12). Samples from the same species tend to cluster, with interacting and monospecific samples intermixed within the species cluster (Figure 3.12). PERMANOVA test showed no significant differences between interacting and monospecific samples of the same species (p-value = 0.374) but showed a highly significant difference between species (p-value = 0.001). Group dispersion test between species was not significant ("betadisper", p-value = 0.154); therefore, there is a significant difference between microbiomes of different species regardless of their interaction status.

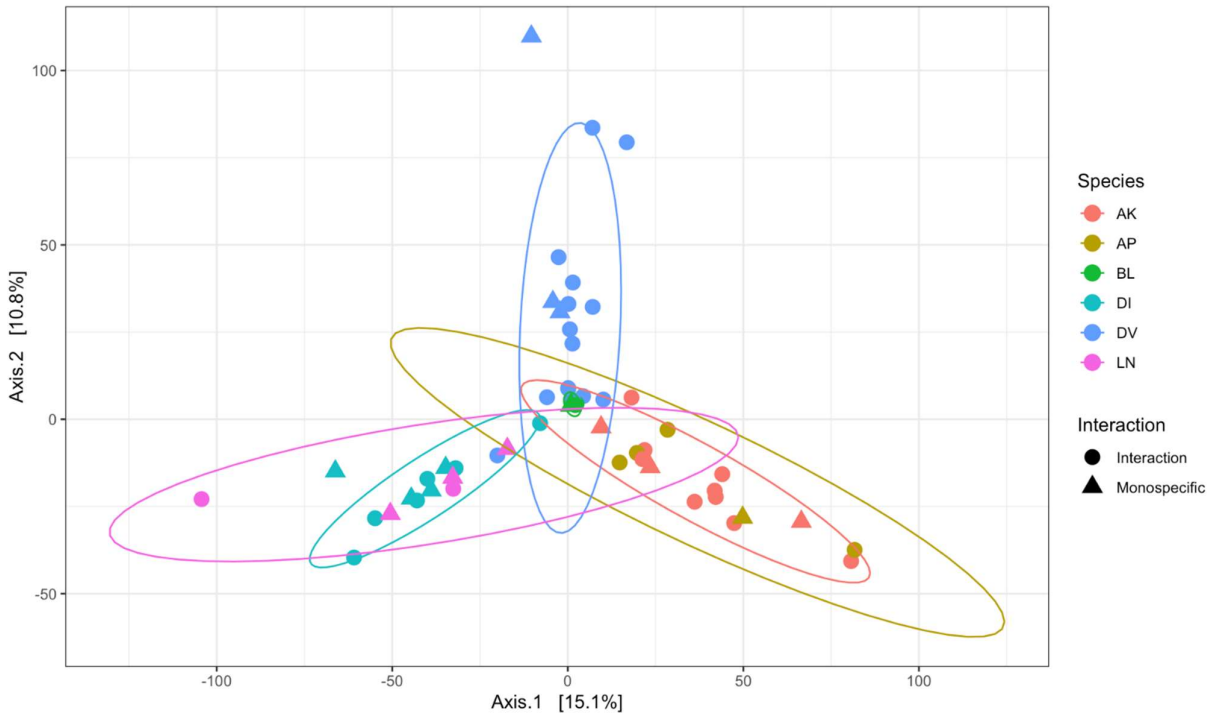


Figure 3.12: PCoA plot showing the similarity between monospecific and interaction samples sorted by species (no IB samples were included). The circles highlight the species clusters and the two intermixing ecological states (monospecific / interacting).

3.5.3 The Effect of Different Species Pairings on the Ascidian Microbiome

Comparisons based on alpha and beta diversity analyses were made between monospecific and different species pairing samples (excluding IB samples) for each ascidian species. The purpose is to explore the possibility that different species pairing may have divergent effects on the ascidian microbiome.

3.5.3.1 Effects of Different Species Pairings on the *Didemnum vexillum* Microbiome

The DV microbiome was examined using monospecific samples and DV samples from five different species pairings (Figure 3.13). The alpha diversity plot (Figure 3.13A) showed no significant difference between samples (Kruskal-Wallis, p-value = 0.57). The PCoA plot some clustering by sample types (Figure 3.13B). PERMANOVA test showed highly significant differences among sample type (PERMANOVA, p-value = 0.009, "betadisper", p-value = 0.95). However, when comparing between sample types using the

pairwise adonis test, no significant difference was observed (PERMANOVA, p-value >0.1).

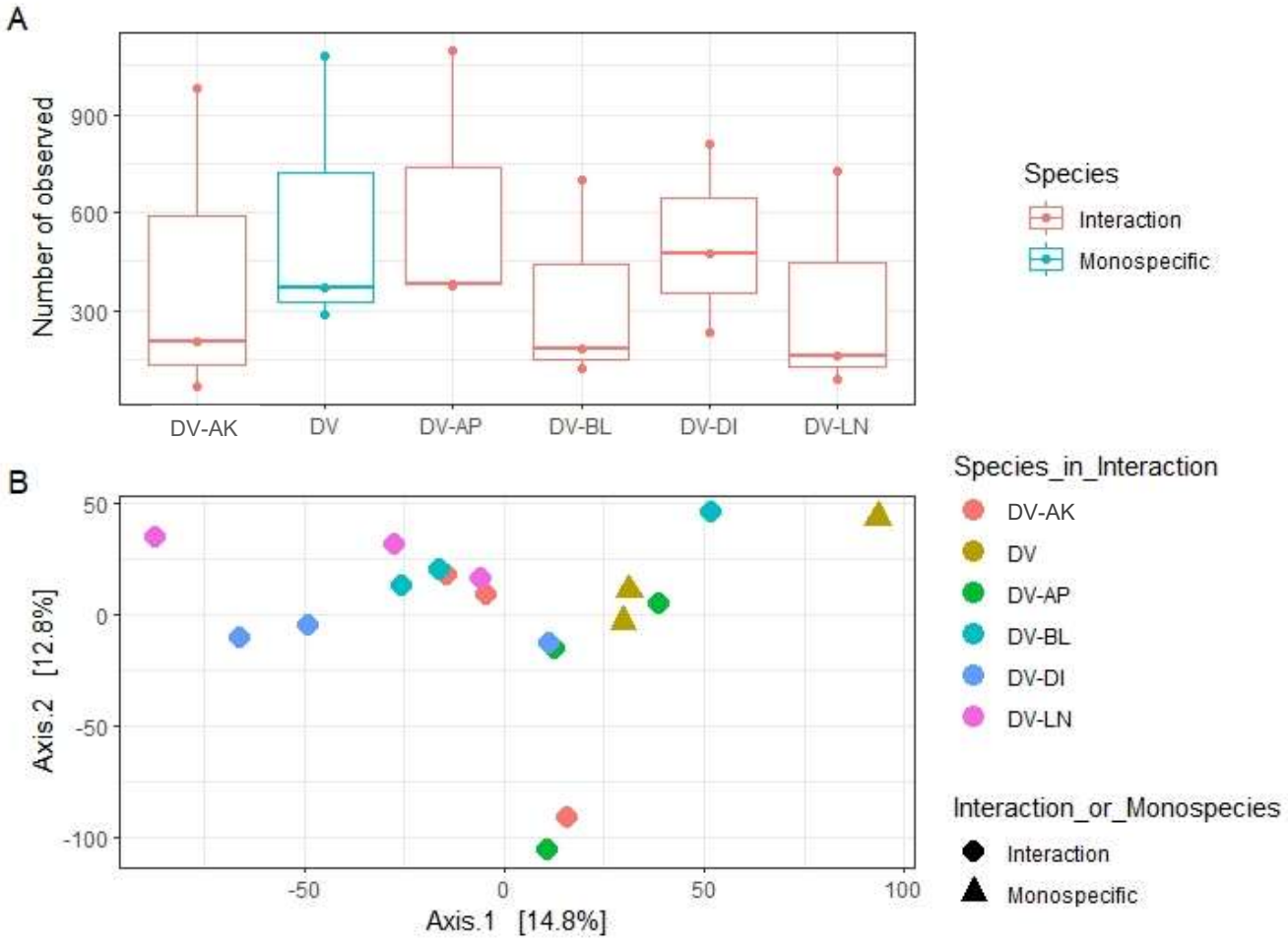


Figure 3.13: Alpha diversity using observed richness (A), and PCoA plot (B) showing the differences between DV monospecific samples and DV samples from five different species pairings.

3.5.3.2 Effects of Different Species Pairings on the *Aplidium kottae* Microbiome

The AK microbiome was examined using monospecific samples and AK samples from three different species pairings (Figure 3.14). The alpha diversity plot (Figure 3.14A) showed no significant difference between samples (Kruskal-Wallis, p-value = 0.89). The PCoA plot showed no difference among the sample types (PERMANOVA, p-value = 0.86) (Figure 3.14B).

