Comparing Competitive Interactions and Settlement Success Among Native and Non-indigenous Species in Marine Hard Bottom Communities of Colonial Ascidians, from the Bay of Plenty, New Zealand

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"We are living in a period of the world's history when the mingling of thousands of kinds of organisms from different parts of the world is setting up terrific dislocations in nature."

- Charles Sutherland Elton (*The Ecology of Invasions by Animals and Plants*- 1958)

ABSTRACT

The requirement for space in marine hard bottom assemblages is paramount for life. Due to the crowded nature and demand for space in these sessile assemblages, bare space that is freed is quickly occupied. The intense push and pull for space within these assemblages is represented by heavy overgrowth interactions. These competitive interactions often result in the displacement of native species and the formation of competitive hierarchies amongst species, with some individuals settling as epibionts and causing the mortality or stress of those beneath them.

Ascidian invasions have become more frequently reported on a global scale, becoming an emerging issue on many coastlines. Colonial ascidians (the key phylogenetic class of this study) and their ability to dominate and occupy vast amounts of space, has landed them their reputation as notorious marine invaders. With urbanization comes the general increase in anthropogenic activity, which in turn has been known to coincide with the increase in the translocations of nonindigenous species. Vectors such as interoceanic trade and travel have contributed heavily to the spread of nonindigenous ascidians, allowing them to overcome geographic barriers. Unmanaged populations of nonindigenous colonial ascidians heavily foul much of the submerged substrate in the Tauranga Harbour, threatening the biodiversity, population structure and function of native communities as well as fouling of marina and port substrates (wharves, pylons, ropes, boat hulls).

The framework of this research infers that the competitive abilities of non-indigenous colonial ascidians through their rapid occupation of substrate is a key determinant in their invasion success. *Ex-situ* manipulative experiments were used to examine potential competition (epibiotic or bare space settlement) recruiting species may have on nonindigenous and native ascidians. Simultaneously, we examined if the status (nonindigenous or native) of the test species impacted the settlement of the nonindigenous and native recruiting species. Few studies have attempted the novel process of rearing ascidian cultures *ex-situ*, where manipulation and control can be maximised. We attempted to develop a robust ascidian culture system to better study ascidian species.

The lines of evidence provided by these experiments revealed that nonindigenous colonial ascidians are often opportunistic settlers and can largely determine the dynamics of native sessile communities, as they settled the most as both epibionts and bare space recruits on the experimental plates in this study.

Experimental results found most recruits to prefer settling on bare substrate than on the surfaces of other organisms, supportive of the concept that surface microtopography and secondary metabolite release may play a role in recruit settlement.

Findings bring focus to the unrivalled ability of nonindigenous species to exert settlement pressures on existing sessile communities, illustrated by their competitive power. Introduced species' ability to settle heavily as both basibionts and epibionts allows them to litter submerged substrates with larvae, illustrative of high propagule pressures found in this study. Experimental results offer managers to better utilise biosecurity management resources when dealing with nonindigenous incursions in the future.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

GLOSSARY

Allelochemical: The chemical release by an organism to inhibit the growth or reproduction of surrounding/nearby organisms.

Aquaria: A glass sided tank or bowl used to keep living aquatic organisms that are kept for exhibit or research.

Assemblage: A functional group of species making up a small community of organisms.

Basibiont: Organisms that can act as a substrate for other epibiont organisms.

Bio-invasion: The expansion of the range of a species through both human and natural mediated introductions.

Biosecurity: A system designed to protect a country's economy, environment, human health and social and cultural values against pests and diseases. Ideally before they arrive and establish.

Culture: The growth of microorganisms in a specifically prepared nutrient medium under supervised and controlled conditions.

Epibiont: Organisms that settle on and occupy the surface of other organisms.

Established: A species that has successfully attained a self-sustaining population.

Incursion: The movement of an introduced organism into a region where it has never known to be previously present.

Inoculation: The introduction of microorganisms to a culture in which they can grow and reproduce in.

Introduction: The human or natural mediated movement of a species into a receiving region.

Invasion: The introduction and arrival of a species that did not evolve in that environment/location.

Invasive species: A species that is introduced, establishes, and can cause economic, social and environmental issues.

Native species: An organism living within its natural environment.

Non-indigenous species (NIS): An organism that is living outside of its natural environment.

Teste: A gelatinous matrix located between zooids.

Zooid: An individual organism 'budding' from another, forming colonial organisms.

CHAPTER 1.

INTRODUCTION

1.1 Non-indigenous introductions of marine species: background and significance

New Zealand is no stranger to the impacts of invasive species and diseases, a topic that has been widely studied and discussed. The geographic isolation of New Zealand and the evolution of its' unique fauna in the absence of mammalian predators, makes it especially susceptible to the introductions of both terrestrial and marine non-indigenous species (NIS) (Marshall & Sullivan, 2010). New Zealand's wealth in endemism is recognized worldwide, yet this is largely based again on terrestrial fauna and flora but is equally attributable to marine biodiversity. By comparison, international research into marine biosecurity is in its infancy within Aotearoa New Zealand, awareness of the scale of the issue has only recently been realised.

Annually, tens of thousands of species both terrestrial and marine are translocated across the world. Costing an estimated $1.4 trillion annually, this adds up to 5% of the world's global economy (Pimentel et al, 2005). Incursions of NIS to new geographical locations threaten the native marine biodiversity and community structure of recipient communities (Rius et al, 2014). Some researchers contend that invasions of non-indigenous species and climate change are the most important issues today (Adams et al, 2014; Burgiel & Hall, 2014). However, other studies argue that trade and travel may present a greater and more immediate threat to global ecosystems than climate change itself (Stohlgren et al, 2014).

Within the marine environment, the translocations of NIS often involve species being picked up through ballast water or attaching to substrates (ship hulls, bilge tanks, propellers etc) and transported to new geographic locations (Cahill et al, 2012). The exponential increase in international oceanic travel can be linked to the increase in spread of non-indigenous species, with oceanic trade as a primary vector for marine NIS (Lambert, 2001).

Despite invasions reported *en masse* across the globe, translocations of NIS rarely result in establishment success, even with large numbers surviving the initial phase of settlement at a foreign coast or harbour. Once a species has been translocated from its native range, multiple abiotic and biotic factors influence its spread (Johnston et al, 2017). Invading species must survive multiple phases in the settlement process to successfully establish in their invaded region (Britton-Simmons & Abbott, 2008). It is here where the 'tens' rule may apply, first introduced by

Williamson & Fitter (1996). The Tens Rule refers to the establishment success of a species whilst highlighting the difficulty and selectivity behind the invasion process, with 1 out of ten species being translocated, of those one out of ten will become established in its new environment. Interestingly, Jarić & Cvijanovic (2012) argue that the Tens Rule obscures the potential damage that NIS can impose and downplays the impacts associated with invasion, however the rule is ideal in elucidating potential of many species to pose as invaders and the rigorous requirements (genotype, phenotype, behavioural or evolutional) required to be a successful NIS.

Invasion ecology, defined here as the investigation of pathways in which a new species is introduced into a new environment, is the focus of this thesis. Invasion ecology research is argued to constitute a major pathway aimed at the prevention of incursions into the future, by identifying biosecurity threats at a higher level through understanding processes underpinning successful recruitment into new habitats (Johnston et al, 2009). Understanding the mechanics behind factors that regulate NIS invasions remains a challenge in ecology (Britton-Simmons & Abbott, 2008). Increased understanding of why only a small percentage of NIS survive the translocation process and then what triggers some of these species to become a problem as an 'invader' will better inform managers, both on existing confirmed marine biosecurity threats and also on the effective management of future NIS incursions.