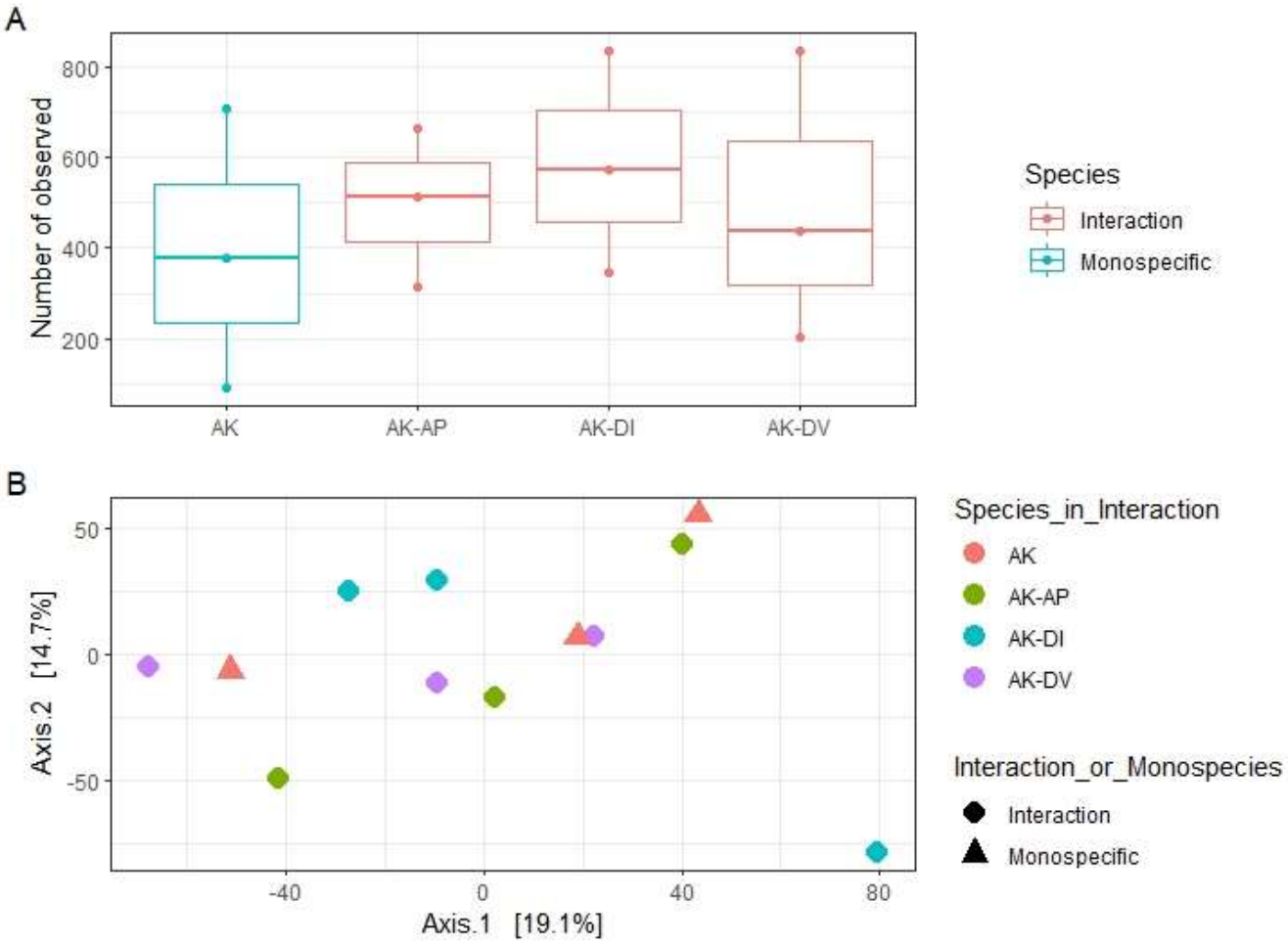


Figure 3.14: Alpha diversity using observed richness (A), and PCoA plot (B) showing the differences between AK monospecific samples and AK samples from three different species pairings.

3.5.3.3 Effects of Interaction with Didemnum vexillum on the *Botrylloides leachi* Microbiome

The BL microbiome was examined using monospecific samples and BL samples from the sole interaction specimen (with DV) (Figure 3.15). The alpha diversity plot (Figure 3.15A) showed no significant difference between sample types (Kruskal-Wallis, p-value = 0.13). The PCoA plot showed clear clustering by sample type (Figure 3.15B), but this was not statistically significant (PERMANOVA, p-value = 0.1).

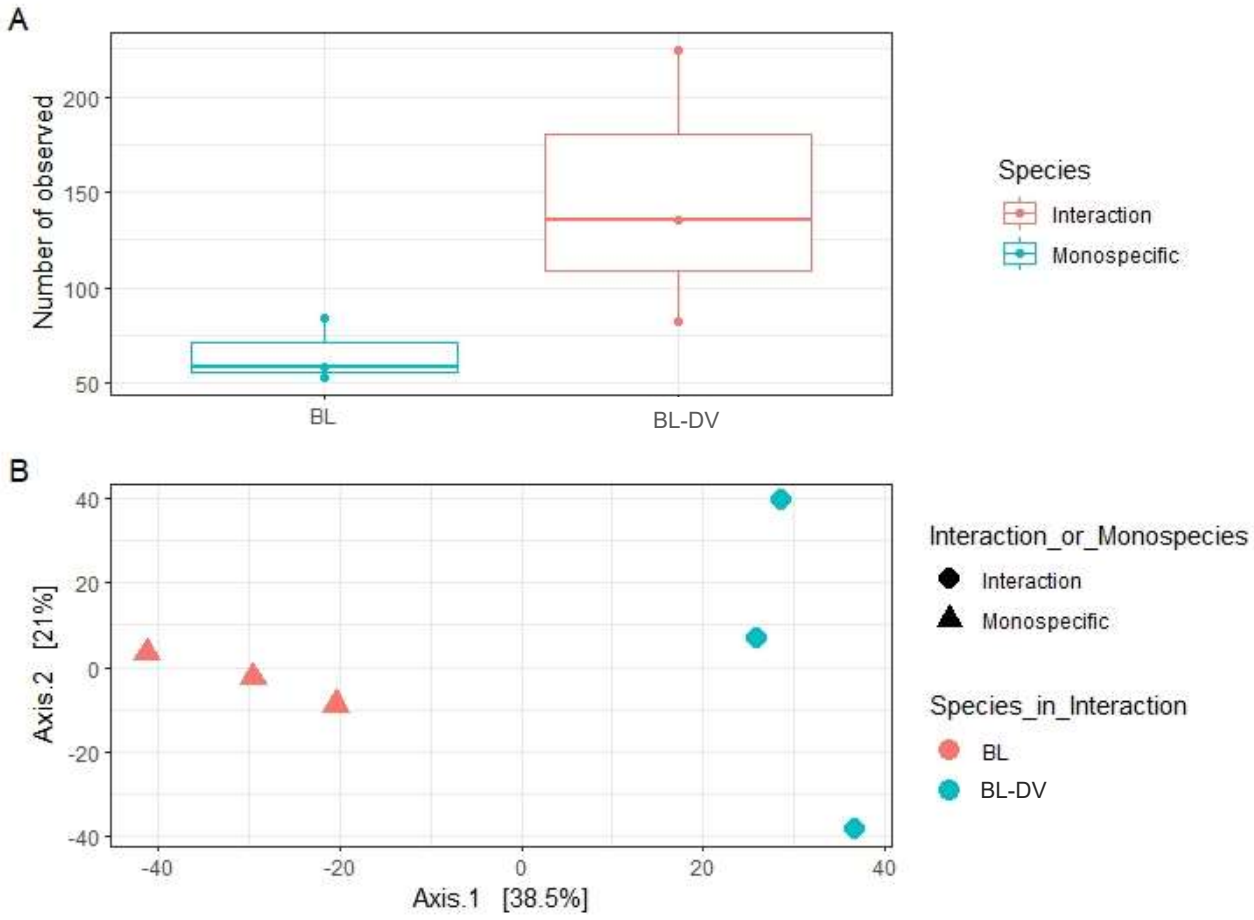


Figure 3.15: Alpha diversity using observed richness (A), and PCoA plot (B) showing the differences between BL monospecific samples and BL samples from BL-DV species pairing.

3.5.3.4 Effects of Different Species Pairings on the *Didemnum incanum* Microbiome

The DI microbiome was examined using monospecific samples and DI samples from two different species pairings (Figure 3.16). The alpha diversity plot (Figure 3.16A) showed no significant difference between samples (Kruskal-Wallis, p-value = 0.58). The PCoA plot showed significant differences in microbial structure (PERMANOVA p-value = 0.02, "betadisper" p-value = 0.87), as the DV-DI samples cluster together and AK-DI and DI intermixed together (Figure 3.16B). However, when comparing between sample types ("pairwise.adonis"), no significant difference was observed (PERMANOVA, p-value ranging from 0.07 to 0.3).

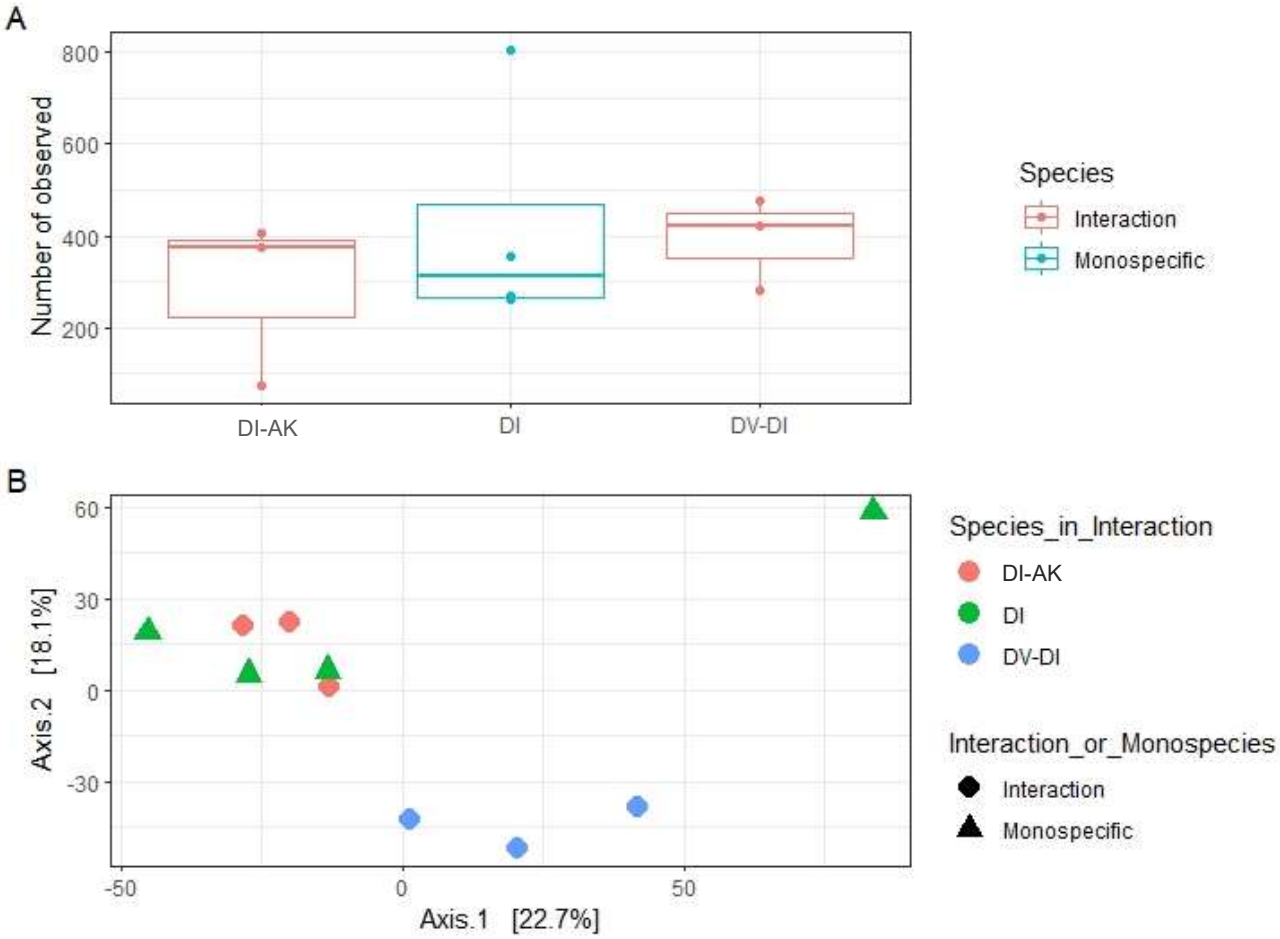


Figure 3.16: Alpha diversity using observed richness (A), and PCoA plot (B) showing the differences between DI monospecific samples and DI samples from two different species pairings.

3.5.3.5 Effects of Interaction with *Didemnum vexillum* on the *Lissoclinum notti* Microbiome

The LN microbiome was examined using monospecific samples and LN samples from the sole interaction specimen (with DV) (Figure 3.17). The alpha diversity plot (Figure 3.17A) showed no significant difference between sample types (Kruskal-Wallis, p-value = 0.083). The PCoA plot showed potential clustering by sample type (Figure 3.17B), but this was not statistically significant (PERMANOVA, p-value = 0.1).