Of relevance to this study, is the accumulation of NIS organisms on a surface, referred to here on as biofouling (Fitrige et al, 2012). Such introductions of biofouling organisms primarily impact bivalve aquaculture in New Zealand (Fitrige et al, 2012). Species of NIS colonial ascidian (the subject phylum of this study),

have led to a reduction in aquaculture stock yields through growth, survival and reproduction limitations on bivalve stock (Dürr & Watson, 2009). Globally, NIS ascidian introductions have become more frequently reported (Lambert, 2001). NIS ascidians are excellent marine invaders and good models for the examination of invasion dynamics (Zhan et al, 2015; Lins et al, 2018).

Invasions of NIS ascidians impact native hard bottom communities by smothering and dominating substrate, altering native biodiversity, and altering the structure and function of community dynamics (Catilla et al, 2004; Lagos & Cerda, 2004; Rius et al, 2014).

The Tauranga Harbour (latitude: 37° 41' 10.00" S, and longitude: 176° 10' 0.01" E) was chosen as the study location, as it is a strong source demographic of NIS due to the nearby Port of Tauranga, the largest shipping harbour and port of New Zealand (Inglis et al, 2006). Located

near Mount Maunganui at the south-eastern end of the Tauranga Harbour, The Port of Tauranga is one of the biggest commercial enterprises in the Bay of Plenty, playing a large role in international oceanic trade (Inglis et al, 2006). Vessels arriving to the port are primarily commercial vessels visiting from Australia (35%), followed by the northwest Pacific (24%), northeast Pacific (15%), south Pacific (13%), east Asian Seas (3%) and other New Zealand ports (Inglis et al, 2006). As NIS are often abundant in urbanized anthropogenic regions (Clark & Johnston, 2009), due to increased hard surfaces. The Tauranga Harbours vessel and anthropogenic activity may increase the propagule pressures of NIS ascidians and contribute to the global issue of NIS as a whole.

1.2 Marine biosecurity

Marine biosecurity is rarely considered by the public as high priority, and as a result, the negligence in management, monitoring and legislation continues despite irreversible deleterious consequences to marine ecosystems. In an ocean that is fast changing due to the influences of global climate shifts, population growth, pollution and unsustainable marine resource harvesting, the urgency to protect remaining biodiversity is imperative (Halpern et al, 2019; Ricciardi & Rasmussen, 2001).

Therefore, research to better understand why and how species invade through invasion ecology research are paramount. Current hull fouling and inspection regimes are inadequate for the large scale of global ship traffic, and hence there is unmanaged international risk inherent in modern maritime activities (Ferreira et al, 2004). Current ‘at source’ monitoring is also inadequate. Added to this is the fact that the marine environment can be highly dynamic and unpredictable. Factors such as hydrodynamic flow, weather events and the size of the ‘receiving’ harbour/water body, means that sufficient and continuous monitoring of potentially invasive species in a 3-dimensional realm proves difficult (Trebitz et al, 2017). Issues caused by constraints on New Zealand marine biosecurity responses, theoretical research tools, technologies and capacities are exacerbated by an absence of prioritized risk assessment and subsequent management (Hewitt et al, 2004).

The biosecurity continuum is a key motif that entails the protection of social, cultural, environmental, and economic values through pre-border, border, and post-border control systems (Sharma et al, 2014). The pre-border prevents the transport of propagules (individuals) outside of their native range and to new regions, preventing the translocation of propagules at the pre-border vector is crucial when attempting to prevent or control invasions of NIS (Johnston et al, 2009). The border acts as a control to prevent propagules from entering the region once arrived, and acts as the last line of defence before post-border management (Johnston et al, 2009, Britton-Simmons & Abbott, 2008). Post-border management is unfortunately the more common area of work in most countries and certainly in New Zealand. When NIS have successfully established, the post-border of the biosecurity continuum carries out the task of future management, surveillance systems and even attempts to eradicate the NIS(Britton-Simmons & Abbott, 2008). During this phase, factors such as disturbance and propagule pressure can contribute to the control of invading species (Britton-Simmons & Abbott, 2008).

1.3 Colonial ascidians: Non-indigenous

The focal phylogenetic class of this study, colonial ascidians are primitive members of our own phylum, Chordata (Lambert, 2005). With over 3000 described species, ascidians are the most diverse and largest class of the subphylum Tunicata (Urochordata) (Shenkar & Swalla, 2011). While many ascidians are solitary, colonial ascidians are composed of morphologically identical individuals (zooids) that are embedded in a common tunic and share a circulatory system (Epelbaum et al, 2009) (see figure 1.1 and 1.2). Colonial ascidians were chosen in this study as they are easily located in the field, can be grown under laboratory conditions, and cloned for experimental manipulations. More specifically, they were selected for this study due to their large consumption of available substrate space and their reputation as notorious marine invaders (Zhan et al, 2015).

Globally, NIS ascidian introductions have become more frequently reported (Lambert, 2001). NIS ascidians are excellent marine invaders and good models for the examination of invasion dynamics (Zhan et al, 2015; Lins et al, 2018). Research recounted in this thesis will focus on ascidians as one of the more aggressive and prevalent NIS (Lins et al, 2018), and thus often lending themselves to experimental research.

Colonial ascidians are highly fecund hermaphrodites and are capable of both self and cross fertilization, a reproductive trait that aids in their quick dispersal (Lambert, 2007). Some studies

suggest efficient reproduction may aid ascidians in their quest for space (Lambert, 2005). Common colonial ascidians can release between 7-20 tadpole larvae per week, with solitary tunicates releasing even more (Lambert, 2005). Asexual reproduction helps colonies expand and occupy more substrate, new zooids can also disperse asexually through the fragmentation of part of the colony body and disperse to a new area (Lambert, 2005). Colonial ascidians can reach sexual maturity and produce a second generation in just a few weeks (Lambert, 2005). Their larvae, despite being non-feeding and short-lived, can postpone their settlement to survive oceanic transportation in the translocation process, therefore optimizing their success from the very beginning of the invasion process (Lambert, 2001).

NIS ascidians can tolerate a broad range of environmental conditions with most ascidian species being able to tolerate and survive in salinities between 25 and 40‰ (Lambert, 2005). *Ciona intestinalis* has been shown to survive in salinities at 12–40 ‰. Additionally, many ascidian species can survive a wide range of temperatures, *C. intestinalis* for example has been recorded to survive water temperatures as high as 35 °C (Dybern, 1965). *Botryllus schlosser*i and *Botrylloides violaceus* have been observed to survive water temperatures between 10–25°C (Epelbaum et al, 2009), and *Didemnum vexillum* has been found to tolerate a wide range of temperatures from 1-24 °C with daily fluctuations of up to 11 °C (Lengeyl et al, 2009).

Ascidians can also survive under polluted conditions, even in environments containing increased loads of heavy metals (Zhan et al, 2005). Tolerance to anthropogenic introductions of heavy metals and pollutants may explain why ascidians can thrive in highly urbanised environments. The adaptation and tolerance of polluted and eutrophic environments may facilitate NIS ascidians to live in fast-changing environments during transportation and introduction stages (e.g ballast tanks) (Zhan et al, 2015).

It is no surprise that Antarctica is the only continent exempt of reported invasive ascidians (Zhan et al. 2015), ascidians are equipped with high success and establishment rates (Zhan et al, 2015) due to their ability to: (1) occupy and dominate surfaces on ships and other structures, (2) spread rapidly from their clonal mode of reproduction/colony enlargement (Manni et al, 2007) and (3) and their propensity for rapid population bloom (Willis & Woods, 2011).

Populations of the non-indigenous *Didemnum vexillum* were found to limit the densities of smaller size classes of *Perna canaliculus* mussels (Fletcher et al, 2013). Losses of shellfish harvest as large as 50% have been reported following invasions of *Styela clava* (a solitary ascidian) in eastern Canada (Colautti et al, 2006). The introduction of *D. vexillum* in Shakespeare Bay in the South Island of New Zealand catalysed a large-scale eradication attempt, eradication

attempts were unsuccessful and failed to eliminate the species entirely from the area despite costs reaching as high as $650,000 ($NZD) (Coutts & Forrest, 2007).