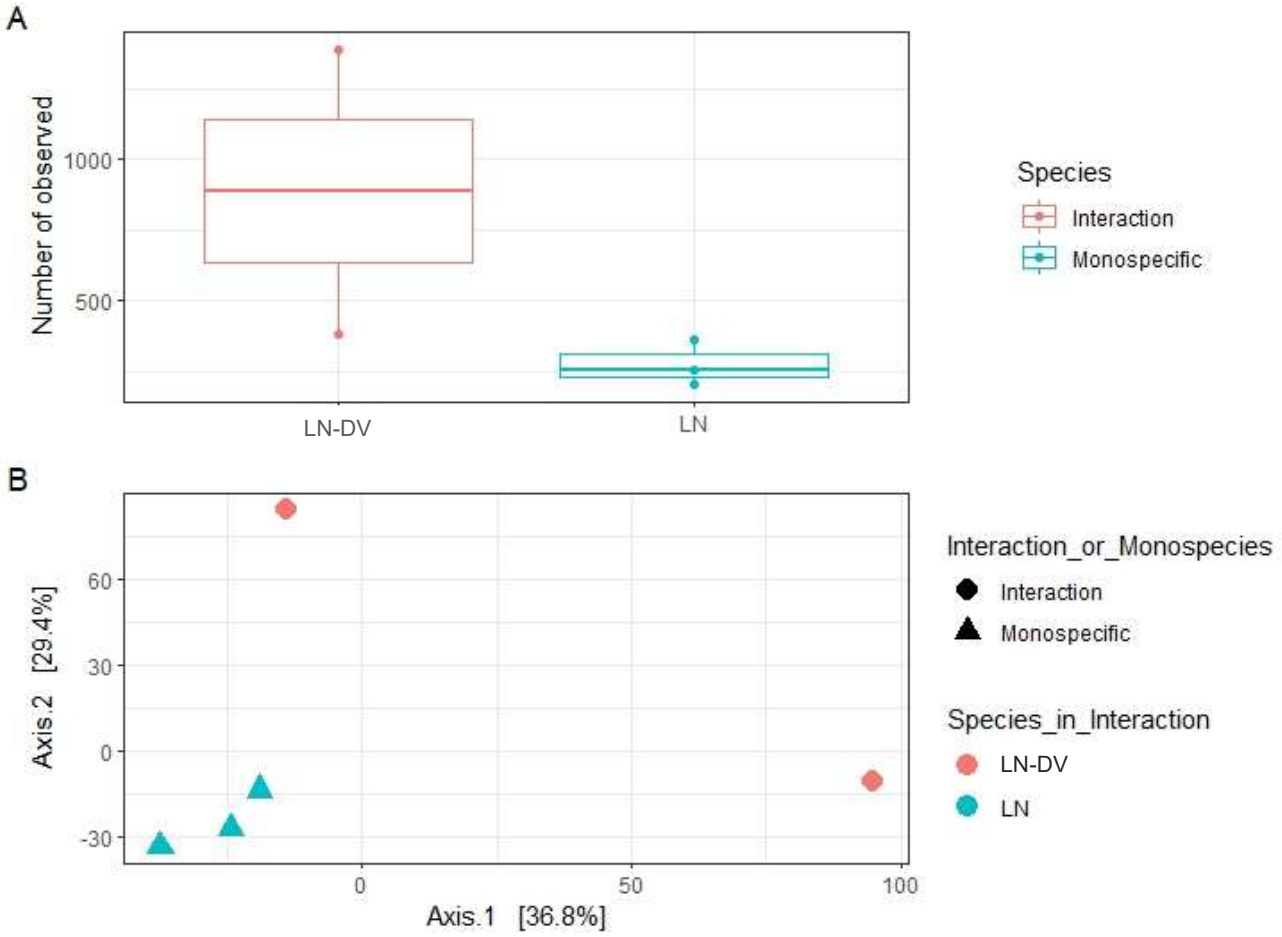


Figure 3.17: Alpha diversity using observed richness (A), and PCoA plot (B) showing the differences between LN monospecific samples and LN samples from LN-DV species pairing.

3.6 Microbial Communities Across the Interaction

To examine whether interactive border (IB) samples are distinct from the interacting species in terms of their microbiome, a PCoA plot and phylum-level taxonomic compositions were generated for each sampled interaction.

3.6.1 All Interactions that Included *Didemnum vexillum* Species

DV was involved with five species-pairing specimen types (Table 3.1, Figure 3.1); therefore, this section presents all the specimens that contained DV.

3.6.1.1 The *Aplidium kottae - Didemnum vexillum* Interaction

The PCoA plot showed an obvious difference in the microbial community structures of samples within the interaction specimens (AK, DV, and IB[AK-DV]) (PERMANOVA, p-value = 0.008, "betadisper", p-value = 0.7), as AK tend to cluster slightly closer to IB[AK-DV] samples (Figure 3.18 A). However, pairwise adonis statistical test shows no significant differences between samples AK & IB[AK-DV] (PERMANOVA, p-value = 0.2), DV & IB[AK-DV] (PERMANOVA, p-value = 0.1), and AK & DV (PERMANOVA, p-value = 0.1). Phylum-level compositions suggest that samples were mainly composed of *Proteobacteria*, with AK and IB[AK-DV] samples exhibited higher relative abundances of the *Bacteroidota* phylum (Figure 3.18 B).

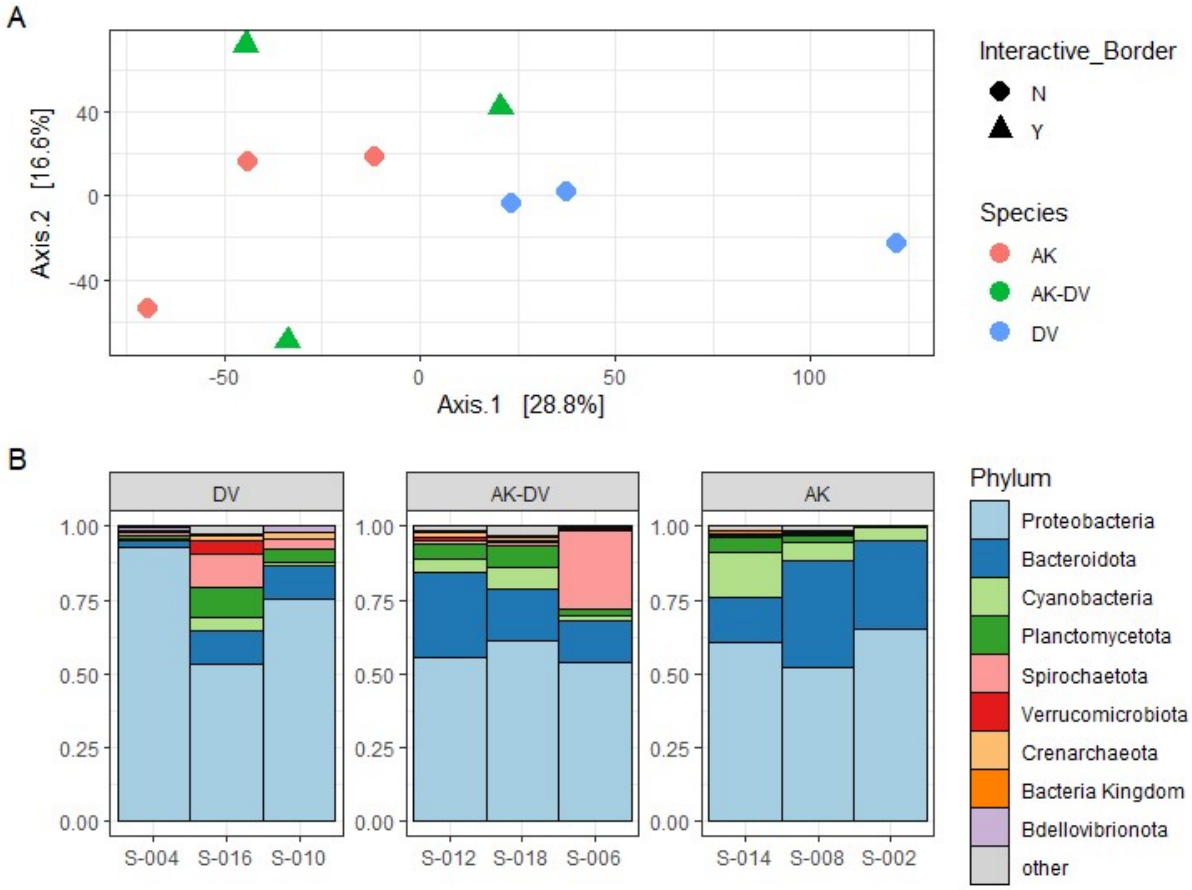


Figure 3.18: PCoA plot (A) and phylum-level microbial taxonomic composition (B), of all samples within the AK-DV pairing interaction (DV, AK, and IB [AK-DV]). Sample groups [S-002, S-004, S-006], [S-008, S-010, S-012], [S-014, S-016, S-018] are from the same specimens.

3.6.1.2 The *Didemnum vexillum - Aplidium phor*tax Interaction

The PCoA plot showed a difference in the microbial community structure of samples within the interaction specimens (DV, AP, and IB[DV-AP]) (PERMANOVA, p-value = 0.008, "betadisper", p-value = 0.79), with AP and IB[DV-AP]) intermixing (Figure 3.19 A). However, pairwise adonis statistical test shows no significant differences between samples AP & IB[DV-AP] (PERMANOVA, p-value = 0.8), DV & IB[DV-AP] (PERMANOVA, p-value = 0.1), and AP & DV (PERMANOVA, p-value = 0.1). Phylum-level compositions suggest that samples were mainly composed of *Proteobacteria*, with AP and IB[DV-AP] samples exhibited higher relative abundance of the *Bacteroidota* phylum (Figure 3.19 B).