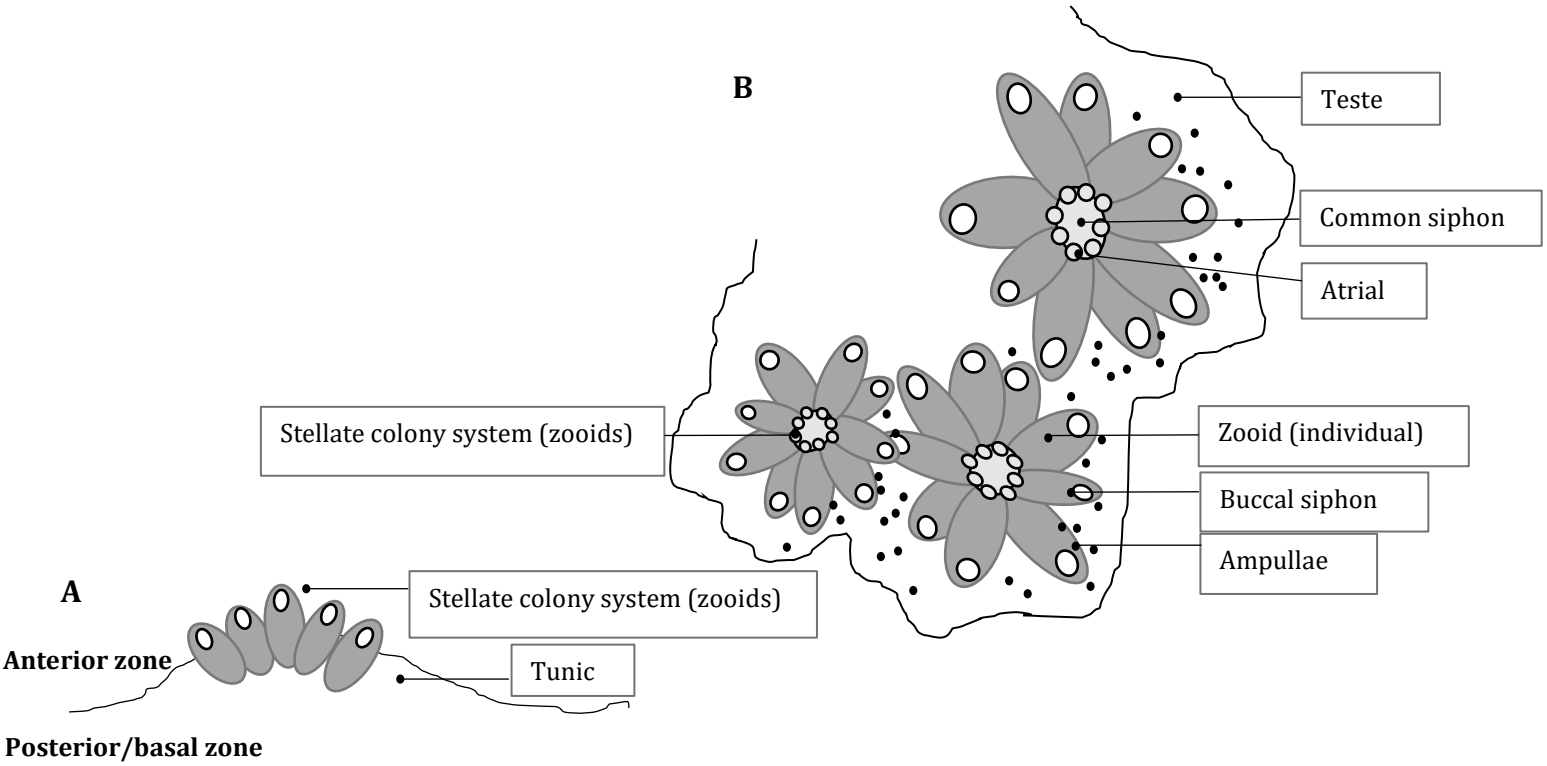

Figure 1.1: (A) Lateral diagram of a *Botryllus schlosseri* colony, showing the anterior and posterior zones. (B) Diagram of *B. schlosseri* colony, zooid, and stellate system anatomy. Adapted and modified from Shirae, 2000.

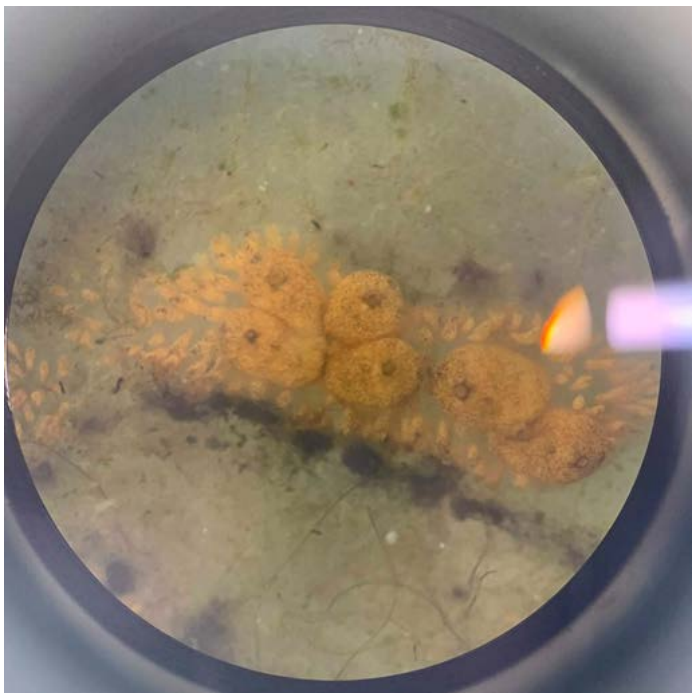

Figure 1.2: Photograph of a *Botrylloides* sp. colony, showing zooids embedded in tunic sac under a compound microscope at 10x10 magnification

NIS ascidians are often referred to as ecosystem engineers, due to their rapid growth and effect on the biodiversity of native ecosystems (Catilla et al, 2004). Colonies of the NIS *Didemnum vexillum* were observed in other studies to deter the foraging behaviours of planktonic predators in Georges Bank (USA), an example of how an invasion can impact the native assemblages of hard bottom communities (Lengeyl et al, 2009). Such alterations to community function may result in food chain shifts in some areas (Mercer et al, 2009). NIS populations of *Botryllus eilatensis* in the coral reefs of Eilat (Israel) have been observed to demonstrate rapid reproduction, growth and spread in the Red sea (Shenkar et al, 2008), and population blooms of *Ciona intestinalis* in San Fransisco Bay (USA) were recounted to decrease the species richness of native species (Blum et al, 2007).

As highly efficient filter feeders, the filtration services that native ascidians offer are often crucial in the assemblages they exist in, controlling pelagic primary production and bacterial populations thus improving local water quality (Stabili et al, 2016). Native ascidian populations also offer filtration services over a broad range of particle sizes, reducing potential harmful algal blooms that can cause fish kills (depletion of oxygen) and often result in beach closures (Worm et al, 2006).

Sessile marine benthic communities are particularly vulnerable to invading species (Zhan et al, 2015). The presence of invading species can interfere with the exchange of nutrients, mass and nutrients between two habitats (Mercer et al, 2009). As an important process in aquatic ecosystems, a reduction in benthic-pelagic coupling could impact nutrient cycling and energy transfer in the food webs of sessile assemblages (Mercer et al, 2009). The geochemical cycling of elements and nutrients are also vulnerable when heavy invasions occur, if an introduced colonial ascidian were to smother an assemblage comprised of native species and act as a physical barrier, the exchanges of dissolved oxygen may be reduced, resulting in alterations to the elemental makeup of native benthic communities (Mercer et al, 2009).