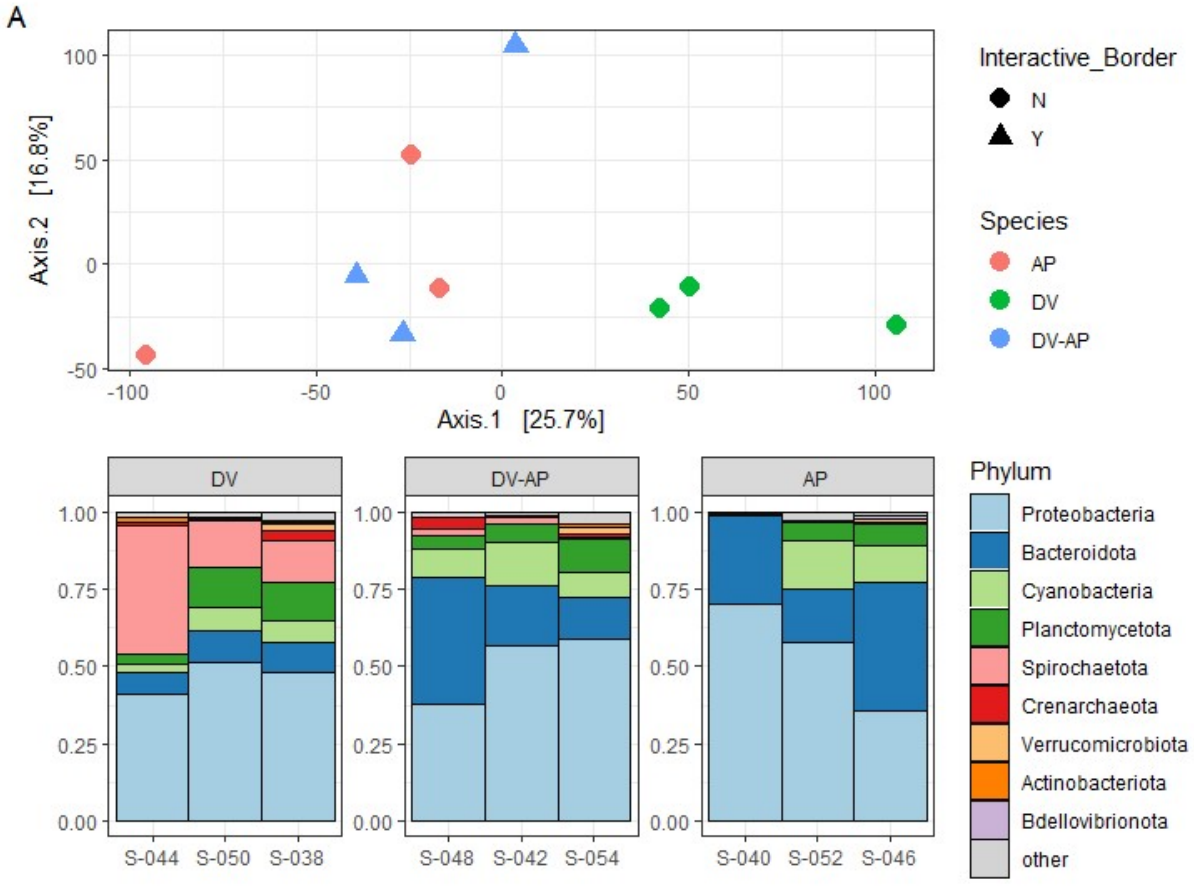


Figure 3.19: PCoA plot (A) and phylum-level microbial taxonomic composition (B), of all samples within the DV-AP pairing interaction (DV, AP, and IB [DV-AP]). Sample groups [S-038, S-040, S-042], [S-044, S-046, S-048], [S-050, S-052, S-054] are from the same specimens.

3.6.1.3 The *Didemnum vexillum - Didemnum incanum* Interaction

The PCoA plot showed a difference in the microbial community structure of samples within the interaction specimens (DV, DI, and IB[DV-DI]) (PERMANOVA, p-value = 0.031, "betadisper", p-value = 0.32), as DI and IB[DV-DI] samples cluster together (Figure 3.20 A). However, pairwise adonis statistical test shows no significant differences between samples DI & IB[DV-DI] (PERMANOVA, p-value = 0.6), DV & IB[DV-DI] (PERMANOVA, p-value = 0.1), and DV & DI (PERMANOVA, p-value = 0.1). Phylum-level compositions suggest that samples were mainly composed of *Proteobacteria*, with DV samples showed slightly lower relative abundance of *Proteobacteria* and higher relative abundance of *Planctomycetota* and *Cyanobacteria* (Figure 3.20 B).

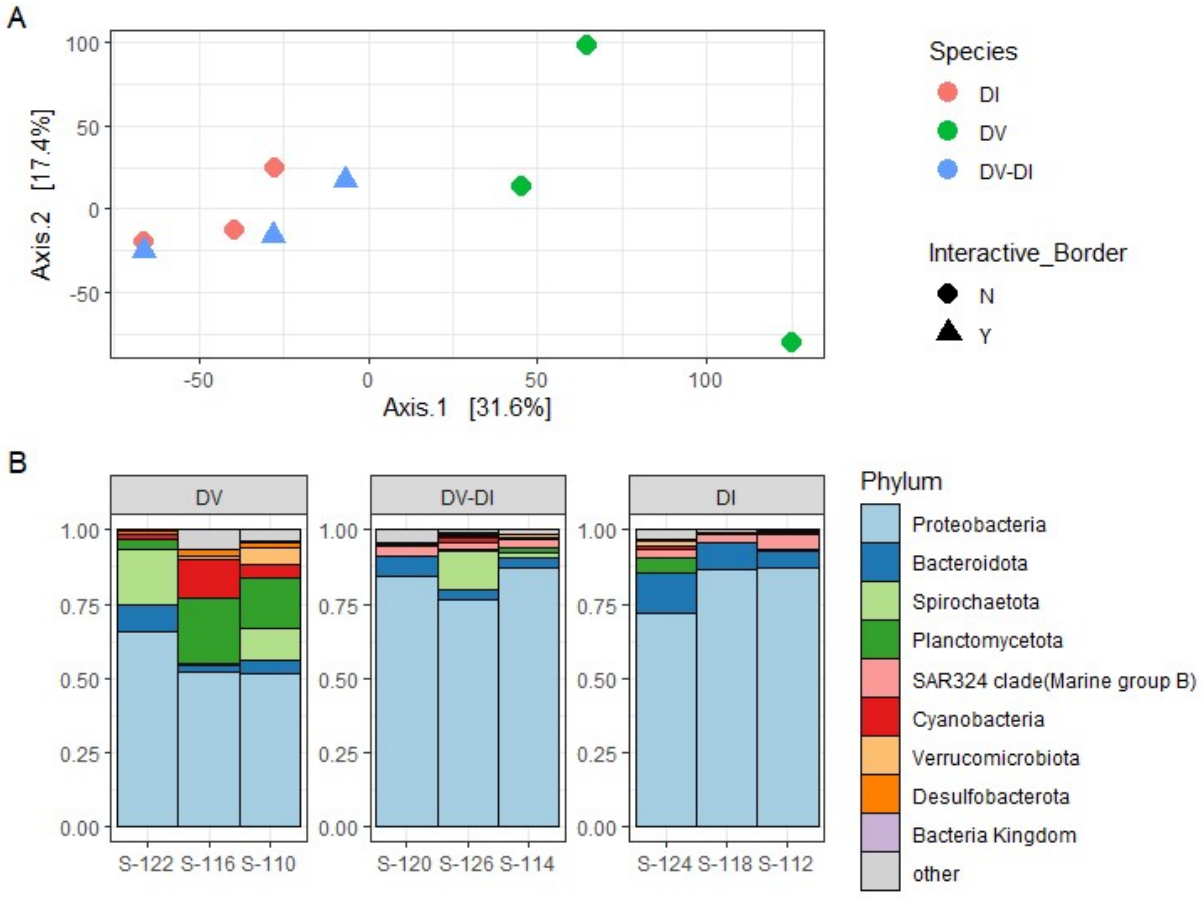


Figure 3.20: PCoA plot (A) and phylum-level microbial taxonomic composition (B), of all samples within the DV-DI pairing interaction (DV, DI, and IB [DV-DI]). Sample groups [S-110, S-112, S-114], [S-116, S-118, S-120], [S-122, S-124, S-126] are from the same specimens.

3.6.1.4 The *Didemnum vexillum - Lissoclinum notti* Interaction

The PCoA plot showed no significant difference in the microbial community structure of samples within the interaction specimens (DV, LN, and IB[DV-LN]) (PERMANOVA, p-value = 0.3) (Figure 3.21 A). Phylum-level compositions suggest that samples were mainly composed of *Proteobacteria* (Figure 3.21 B). Due to lower number of sample replicates these trends could not be fully determined (Figure 3.21).

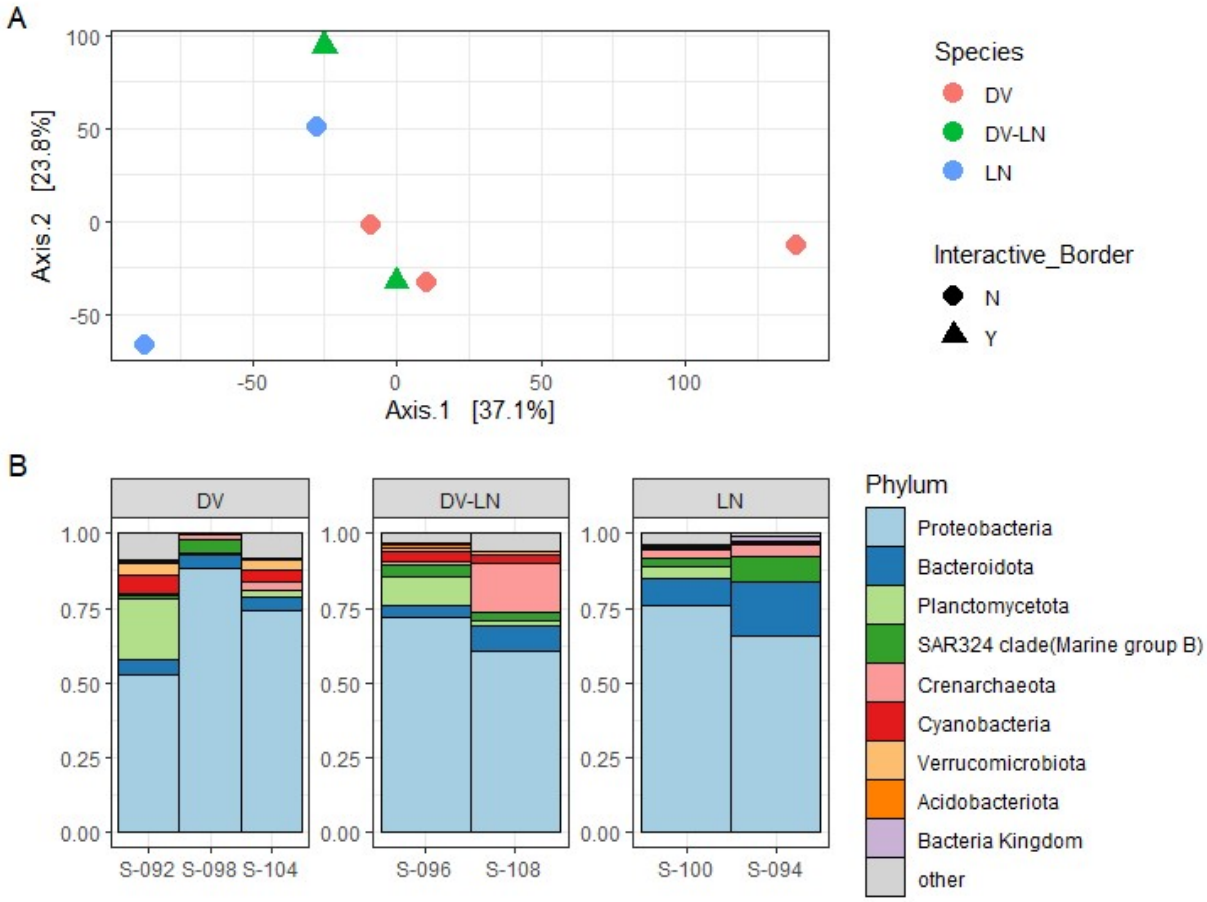


Figure 3.21 : PCoA plot (A) and phylum-level microbial taxonomic composition (B), of all samples within the DV-LN pairing interaction (DV, LN, and IB [DV-LN]). Sample groups [S-092, S-094, S-096], [S-098, S-100], [S-104, S-108] are from the same specimens.

3.6.1.5 The *Didemnum vexillum - Botrylloides leachi* Interaction

The PCoA plot showed no difference in the microbial community structure of samples within the interaction specimens (DV, BL, and IB[DV-BL]) (PERMANOVA, p-value = 0.12) (Figure 3.22 A). Phylum-level compositions suggest that samples were mainly composed of *Proteobacteria*, with DV exhibited slightly lower relative abundance of

Proteobacteria and higher relative abundance of *Planctomycetota* and *Cyanobacteria* (Figure 3.22 B).

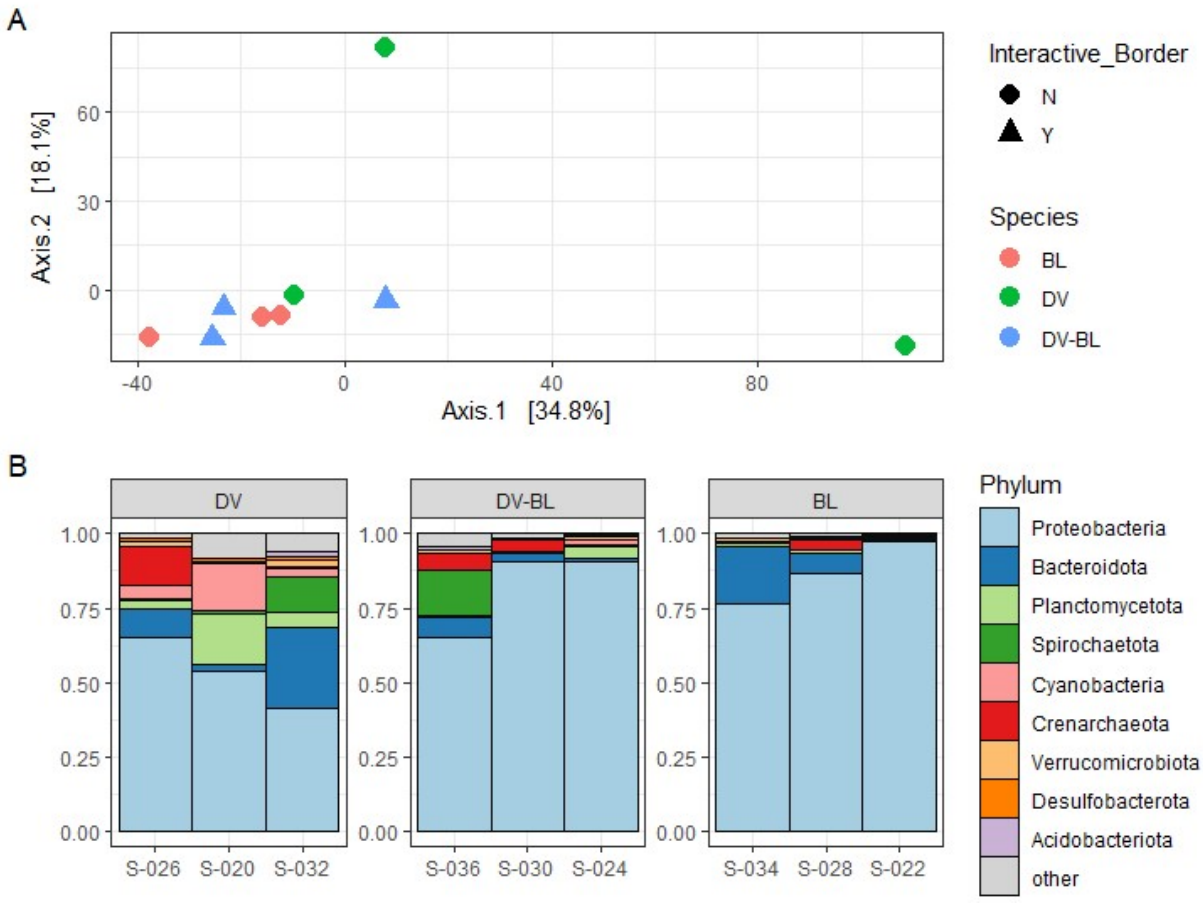


Figure 3.22: PCoA plot (A) and phylum-level microbial taxonomic composition (B), of all samples within the DV-BL pairing interaction (DV, BL, and IB [DV-BL]). Sample groups [S-020, S-022, S-024], [S-026, S-028, S-030], [S-032, S-034, S-036] are from the same specimens.

3.6.2 All Interactions that Included *Aplidium kottae*

Excluding the DV-AK species pairing, which was discussed above, AK was involved in interactions with two other species pairings (Table 3.1, Figure 3.1).

3.6.2.1 The *Aplidium kottae- Aplidium phortax* Interaction

The PCoA plot showed no difference in the microbial community structure of samples within the interaction specimens (AK, AP, and IB[AK-AP]) (PERMANOVA, p-value =

0.9) (Figure 3.23 A). Phylum-level compositions suggest that samples were mainly composed of *Proteobacteria*, and *Bacteroidota* to follow (Figure 3.23 B). However, due to the low number of sample replicates, these trends cannot be interpreted with confidence (Figure 3.23).

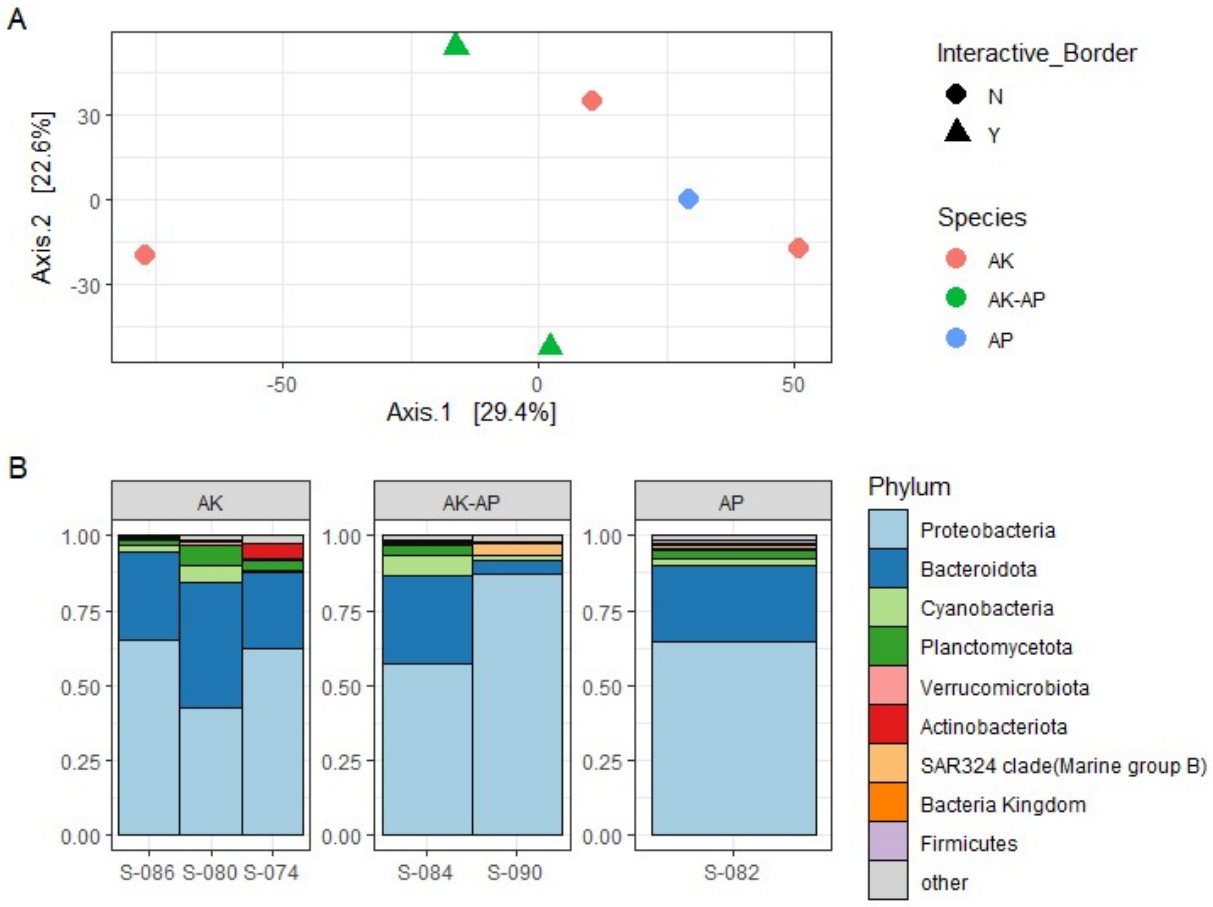


Figure 3.23: PCoA plot (A) and phylum-level microbial taxonomic composition (B), of all samples within the AK-AP pairing interaction (AK, AP, and IB [AK-AP]). Sample groups [S-074], [S-080, S-082, S-084], [S-086, S-090] are from the same specimens.

3.6.2.2 The *Aplidium kottae - Didemnum incanum* Interaction

The PCoA plot showed a difference in the microbial community structure of samples within the interaction specimens (AK, DI, and IB[AK-DI]) (PERMANOVA, p-value = 0.026, "betadisper", p-value = 0.075), as DI slightly intermixed with IB[AK-DI] samples (Figure 3.24A). However, pairwise adonis statistical test shows no significant differences between samples DI & IB[AK-DI] (PERMANOVA, p-value = 0.3), AK & IB[AK-DI] (PERMANOVA, p-value = 0.1), and AK & DI (PERMANOVA, p-value = 0.1). Phylum-level composition plot suggest that samples were mainly composed of *Proteobacteria*,

while AK and IB[AK-DI] samples possess a greater relative abundance of *Bacteroidota* and *Cyanobacteria* (Figure 3.24B).