1.3.1 Vectors of dispersal

The spread of introduced colonial ascidians is believed to be occurring exponentially (Zhan et al, 2015). Globally, coastal marine waters are one of the most invaded habitats (Zhan et al, 2015;Rius et al, 2014). There are multiple anthropogenic dispersal vectors that allow ascidians to overcome geography barriers.

It is widely recognised that submerged anthropogenic substrates (wharves, pylons, moorings, ropes, and concretes) act as novel and desirable settlement substratum for NIS ascidians (Chase et al, 2016). This leads to such substrates becoming common vectors, facilitating ascidians to overcome geographic barriers, and include but are not limited to ballast water release, hull fouling, aquaculture, recreational and fisheries vessel movement (Zhan et al, 2015). Encrusting and fouling organisms can attach to a multitude of areas on the hull of barge shipping containers; sea chests, propellers, bridge keels, bow thrusters and other submerged surfaces, and be translocated to new geographic locations (Cahill et al, 2012). Additional natural vectors consist of biological movement (shelled organisms such as crabs with settled ascidians on their outer shell), currents and the breaking off and rafting of colony parts (Zhan et al, 2015).

The exponential increase in international oceanic travel can be linked to the increase in spread of non-indigenous species, with oceanic trade as a primary vector for marine NIS (Lambert, 2001). Domestic dispersals of NIS ascidians are broken up into two groups, naturally mediated and human mediated (Zhan et al, 2015). Natural mediated vectors are presumed the least common throughout literature, colonial ascidians have short planktonic larval phases, they quickly mature to adults after settlement (Svane & Young, 1989), and ascidian larvae are can only disperse

within meters of their original colony, this indicates that large scale dispersal of NIS ascidians can only be associated with human mediated vectors (Zhan et al, 2015.)

1.4 Competition

Marine encrusting communities consist of a cosmopolitan array of sessile and sedentary invertebrate species such as sponges, ascidians, and bryozoans (see figure 1.3). Substrate preference plays a critical role in the life cycle of ascidians (Lopez Gappa, 1989). New substrate that is suitable for settlement is rare and quickly colonised (Wahl and Lafargue, 1990). Here, competitive displacement by overgrowth interactions are common and competitive hierarchies are formed between organisms within these communities (Quinn, 1982). Acquiring and holding space in these systems are fundamental for sessile organisms (Lopez Gappa, 1989), as all required resources are transported across the sessile organisms when they are attached to a surface (Wahl, 2009). The “push and pull” of competition is an imperative and recurring motif in this research, in particular the competition between native and non-indigenous species.

Ascidians predominantly occupy cryptic environments such as submerged substrates, both natural and anthropogenic (Sorokin, 1995). Within these hard-bottom communities, organisms are at constant battle with one another for space, engaging in intense overgrowth interactions (Lopez Gappa, 1989), often with a dominant species heavily occupying space (Chase et al, 2016). Competition as a biotic process can either limit or promote invasion (Johnston et al, 2017), as competition can be influenced by anthropogenic activity such as dredging, disturbance, development, transport, and travel and can result in the mortality of organisms and therefore the opening of space (Levin & Paine, 1974). This space, which quickly becomes occupied and resulting in competitive processes are enhanced (Lambert, 2007).

However, the urgency to settle as quickly as possible is vital. The metamorphosis of ascidian larvae can only occur after settlement on the appropriate substrate (Feng et al, 2010; Young & Braithwaite, 1980), a vulnerability exists between the larval and settlement phase. Many benthic species have a biphasic life cycle, consisting of a pelagic larval phase and demersal/sedentary adult phase (Chase et al, 2016). Pelagic larvae can remain in the water column for periods ranging from weeks to months before metamorphosing and settling on a surface (Chase et al, 2016). Ascidian larvae experience negative phototropism and positive geotropism when the attachment process begins (Reid et al, 2016), larvae attach to a substrate using adhesives secreted

from their anterior papillae begin metamorphosis (Torrence & Cloney, 1981). These once non-feeding and mobile larvae are now filter-feeding sessile juvenile ascidians (Reid et al, 2016).

The suitability of the settlement site is crucial, as once attached larvae are unable to relocate due to sessile nature (Chase et al, 2016). Light intensity, proximity of prey or host, surface microtopography and substrate chemical composition are factors known to influence the settlement of sessile organisms (Lapointe & Bourget, 1999; Chase et al, 2016). Indeed, previous research has shown that the colonial ascidians *Ciona intestinalis* and *Botrylloides violaceus* have exhibited larval substrate preference, with a tendency to settle more frequently on concrete plates than granite (naturally occurring), PVC (man-made) and high-density polyethylene (man-made) (Chase et al, 2016). Other species of sessile organisms such as hydroids and barnacles also exhibit active settlement preferences in relation to surface texture, opting for rougher substrates which may aid in security in fluid environments (Lapointe & Bourget, 1999).

Figure 1.3: A settlement plate that had been deployed in the water for 3-6 months, illustrating heavy biofouling and the competition for space, through epibiosis and overgrowth interactions.

1.5 Epibiotic interactions

Due to the limitation of space in marine benthic systems, organisms can experience epibiosis, a direct consequence of limited settlement substrate and the exploitation of space by one species settling on the surface of another (Harder, 2009). Epibiosis can also be referred to as the non-

symbiotic, facultative association between two organisms; one the epibiont (organism that settles on another) and two the basibiont (organism settled on) (Wahl, 1989).

Sessile species in hard bottom communities rely on their outer surfaces for vital life processes, such as gas and nutrient exchanges, waste expulsion of larvae and defence metabolite release (Zhan et al, 2015; Wahl, 2009), making them particularly vulnerable to epibiosis. Sessile invertebrates are often subject to epibionts as they are bound to the substrate in which they reside, therefore overgrowth interactions as a result of intense competition can result in the mortality of the outcompeted (Wahl, 2009). The costs of epibiosis in sessile invertebrates highlights the importance of substrate space in hard bottom communities. Non-indigenous species as opportunistic epibionts such as *D. vexillum* (Stefaniak et al, 2009), pose a threat to native sessile communities and their existence.

There are two fundamental forms of epibiosis; (1) obligatory epibiosis, the requirement to settle on a surface to carry out life processes, and (2) opportunistic, the settlement of an individual on others (Fernandez-Leborans, 2010; Leonard, 2015). We can assume that opportunistic epibionts possess a form of settling 'power', as they have access to a greater amount of settlement space and a reduced preference for substrate type (Leonard, 2015). Opportunistic epibionts may experience invasion advantages; prime access to hydrodynamic flow and nutrients, experience improved irradiation, and occasional shelter (Wahl, 1989).

1.6 Allelochemistry in ascidians

Many organisms both marine and terrestrial have employed chemical defence mechanisms to maintain a successful position in their community (Braekman et al, 1978; Davis, 1991). There are three mechanisms that basibiont organisms have been cited to use to reduce the pressures of epibiosis: (1) tolerance, (2) avoidance and (3) defence (Wahl, 1989).

Of relevance to this study, chemical defence release by basibionts may contribute to a life free of overgrowth interactions (Davis, 1991). Studies report that ascidians can produce secondary metabolites to deter epibionts and avoid predation (Watters, 2018). The synthesis of secondary metabolites in sessile communities may be key in maintaining space under highly competitive pressures (Green et al, 2002). Colonial ascidians are particularly vulnerable to epibiosis (Koplovitz et al, 2009). Their surfaces are softer and less protected than the harder outer tunics of solitary ascidians and subsequently they may be under greater selective pressures to evolve alternative chemical defences (Koplovitz et al, 2009) (see figure 1.1).

The biosynthesis and exudation of secondary metabolites (Blunt et al, 2013), mucus production and the sloughing of epidermal tissues are all examples of described chemical defence mechanisms (Davis, 1991). It has been observed that NIS such as *D. vexillum* are often free from epibionts (Coutts & Forrest, 2007). Previous studies attributed traits such as size, aggression, phenotype and genotype to explain epibiont-free living, however such existence may be indicative of complex allelochemical release by the individual (McClintock & Baker, 1997).

Natural products produced by ascidians have been a point of discussion within the last 10 years, with potential as a source of drug candidates and novel bioactive leads (Lekha et al, 2001; Cragg & Newman et al, 2013). Reports have suggested that ascidians can produce nitrogenous metabolites derived from amino acids (Fenical et al, 2003). More relevant to this study is the plausible chemical defence mechanisms employed by ascidian species that may contribute to their invasion success. Despite global interest, marine invertebrate chemical ecology is in its infancy as a field. For example, there are many ascidian species present in Antarctic waters, yet McClintock and Baker, 1997 stated that at the time of publication, only one species had been chemically investigated: the solitary *Cnemidocarpa verrucosa*. However chemical release as a defence in ascidians is not the lead point of this study, it merely may be a factor that promotes their invasion success.

1.7 Research aims

The theoretical framework of this research presumes that the ability for non-indigenous colonial ascidians to locate suitable substrate and establish quickly is the sole determinant of their invasion success. Research has illustrated that the settlement within highly competitive hard bottom marine communities may not be random nor based on chance, organisms have been observed to actively seek substrates in relation to texture with notable preferences for rougher substrates (Lapointe & Bourget, 1999, Chase et al, 2016). Within this, mechanisms such as tolerance, avoidance and defence have been reported in basibiont organisms to reduce the settlement pressures of epibiont organisms (Wahl, 1989). Such mechanisms and advantages have been observed predominantly in non-indigenous species that become established in native communities (Shenkar et al, 2008; Lambert, 2001, 2005, 2007).This infers that there is a correlation between the status of a species (non-indigenous or native) and their ability to locate a stronghold on a surface. When non-indigenous species are introduced to native communities of sessile marine invertebrates, I propose that they possess competitive advantages that contribute to their invasion success and ability to heavily occupy and dominate such communities.

In view of this, I hypothesize that in lieu of coevolution, NIS colonial ascidians possess competitive advantages that aid in their establishment success in their invaded range. In this study, I aim to establish:

- If there are possible settlement pressures next to test species (NIS and native)
- The possible settlement preferences of larvae settling as epibionts and on bare space
- If there are differential settlement distances between NIS and native recruits from the test species.

1.8 Organisation of thesis

In this study, I used the lines of evidence derived from *ex-situ* manipulative experiments to examine the potential competition (epibiotic or bare space settlement) recruiting species may have on NIS and native ascidians. I examined if the status (NIS or native) of test species impacted the settlement of the NIS and native recruiting species.

Chapter 1 provides an introductory overview of marine non-indigenous species and the importance of marine biosecurity in New Zealand.

Chapter 2 presents the development of a robust experimental ascidian culture system. This is needed in order to sustain subsequent experimental work. The difficulty and novel process of rearing ascidians *ex-situ* is presented as are current methods of ascidian culture, and methods used in this study are presented in detail.

Chapter 3 presents the observational and manipulative experiments. This chapter attempts to test the given hypotheses through; (1) the experimental design, (2) and methods of the observational and manipulative experiments in this study, (3) the deployment of the test species in experimental containers, (4) data analysis, (5) results and (6) discussions and conclusions.

Chapter 4 draws conclusions from the findings and provides recommendations for future research.

CHAPTER 2.

DEVELOPING A ROBUST EXPERIMENTAL ASCIDIAN CULTURE SYSTEM

2.1 Introduction

Rearing colonies under controlled conditions reduces the specimens' exposure to disease, disturbance, natural disasters, and environmental variabilities (Leal et al, 2014). When successful, colonies can survive up to one month under such conditions at temperatures around 13°C to 16°C (Lambert, 1979). Rearing healthy test species of ascidians *ex-situ* required experimentation of different rearing techniques over a 12-month period. Rearing the colonies *ex-situ* was paramount, as test species needed to (1) reach a sufficient surface area (5cm^2 or larger) in order to be large enough to be correctly identified; (2) monitored for health and confirm recruit settlement; (3) allow for time for test species to naturally attach to the experimental plate.

There is a need to develop ascidian culture methodologies and techniques, as it will aid in the understanding of the traits that contribute to the invasion success of NIS ascidians (Rinkevich & Fidler, 2014). Ascidians occupy water depths from 0.3-2m deep (Rinkevich & Fidler, 2014). *In-situ*, ascidians have been known to survive in eutrophic waters (Zhan et al, 2015), as well as preferring cryptic environments (Carlton, 2007). Previous literature reports that ascidians are more prominent in tropical regions, where water temperature is higher (Lambert, 2007). Ascidians can tolerate a wide range of salinities (between 25 and 40‰) (Lambert, 2005), a broad range of temperatures with some species such as *Ciona intestinalis* tolerating water temperatures as high as 35 °C (Dybern, 1965).

Under *ex-situ* conditions, stocking density must be considered in accordance with feeding (Keough, 2003). Too many colonies in one tank may reduce the metabolisms of the test species and result in stress responses (Joly et al, 2007). Ascidians are efficient filter feeders (Stabili et al, 2016), and can filtrate a broad range of particle size classes (Worm et al, 2006). The feeding of microalgae to ascidian cultures has been used to rear ascidians in previous work. The green alga *Dunaliella primolecta* has been fed to the solitary ascidian *Pyura stolonifera* in previous studies (Klum, 1984). Other studies have fed A 1:2 ratio mixture of the algae *Chaetoceros gracilis* (IFREMER strain from Argenton) and *Isochrysis galbana* (Tahitian strain from Roscoff Culture Collection) once every two days to sustain ascidian colonies (Kenworthy et al, 2018). Rinkevich & Fidler (2014) fed colonies of *Didemnum vexillum* an algal mix of (~1:1 ratio) of *Isochrysis*

galbana. A species of Haptophyta that is commonly used to feed bivalve larvae, additionally the mixed diet approach has been reported as the best option for a monotype diet in aquaria (Rinkevich & Fidler, 2014). However, the feed chosen to sustain the ascidian colonies in this study was the red microalgae *Rhodomans salina* (Superclass, Cryptomonoda), chosen as it has been cited as the preferred food for the colonial ascidians in previous research (Berrill, 1947).

The use of unfiltered seawater and tanks with circulating water flow is a popular method used in many research experiments (Mackie et al, 2006). The use of unfiltered and renewed seawater from the specimen collection site for rearing colonies in aquaria has been used in previous research, which was proven to extend in-tank residency and survivability (Kenworthy et al, 2018). Literature suggests that using flow through systems may reduce environmental oxidative stress (Tasselli et al, 2017). *Pyura stolonifera* has been reared in filtration and respiration systems also using filtered seawater, however water was exchanged once a day (Klum, 1984), more frequently compared to other studies.

Other studies reared colonies at a smaller scale, using smaller fragments, Rinkevich & Fidler (2014) grew colonies of *Didemnum vexillum* on glass slides, using thin cotton threads to aid the fragments in natural settlement (firmly attaching the fragments to the glass). Some specimens underwent the cleaning of their surfaces with small paint brushes, to remove debris (Rinkevich & Fidler, 2014).

The rearing of solitary ascidian species of *Corella* has been shown in previous research to survive without the need of additional food using unfiltered flow through seawater systems (Mackie et al, 2006). A cost-effective seawater tank system at Marine Laboratories UC Santa Barbara was developed and used raw and unfiltered seawater to feed and maintain colonies of two species of solitary ascidian, *Ciona intestinalis* and *Ciona savignyi* (Moody et al, 1999). This aquaria system was named ‘The Raw Seawater System’ and suggested that solitary ascidians could grow and survive on the unfiltered seawater alone as a food source (Moody et al, 1999).