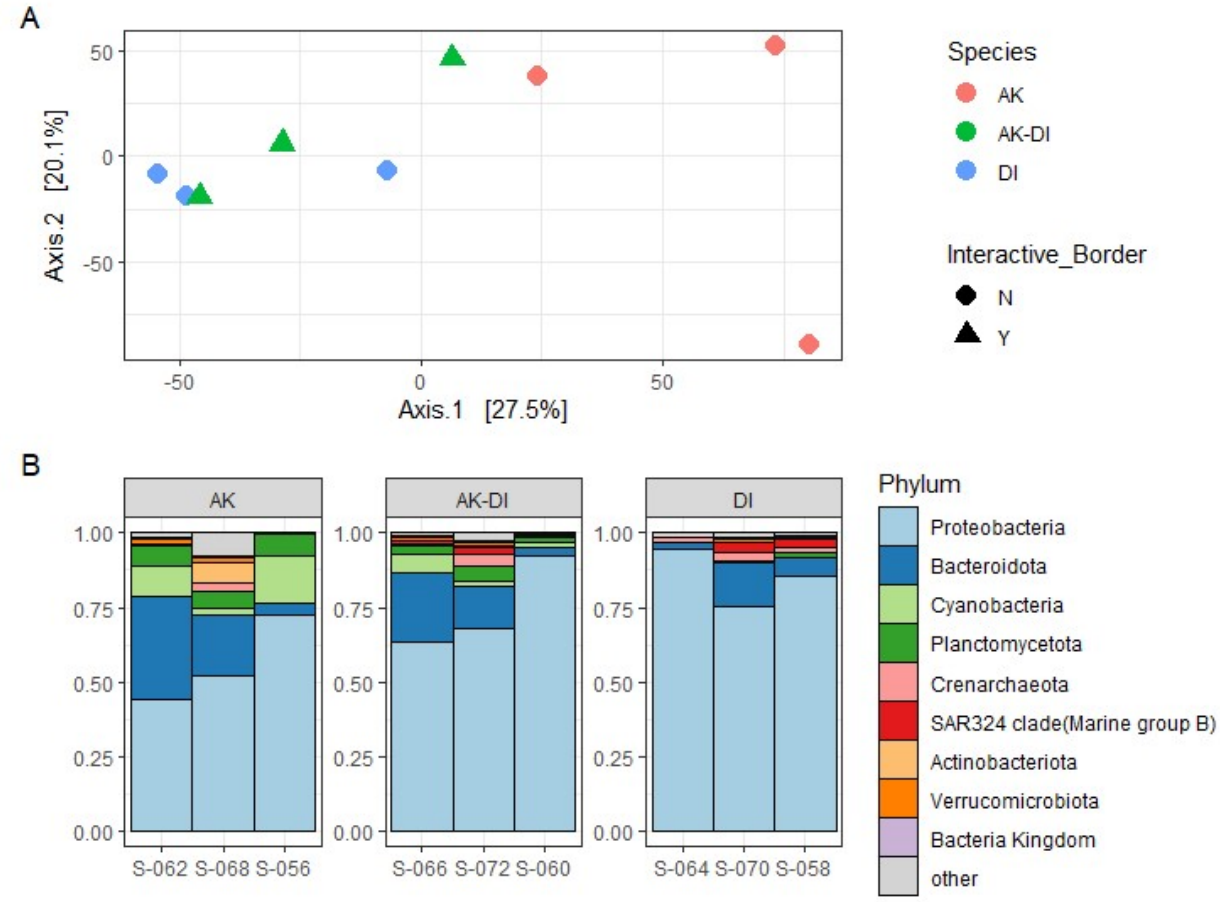


Figure 3.24: PCoA plot (A) and phylum-level microbial taxonomic composition (B), of all samples within the AK-DI pairing interaction (AK, DI, and IB [AK-DI]). Sample groups [S-056, S-058, S-060], [S-062, S-064, S-066], [S-068, S-070, S-072] are from the same specimens.

3.7 Microbiomes of Native Versus Invasive Colonial Ascidians.

3.7.1 Community Structure

Since no significant difference was found between the microbiomes of monospecific and interaction specimens (Figure 3.13), the following analysis was conducted using both specimen types (excluding IB samples) to increase statistical power. PCoA plot showed a significant difference in the microbial community structure between native and invasive ascidian species (PERMANOVA p-value = 0.001, "betadisper", p-value = 0.39) (Figure 3.25). An apparent clustering is observed in the bottom half of the graph between native species DI and LN, and some clustering in the upper half of the graph between invasive species DV and BL. However, in the right half of the graph, the invasive species

AK intermix with the native species AP (Figure 3.25). Nonetheless, when examining the plot by species, a significant difference was observed as well (PERMANOVA, p-value = 0.001) (Figure 3.25), suggesting that the apparent clustering could also be associated with species-specific microbiome regardless of the ascidian's ecological status (i.e., invasive or native). However, group dispersion test for species groups showed significant difference ("betadisper", p-value = 0.019), implying that this pattern might associated with the native or invasive ecological status of the species. When comparing between pairs using the pairwise adonis statistical test, no significant difference was observed between the invasives DV and BL (PERMANOVA, p-value = 0.78), the natives DI and LN (PERMANOVA, p-value = 1.0), the native AP and the invasive AK (PERMANOVA, p-value = 1.0) and the natives AP and LN (PERMANOVA, p-value = 0.12), while all the pairs are significantly different (Table 3.4).

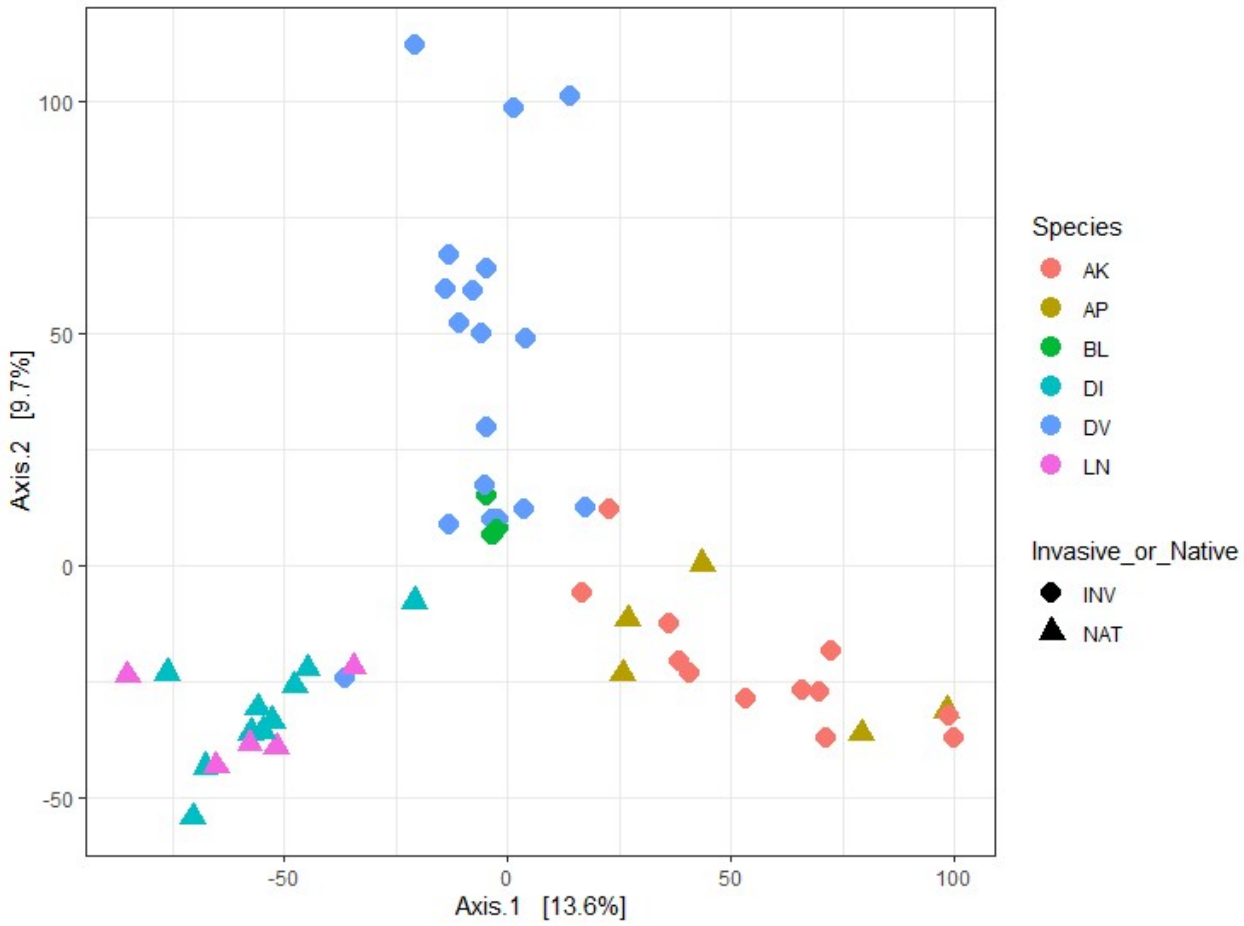


Figure 3.25: PCoA plot showing the dissimilarity between native and invasive species (including interacting and monospecific samples but no IB samples).

Table 3.4: P-values calculated in the PERMANOVA Adonis pairwise test, showing the differences between the native and invasive species (no IB samples). The blue colour represents the natives, whereas the red colour represents the invasives.

Species	P-value
AK vs DV	0.015
AK vs BL	0.030
DV vs BL	**0.780**
AK vs AP	**1.000**
AK vs DI	0.015
AK vs LN	0.015
DV vs AP	0.015
DV vs DI	0.015
DV vs LN	0.015
BL vs AP	0.045
BL vs DI	0.015
BL vs LN	0.045
AP vs DI	0.015
AP vs LN	**0.120**
DI vs LN	**1.000**

3.8 Collection Site Influence

PCoA plot was used to check the possible influence of collection site on samples containing DV (excluding the IB samples) (Figure 3.26), since it is the only species that was collected in all three sampling sites: Bridge (1802); Pier A (1503) and Mount (2503) (Table 3.1). The plot showed some clustering by sites; however, in the middle of the graph, many data points intermix with one another regardless of collection site. PERMANOVA test showed significant differences between collection sites (PERMANOVA, p-value = 0.003, "betadisper", p-value = 0.47). When comparing between the locations using the pairwise adonis statistical test, the Mount was significantly different from Pier A (PERMANOVA, p-value = 0.003) and Bridge (PERMANOVA, p-value = 0.03), while Pier A and Bridge were not significantly different (PERMANOVA, p-value = 0.066).