The use of flow through tanks was not possible in this study, the availability and access to seawater in this study was limited. The laboratory lacked the resources to construct large flow through seawater tanks, in addition, funding such construction would exceed the research allowance. Therefore, tank-based rearing was undertaken, with manual operations of water exchange, feeding and monitoring of temperature, salinity and light.

Despite many studies on ascidians under the umbrella of biology, few studies have explored the techniques used to culture ascidians as well as accompanying experimental procedures. No

solidified and widely adapted method for ascidian rearing exists, therefore developing a robust ascidian culture system is a novel process. Previous studies have shared ascidian culture methodologies yet vary in technique and approach (Rinkevich & Fidler, 2014) and (Lambert, 1979). Due to the lack of shared information on the culturing and maintenance of healthy ascidians, the establishment and development of a satisfactory methodology required time. This emphasizes the rarity of this thesis and the contribution it will have to the field of marine invasion ecology.

The following chapter will present the culture techniques and methods undertaken to develop a robust, repeatable methodology for further research. This will include the methodologies of the following (figure 2.1):

- The field collection and processing (removal from substrate and transplantation onto artificial substrate) of ascidian colonies from the Tauranga Harbour
- The culture techniques used for ascidian colonies under temperature and light controlled conditions
- The techniques of culturing of *Rhodomonas salina* (a red microalgae) as a feed
- The construction and rationale of deployment apparatus, as well as the protocol and itinerary of the deployment of the biosecure environmentally controlled container apparatus

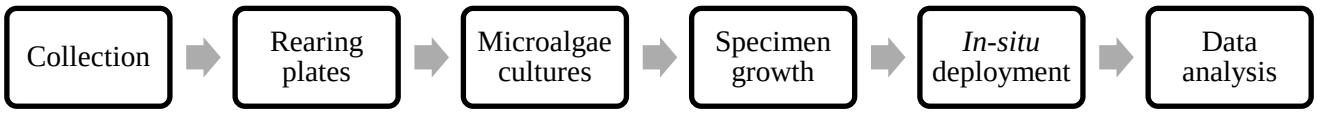

Figure 2.1: Schematic diagram displaying the general methodology undertaken in this study

2.2 Field collection methods

Approximately 100 individual colonies of the four test species: (1) *D. vexillum* (NIS), (2) *Didemnum incanum* (native), (3) *Botrylloides leachi* (NIS) and (4) *Botrylloides* sp. (NIS) were collected and grown *ex-situ* over the course of this trial. The Marine Biosecurity Porthole (an online database containing information and locations of NIS in New Zealand, a NIWA and MPI collaboration) was also used to identify sites within the Tauranga Harbour where species may be located. Native species were identified and collected first, with the notion that they will be more difficult to find and keep alive in aquaria.

Native and non-indigenous colonial ascidians were collected from the Bay of Plenty coastal region in the North Island of New Zealand, between January 2019 and August 2019 (Austral summer to late winter) (figure 2.2). Ascidians were collected by means of SCUBA diving, snorkelling or by hand from wharves. These collection methods varied depending on the species and location. To illustrate, test species were collected by hand manually from the wharf sides and pylons at Sulphur Point Marina and Tauranga Bridge Marinas, while test species at Salisbury Wharf were collected on snorkel as the colonies were located 1-2 metres below the water surface.

Specimens were identified briefly in the water, then scraped gently from the substrate and placed into a cool box filled with seawater from the collection site. Between 200-500mL of *R. salina* (cells/ml) (figure 2.4) was added to the cool box. Aeration was used to maintain dissolved oxygen between 5-7 m, and the lid was closed throughout transport to reduce solar stress. The cool box containing the specimens was then transported to the University of Waikato Coastal Marine Field Station (CMFS). Care was taken during transport to minimise stress induced movement by light, increase in temperature and contact disturbance, and all transport periods were limited to less than 40 minutes.

Once transported back to the CMFS, the cool box with the specimens was placed into a biosecure air-conditioned container (20 °C) to acclimate for 24 hours prior to further processing. All collected species and numbers were recorded in order to provide information for The Ministry of Primary Industries (MPI) if needed

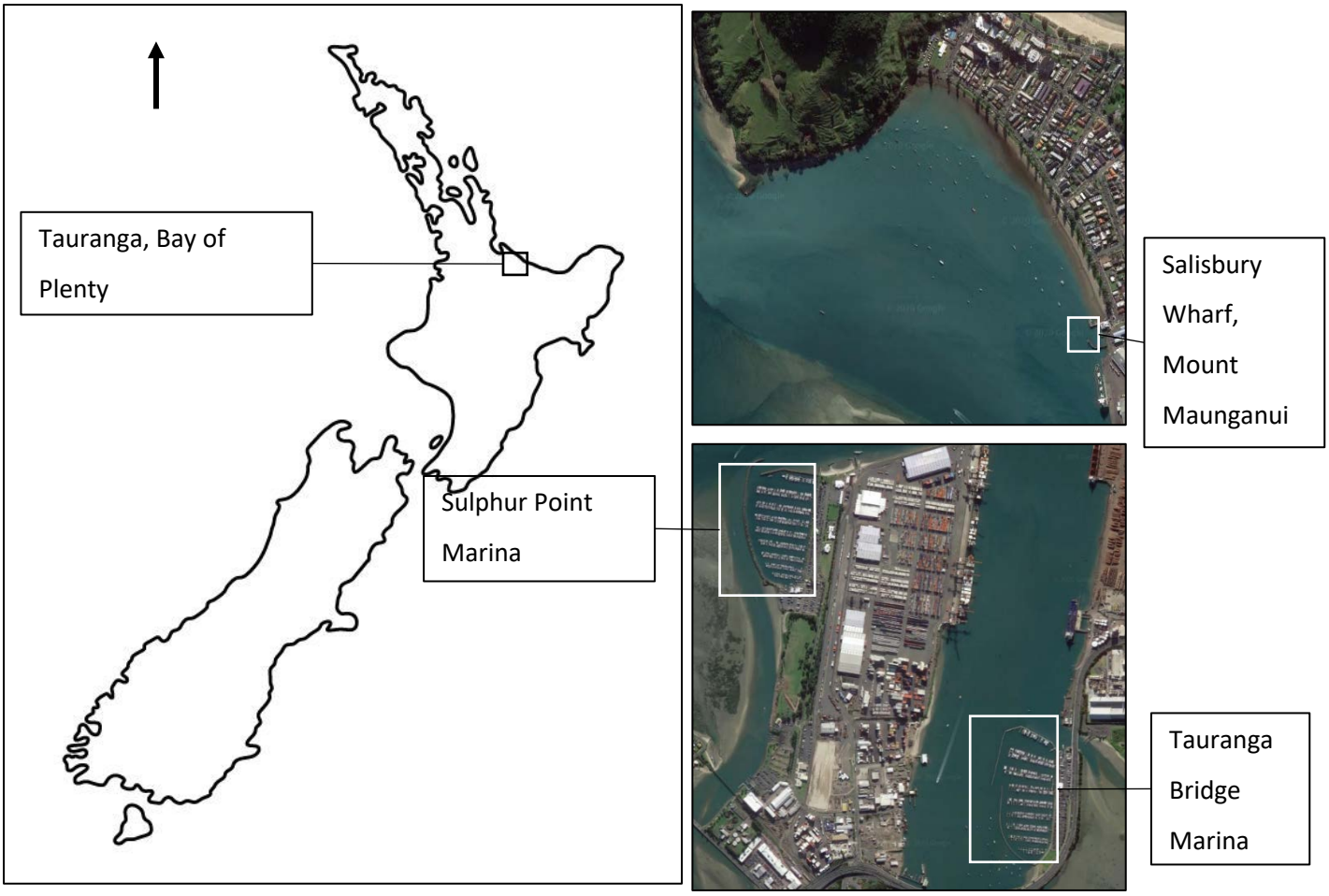

Figure 2.2: GIS map of the study site locations within the Bay of Plenty Tauranga Harbour: Matakana Island Wharf (Matakana Island), Pilot Bay Stone Jetty (Mount Maunganui), Salisbury Wharf (Mount Maunganui), Sulphur Point Marina (Mount Maunganui) and Tauranga Bridge Marina (Tauranga).

2.3 Aquaria methods

Ten 20 L glass tanks (W=25 x L=40 x H =20cm) were filled with filtered seawater (FSW), and kept in specifically fitted-out, biosecure, temperature-controlled shipping containers. Salinity was monitored using a refractometer with measures taken to maintain a salinity of approximately 35 ± 0.1 t. The pH (7.90 ± 0.1) of all tanks was monitored using the API water quality testing kit, the temperature (18°C ± 1°C) of all tanks was monitored HOBO (UA-002-64) data loggers. In order to obtain FSW, sea water was collected from the Sulphur Point channel in Tauranga Harbour, collected at a depth of 1-2m below surface. This water was kept indoors in 1000 L intermediate bulk containers (IBC), before the aeration by several aerator stones and constantly pumped across a 36 W ultraviolet lamp, then filtered through a 20 µm mesh filter followed by a 0.5 µm mesh filter. Two weeks before the observational and manipulative experiments (in harbour deployments), the tanks were linearly acclimated to 23 °C from 20 °C over the span of

two weeks, this was to ensure tank temperatures were similar to harbour temperatures before deployment.

The 20 L glass tanks in the biosecure container were open and exposed to air yet isolated from any dust and particle sources, to ensure water quality. The tanks were aerated constantly with single pipeline aerator stones, which were replaced every three weeks to ensure maximum oxygenation and flow of the water. Tanks were subject to natural light conditions, three large windows in the laboratory allowed for natural light and dark phases. The glass tanks were located on a perpendicular wall to reduce solar exposure. Tank conditions such as water pH, temperature, salinity, and chlorine content were monitored once every two weeks, throughout the experiment. Tanks were briefly cleaned weekly to deter biofilm build up. Once a week four 20L containers were filled with FSW from the 1000 L IBC tanks and stored in the laboratory to acclimate them for 24 hours. This water was then used to exchange 50% of the water of each tank with specimens.

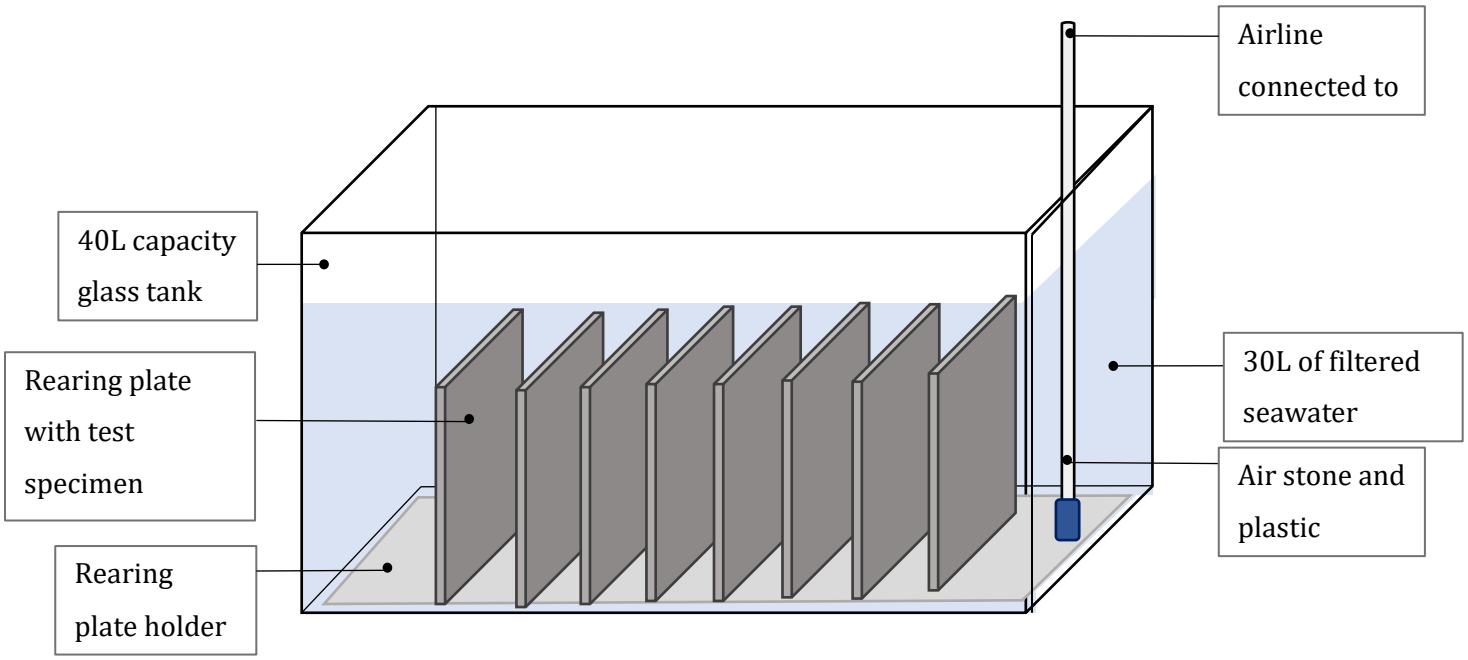

Figure 2.3: Schematic diagram of a 40L tank set up, with a plate holder, rearing plates containing test species, air tubing and airline organisation and equipment.

2.4 Microalgae culture methods

The feed chosen to sustain the ascidian colonies was the red microalgae *R. salina* (Superclass, Cryptomonoda). *R. salina* was chosen as it is one of few that is available in New Zealand and the only genus represented in literature to successfully grow ascidians (Berrill, 1947).

In a temperature and light controlled laboratory (20°C ± 1°C), flasks of *R. salina* cultures were kept next to light emitting-diode lamps (LEDs) emitting 20 Watts of LED light constantly. The three step culture process used to culture appropriate cell densities of *R. salina* microalgae to feed colonial ascidians consisted of; (1) Inoculation of 250mL flask, (2) Inoculation from 250mL flasks to 2L aerated flasks, and (3) Inoculation from 2L aerated flasks to a 20L aerated carboy (figure 2.4). Sterile conditions were maintained when opening and closing flasks during inoculation using ethanol spray (70% ethanol and 30% freshwater) to sterilise the room and equipment.

2.4.1 500mL flask inoculation

R.salina colonies were first inoculated in 500mL flasks. 150mL of FSW was added to four empty flasks, the four flasks were sealed with paper bungs, the top wrapped in aluminium foil and taped with one strip of autoclave tape. These four flasks were then placed into an autoclave and set to sterilise through pressurised steam as a sterilization agent. Once this sterilisation was complete, the four 150mL flasks with now sterilised FSW were left in the biosecure container for 24 hours to acclimate to laboratory temperature (20°C).

Once acclimated, the flasks were set in a fume hood, and both the fume hood and flasks were sterilised with ethanol spray. A Bunsen burner was prepared and lit; the rim of each flask was sterilised with the flame before adding 250 µm of nutrient (F/2 medium) with a pipette into each flask. The flasks were quickly sealed between the addition of F/2 medium to reduce exposure to the air. Once all flasks contained nutrient, one flask containing 250mL of mature (high cell density) *R. salina* was divided into the four flasks, adding approximately 60mL of *R. salina* to each flask. Once inoculation was complete, all flasks were sealed and returned to the biosecure container. The newly inoculated flasks were then labelled with the date and contents (*R. salina*) and set by the LED lamps for approximately 7 days to duplicate and increase in cell density, eventually turning red in colour.