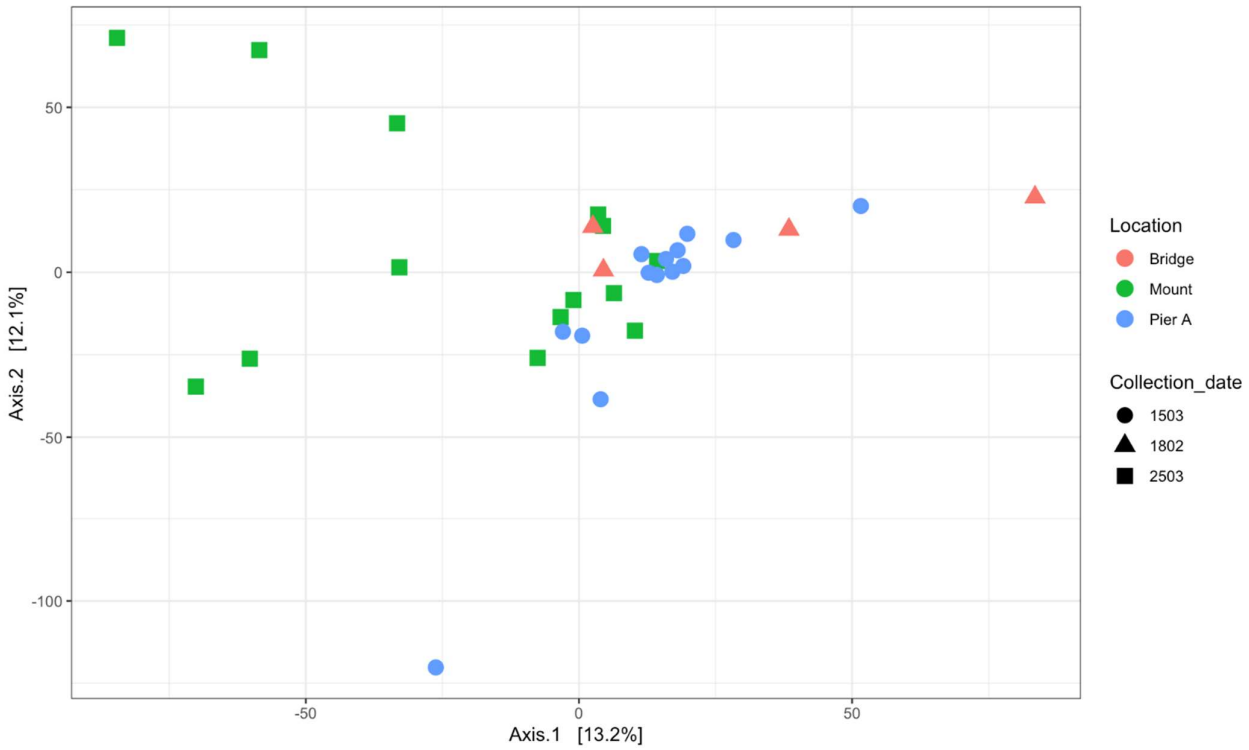


Figure 3.26: PCoA plot showing the similarity between collection sites and collection dates by samples containing DV species (excluding the IB samples).

Chapter 4

Discussion

4.1 Zooids and Tunic Harbour Similar Microbiomes, which Are Distinct from Seawater Microbial Community

Although the available literature hints at tissue-specific microbiomes in ascidians (Chen et al., 2017; Utermann et al., 2020), this assumption has never been tested on zooids - the feeding vessel of colonial ascidians (Chen et al., 2017). Without any empirical evidence, zooids were consistently excluded in previous studies on the basis that they contain mostly bacterioplankton (Erwin et al., 2013, 2014; Goddard-Dwyer et al., 2020; Martinez Garcia et al., 2009). We conducted the first microbial analysis of zooids and tunic in colonial ascidians to examine this longstanding assumption with the hypothesis that colonial ascidians harbour tissue-specific microbiomes in the zooids and tunic.

Surprisingly, our findings rejected this hypothesis and indicated that zooid microbiomes were not drastically different from those in the tunic, based on microbial community structure (Figure 3.5) as well as phylum-level composition (Figure 3.7). We note that the p-value for the PERMANOVA test performed on beta-diversity is technically below the common threshold of 0.05, and both zooid and tunic microbiomes are significantly different from those of seawater samples (PERMANOVA, p-values = 0.01 & 0.029 respectively). The data overall thus rejected the common assumption that the zooid microbiomes are dominated by bacterioplankton from the surrounding seawater. Moreover, a Venn diagram of ASVs found in the three sample types showed that tunic samples harbour more than twice as many ASVs in common with seawater samples (37 ASVs) compared with zooid samples (15 ASVs in common with seawater samples). Tunic samples collectively harboured the most diverse bacterial community, with 12,490 unique ASVs and 4,541 ASVs shared with zooid samples (Figure 3.6). This suggests that the similarity between zooid and tunic microbiomes is not only in their overall structure and high-level taxonomic composition (Figures 3.5, 3.7), but at the ASVs level to a certain extent (Figure 3.6). To allow our findings to be more directly compared with those from previous studies, most of which excluded zooids, we tested our remaining hypotheses using only the tunic samples.

4.2 Ascidian Microbiomes Exhibit Strong Species Microbial Specificity

Based on previous studies, we hypothesised that each ascidian species harbours a species-specific microbiome (Evans et al., 2017). To reduce ecological stress and epibiosis influence on the ascidian microbiome (A. R. Davis et al., 1989; Núñez-Pons et al., 2012; Wahl, 2008), we tested species specificity by limiting the analysis to monospecific specimens. Microbial richness showed some variability between the species with non-significant difference (Kruskal-Wallis, p=0.067 - 0.068), which might be attributed to the high variability around the mean (Figure 3.8). However, the PCoA plot presented highly significant variabilities (PERMANOVA, p-value = 0.002) in microbial structure, implying a high degree of species specificity in the microbiomes of colonial ascidian hosts (Figure 3.9). This was also demonstrated in microbial taxonomic composition clearly specific to each species despite the high intra-species heterogeneity observed between replicate samples (Figure 3.10). Since samples that contained species LN & DI, and AK & AP intermixed in the PCoA plot (Figure 3.9), the phylum-level compositions supported these trends with some compositional similarity (Figure 3.10).

However, when examining statistical differences in community structure between the monospecific samples, no significant difference was observed (Table 3.3) with the exception of the DI species, which exhibited a significant difference from DV (PERMANOVA, p-value = 0.033), BL (PERMANOVA, p-value = 0.002), and AK (PERMANOVA, p-value = 0.035). Nonetheless, when examining both interacting and monospecific samples (Figure 3.25), a significant difference was observed between the majority of the species (Table 3.4). This might be attributed to increased statistical power due to a higher number of samples included in the analysis, but the results nevertheless suggesting a strong species-specificity pattern.

The high degree of species specificity in ascidian microbiomes found in this study further supports previous studies showing that ascidian species-specific microbiome appears to be stable across time, space, and environments (Cahill et al., 2016; Erwin et al., 2014; Evans et al., 2017; López-Legentil et al., 2015; Tianero et al., 2015). In this study, we also observed some level of compositional heterogeneities among replicates of the same species, indicating some degree of host specificity (Figure 3.10). This host-specificity might be explained by previous studies indicating that each host possesses unique features and physiological traits to support microniches for different microbial communities

(Evans et al., 2017), and that some level of vertical transmission may occur in colonial ascidians microbiomes (López-Legentil et al., 2015).

Even though the metabolic role of many ascidian-associated microbiomes remains unknown, we know today that it may supply critical metabolic needs of the host (Martínez-García et al., 2007; Schmidt, 2015). Different metabolic requirements of different species may be reflected in the different phylum-level taxonomic composition of each species and/or individual host (Figure 3.10). The phylum-level composition across most species and samples were mainly dominated by the phylum *Proteobacteria. Proteobacteria* is represented by 13 genera, which belong to nine families (Chen et al., 2018). As demonstrated in this study, *Proteobacteria* are known to dominate the microbiome of a large variety of host species (Chen et al., 2018), and a few *Proteobacteria* are known for their ability to synthesis toxic secondary metabolites as well, for example the North Sea *alphaproteobacterium*, *Oceanibulbus indolifex* (Wagner-Döbler et al., 2004). The Phylum *Bacteroidota* was also present in relatively high abundance specifically in species AK, AP, DV, BL.

LN and DI harbour a relatively similar phylum-level composition dominated by *Proteobacteria* (approximately 80%). DV and BL both harbour relatively unique microbial communities. DV was composed of only roughly 50% *Proteobacteria,* and roughly 50% of *Bacteroidota*, *Crenarchaeota,* and *Spirochaetota* which is known to be relate to brominated compounds, possess interesting properties potentially linked to the production of halogenated pharmaceuticals (Gutleben et al., 2019). BL is the only species which exhibited a high abundance of unknown phylum (>50%) across most of the replicates of that species (Figure 3.10). AK and AP harbour approximately 25% relative abundance of the phylum *Cyanobacteria* (Figure 3.10), a phylum that is known to produce oxygen, provide nutrients, and synthesise secondary metabolites for their chemical defence (Chen et al., 2018). The similar microbial composition AK and AP exhibit might be related to the fact they both belong to the *Aplidium* genus (Shenkar et al., 2022).

4.3 Ecological Status of the Ascidian Host Does Not Consistently Correlate with Its Microbiome

This study is the first to compare the microbiomes between the interacting and monospecific counterparts of colonial ascidians. We hypothesised that the interacting

ascidians (i.e., competing chemically) harbour a different microbiome compared to their monospecific counterparts, to enhance their allelochemical capacities and ecological success probabilities (López-Legentil et al., 2006; Núñez-Pons et al., 2012; Zhan et al., 2015). Surprisingly, no consistent difference was found between the monospecific and interacting samples from the same ascidian species (Figures 3.11, 3.12). Community richness (Figure 3.11) showed relatively similar alpha diversity for both interacting and monospecific samples per species. That was also demonstrated in community structure, with a clear overlap between the monospecific and interacting samples within their species cluster (Figure 13.12).

In order to examine whether this trend is consistent across the different species pairings; we analysed each species' microbiome across the varied interactions it was involved in. No significant differences between the interacting and monospecific samples were observed across most species-pairing. AK, BL, and LN samples exhibited no significant difference in community richness and community structure (Kruskal-Wallis, PERMANOVA p-value > 0.05) (Figures 3.14, 3.15, 3.17). On the other hand, DV samples showed high significant differences in community structure (PERMANOVA p-value = 0.009), however no significant differences were found between samples (PERMANOVA, p-value > 0.1) (Figure 13.3 B). A similar trend was also found in DI samples, with high significant differences in community structure (PERMANOVA, p-value = 0.02), however no significant differences between samples (PERMANOVA, p-value > 0.05) (Figure 3.16 B).

The uneven number of species-pairing per species may have caused bias in the observed statistical tests: BL and LN both have only a single species-pairing to compare to their monospecific samples, whereas DI have two, and AK and DV have multiple (Figure 3.1). In addition, the statistical test could not be evaluated properly for species AP (Figure S5), since two monospecific samples (S-146, S-150), and two interaction samples (S-076, S-088) were removed during the trimming and filtration process conducted initially (Table 3.2). Thus, the similar microbial structure and diversity of both ecological states (i.e., interacting, or monospecific) might be explained by the high species-specificity that was found in this study (Figures 3.9, 3.25). And despite the inconsistent difference between the interacting and monospecific microbiomes, some correlations (i.e., DV & DI samples) were observed that need to be further examined.

4.4 Some Ascidian Microbiomes Dominate the Interactive Border

In this study we aimed to examine not only the monospecific and interacting species microbiomes, but also the microbial community located in the interactive border between two competing species (Figure 2.4). Our hypothesis was that the interactive border (IB) may harbour different microbiome than each of the interacting species due to the physical proximity to the naturally induced ecological challenge (A. R. Davis et al., 1989; Núñez-Pons et al., 2012; Wahl, 2008). Interaction samples were microbially analysed individually to minimise the observed species-specific influence (Figure 3.9, 3.26). The interaction analyses were divided into two sections: all interactions that contained DV (Section 3.6.1), and all interactions that contained AK (Section 3.6.2).

Most of the interactions involving DV (e.g., AK-DV, DV-AP, DV-DI) exhibit a significant difference in microbial community structure (PERMANOVA, p-value < 0.05) between each of the fragments within the interaction (species 1, species 2, IB) (Figures 3.18 A, 3.19 A, 3.20 A, respectively). The only interactions not showing significant difference between the interaction samples were DV-LN (Figure 3.21) (PERMANOVA, p-value = 0.3) and DV-BL (Figure 3.22) (PERMANOVA, p-value = 0.12). However, the interaction DV-LN (Figure 3.21) had only two samples of the interacting LN samples, which might explain that insignificant difference. The non-significant difference between the interaction DV-BL was unexpected, however the PCoA and taxonomical composition bar plots (Figure 3.22 B) showed that the DV species showed relatively high taxonomic variability, which might explain the non-significant result.

Among interactions containing AK (Section 3.6.2), the interaction AK-DI exhibits a significant difference in microbial community structure (PERMANOVA, p-value = 0.026) and composition (Figure 3.24) between each of the interacting AK, DI, and the IB[AK-DI]. However, the interaction between AK-AP exhibited no significant difference in community structure (PERMANOVA, p-value = 0.9) (Figure 3.23 A), and phylum-level composition analysis between samples (Figure 3.23 B). Nonetheless, the high level compositional similarity between the samples within the AK-AP interaction might be also attributed to the fact that both AK & AP species belong to the same genus (*Aplidium*), as discussed previously (Shenkar et al., 2022).

When examining the IB microbiome within all interactions that included DV (DV-AP, DV-BL, AK-DV, DV-DI, DV-LN), An interesting trend was observed: all IB samples

were microbially dominated (according to PCoA plots and taxonomic composition) by the other species' microbiome, regardless of whether that species is native or invasive. For example, DI overlapped with IB[DV-DI] , while DV tended to cluster more distinctly (Figure 3.21). The same trend was also observed within the interaction that included the invasive AK, as the IB[AK-DI] samples were more dominated by the native DI microbiome (Figure 3.25). AK-AP interaction had lower amounts of replicates, which may affect the ability to observe a similar trend. However, this trend was not statistically significant, which probably relates to the small datasets analysed, and consequently low statistical power.

Interestingly, in the interaction between the invasives AK & DV, the IB[AK-DV] was microbially dominated by AK species, as AK and IB[AK-DV] clustered on the left part of the PCoA plot (Figure 3.19A). This may imply that DV species associated microbiome is consistently showing a lower microbial dominance within the IB when competing with all examined species in this study. This finding is quite surprising, since the invasive DV species (*D. vexillum*) is considered a major ecological threat in many marine ecosystems, to native sessile communities (Casso et al., 2020; Stefaniak, 2009). This was assumed to be attributed to DV stronger chemical abilities induced by its associated microbiome (Casso et al., 2020). However, DV microbiome does not seem to be predominant in the IB samples in this study. Nonetheless, limited by the low number of replicates per interaction specimen in this study, this trend must be further examined in future studies to be fully supported.

Overall, most of the interactions exhibited significant differences between the different fragments within the interactions as expected. The interesting IB microbial dominance trends that were found in this study, must be further examined in future studies.

4.5 Invasive and Native Ascidians (Mostly) Harbour Distinct Microbiomes

The examined species we tested in this study are defined as native or invasive to New-Zealand oceans (Awesome Ascidians, 2012). The hypothesis was that invasive ascidians harbour different microbiome to native ones, due to their competitive advantage that enables them to overcome the native species (Casso et al., 2020; Evans et al., 2018; Rpm

et al., 1996). In this study we found distinct microbiomes associated with host ecological status (i.e., invasive and native), with a clear influence of species specificity (Figure 3.25).

PCoA plot showed a clear grouping of some of the native and invasive species with a significant difference between the groups (PERMANOVA, p-value = 0.001) (Figure 3.25). The invasive BL and DV species exhibit similarity with overlapping data points (PEMANOVA, p-value = 0.78), and the native DI and LN show similar community structure, with overlapping and intermixing data points (PERMANOVA, p-value = 1.0). Nonetheless, the invasives BL and DV are significantly different from the invasive AK (PERMANOVA, p-value = 0.03, 0.015 respectively) (Table 3.4). Similarly, the native DI is significantly different from the native AP (PERMANOVA, p-value = 0.015), while the native LN shows similarity to the native AP (PERMANOVA, p-value = 0.12) (Table 3.4). However, the native AP and the invasive AK exhibit similar microbial structure (PERMANOVA, p-value = 1.0) despite being one native and one invasive to this habitat. Since AK and AP belong to the same genus (*Aplidium*) (Shenkar et al., 2022), this interesting finding may suggest that species specificity might have stronger microbial impact on a host microbiome rather than host ecological status. However, this trend was not observed for DI and DV, both of which belong to the *Didemnum* genus yet harbour significantly different microbiome (PERMANOVA, p-value = 0.015) (Table 3.4).

When comparing the microbial structure of interacting versus monospecific counterparts of the invasive ascidians (DV, AK, BL), the microbiome exhibits a high stability across both ecological states (Figure 3.12). This may be attributed to the high invasion capabilities that allow them to thrive in a wide range of habitats and environments as suggested in previous studies (Evans et al., 2018). However, the microbiome stability was also observed across the monospecific and interacting states of the native species (Figure 3.12). This reinforces the observed trend that species- specificity may play a bigger role in microbiome stability, than the invasion capabilities of the host (Cahill et al., 2016; López-Legentil et al., 2015; Tianero et al., 2015). This was also shown in the lack of dominance within the examined interactions, where surprisingly the invasive species DV and AK exhibited a low microbial dominance within the IB samples when competing with all examined species in the study and regardless of if they compete with natives or invasive species (Section 3.6).

All the above-mentioned suggests that invasive and native species might have a stable microbial core associated with their ecological status (native/invasive), which is also species-specific. However, it does not necessarily mean that invasive hosts rely on restructuring specific microbial communities to invade, survive, or compete under environmental conditions like epibiosis (A. R. Davis et al., 1989; Wahl, 2008). This may also suggest that epibiosis is not a sufficient stressful ecological state to require the host to recruit other symbionts, like for example in an increased environmental pollution (Evans et al., 2018).

4.6 Location Variability

Despite the geographical and temporal proximity of the collection sites and dates to one another (Figure 2.1, Table 3.1), and despite the compositional similarity of the collected seawater samples from each site (Figure S6), the PCoA plot showed significant difference between collection sites (PERMANOVA, p-value = 0.003) (Figure 3.26). While Pier A and Bridge seemed to be similar habitats (PERMANOVA, p-value = 0.066), the Mount was significantly different from both Pier A (PERMANOVA, p-value = 0.003) and Bridge (PERMANOVA, p-value = 0.03). When looking at the aerial images (Figure 2.1) the Mount is indeed a more rural environment with larger access to the open sea and a larger wave exposure area, while Pier A and Bridge are both located in closer proximity to one another and closer to the marina port, with possibly lower hydrodynamic exposure. The hydrodynamic energy regime has been demonstrated as an important factor influencing light availability, water motion, therefore nutrients and oxygen availability (Rattray et al., 2015). These environmental factors may affect microbial composition of the host in response to changes in the environment (Littman et al., 2009). Therefore, there is some effect of collection site on the microbial communities, however the data did not show any specific trend associated with either of the collection sites, perhaps due to the high species-specificity found in this study.

Chapter 5
Conclusion and Future Directions

In this study, 40 wild specimens of colonial ascidian species were analysed to reveal their associated microbiome across varied ecological states. To date, most studies of colonial ascidian microbiome have analysed only the tunic tissues while excluding the zooids on the grounds that they are presumed to mostly contain planktonic microorganism (Chen et al., 2017; Erwin et al., 2013, 2014; Goddard-Dwyer et al., 2020; Martinez Garcia et al., 2009). This study was the first to acknowledge that zooids microbiome might play an essential role in the chemical defence and offence mechanisms of ascidians through the biosynthesis of the potent secondary metabolites. This study confirmed our hypothesis that colonial ascidians harbour a tissue-specific microbiome, as both tissue types demonstrated a clear distinction between ascidian and seawater communities (Figures 3.5, 3.6). However, the most remarkable finding is that zooids and tunic share a mutual core (Figure 3.6) and demonstrated similar microbial structure (Figure 3.5) and composition (Figure 3.7). Based on these findings, zooids should not be eliminated out of future microbial analyses, so the ascidian microbiome could be explored aligned with the hologenome theory (Rosenberg & Zilber-Rosenberg, 2018).

This study also confirms the hypothesis that colonial ascidian species harbour a species-specific microbiome (Figures 3.9, 3.25). This finding was consistent across the interacting and monospecific states of the host (Figure 3.12). Interestingly, the species-specificity appears to be a factor determining the microbiome of the native or invasive host as well (Figure 3.25). The classic example of that was demonstrated when species AK and AP, which belong to the same genus (*Aplidium*) (Shenkar et al., 2022), exhibited microbial similarly in structure (PERMANOVA, p-value = 1) despite the fact the AP is native, and AK is invasive (Figure 3.25) (Table 3.4). This study has also suggested that native and invasive species exhibit a significant difference in their microbiomes (Table 3.4, Figure 3.26).

Interestingly, most interacting ascidian species did not seem to harbour a distinct microbiome compared to their monospecific counterparts, neither in community richness (Figure 3.11) nor structure (Figure 3.12). However, when comparing each species and its associated pairings, this finding was influenced by the number of replicates (Figures 3.17,

S5), or a higher number of species pairings (e.g., DV, AK) (Figures 3.13, 3.14). Surprisingly, in most interactions involving the invasive DV species, the interactive border samples showed overlap with the competing species (regardless of if it was native or invasive) (Section 3.6.1).

So far, ascidians have provided the most significant number of marine-derived secondary metabolites, including some of the most interesting drug candidates (Proksch et al., 2003). Yet, it is almost certain that many bioactive products have not yet been discovered (Palanisamy et al., 2017). Our initial investigation into zooids microbiome, and the finding that zooids share a mutual core with the tunic (Figures 3.5, 3.6) is an initial milestone to reveal the unresolved biosynthetic mechanisms, for many metabolites, that have yet been identified (Chen et al., 2018; Palanisamy et al., 2017), as that type of detailed and targeted approach was yet conducted. Correspondingly, further research should be done on colonial ascidian zooid tissue to gain better understanding on the associated microbiome and its role with secondary metabolite synthesis and ecological adaptation.

Moreover, only a few studies have examined the microbial symbionts of invasive colonial ascidians, specifically in the bioactive detection context (Casso et al., 2020; Evans et al., 2018, 2021; Goddard-Dwyer et al., 2020). That is despite that their specific and unique microbiome is related to their ability to invade new environments and defeat the endemic species by producing stronger chemical compounds (bioactives) (Amsellem et al., 2017; Evans et al., 2018; Schmidt & Donia, 2010). In this study, we revealed the microbiome of invasive colonial ascidians species and parallel; we coupled it with bioactive detection on a separate project. This can potentially lead to a fast-track of stronger and improved marine natural products for human applications.

Nonetheless, further study should be conducted to correlate the revealed microbiome to the produced bioactive compounds. Based on these study outcomes, specific samples will be targeted for future metagenomic analysis to identify shifts in functional diversity and relative abundances of functional genes. This will help to reveal the relevant gene expression during the bioactive synthesis, and a correlation between microbial composition shifts and allelochemicals synthesis.

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