2.4.2 2L aerated flask inoculation

After 7 days, once the four 250mL flasks were red in colour, indicating high cell density of *R. salina*, they were upscaled into 2L aerated flasks. Of the four 250mL flasks, only two were upscaled, leaving two 250mL flasks as a contingency.

Two 2L flasks were filled with autoclaved 1.5L of FSW, again left to acclimate to the laboratory temperature of 20°C. Once acclimated, the two 1.5L flasks and two 250mL flasks were set in the fume hood (both the flasks and the fume hood were sterilised with ethanol spray). The rims of all flasks were sterilised by flame before adding 2.5mL of nutrient (F/2 medium) with a sterile syringe into each 1.5L flask. All flasks were bunged between the addition of F/2 medium to reduce exposure to the air. Once the 1.5L flasks contained nutrient, one whole flask of 250mL *R. salina* was added to each 1.5L flask. The newly upscaled 1.5L flasks were labelled and sealed with an aerated bung containing three glass pipes (figure2.5).

One longer pipe reached to the bottom of the flasks as an airline, connected by a tube to an airline to promote flow and oxygen to the culture. One shorter glass pipe was connected to tubing with a syringe barrel at the end, filled with an absorbent material used to release CO^2 and moisture. The other shorter glass pipe was connected to tubing that was tied off at the end, this was used to remove algae from the flasks when feeding so that the entire bung did not need to be removed. The two flasks were then set next to the LED duplicate to reproduce and duplicate for a further 7 days.

2.4.3 10L carboy inoculation

Once the 1.5L flasks indicated a desirable cell density (by red colour), they were then able to upscale into large 10L carboy containers, their final phase before feeding. Two 20L carboys were filled with 10L of FSW each, due to the size and volume of liquid the water could not be autoclaved in this instance for sterilisation, therefore using a sterile syringe, 10 mL of bleach (MaxKleen Pure Hospital Grade Sanitising Bleach) was added to each carboy (1:1000 ratio). The two carboys were set aside in the laboratory for 24 hours to both acclimate and thoroughly sterilise. Following the sterilisation, 20 mL of sodium thiosulfate was then added to the bleached water and the carboy was then set aside for one hour to allow for the chlorine content to decrease.

Aquacheck by Hach Chlorine strips were used to check the total chlorine and free chlorine (it is desirable to have 0-50 chlorine parts per million). Once suitable, an aerated bung was placed into each carboy. 10 mL of F/2 medium was added, using ethanol spray to sterilise the carboy opening. 1.5L of a ready culture was poured into each carboy. Bungs were returned and the carboys were connected to the air line and set by the LED light for a further 10 days.

When cultures of algae were ready to feed the ascidians (indicated by a deep red colour), 500mL was extracted from the carboy each day and poured into the ascidian tanks. To maintain carboy cultures, 10mL of F/2 medium was added once per week.

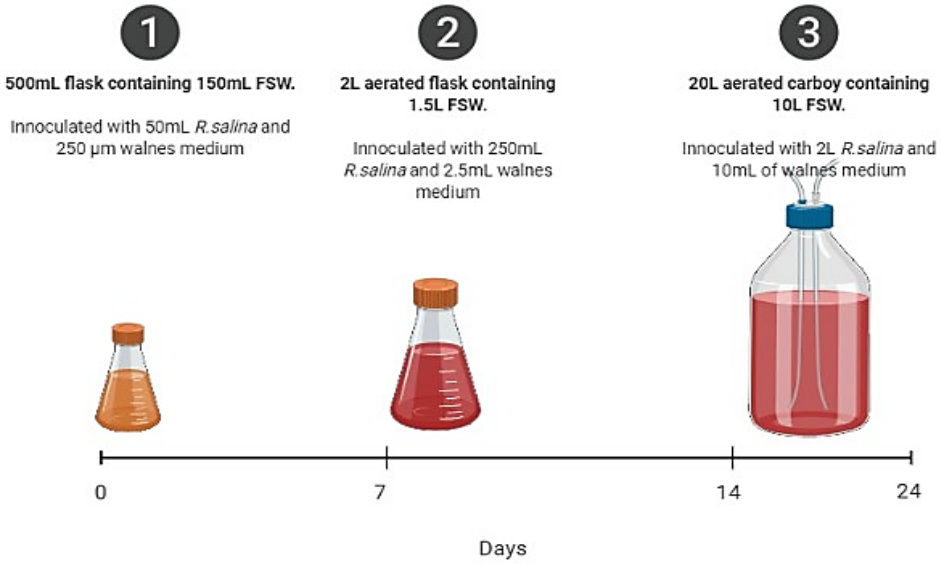

Figure 2.4: Schematic diagram illustrating the microalgae culture process across a daily timeline (created with BioRender.com)

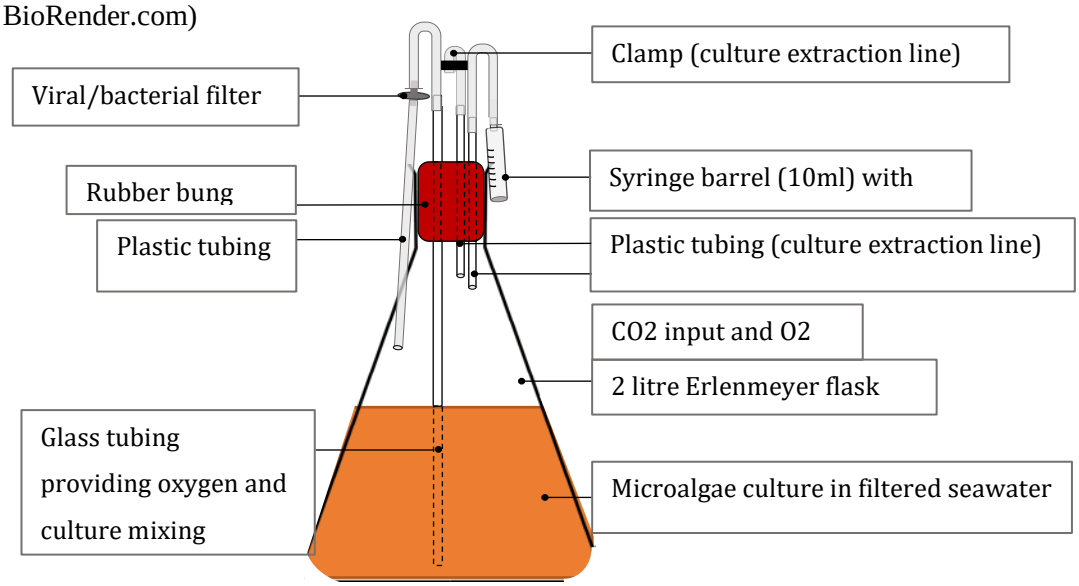

Figure 2.5: Schematic diagram illustrating the crude organisation of a 2L flask holding 1.5L of an aerated microalgae culture.

2.5 Colony transplantation to an experimental plate

Collected colonies of NIS and native ascidians were placed immediately into containers with aerator stones, seawater, and *R.salina* once removed from their substrate in the field. These containers were kept in a large cool box with the lid closed to reduce solar exposure and temperature increase of the water. Upon arrival to the CMFS laboratory, the test species were placed in their containers into glass acclimation tanks (maintained at a similar temperature to that of their collection site) located in the biosecure container, aerator stones were placed into the tanks and the test species were left to acclimate over a 24 hour period.

To attach each test species to an experimental plate, they needed to be removed with care from substrate they had settled on. The fracturing of individual colonies was dependent on original size, health, extent of teste and location of the growing edge. Once fully acclimated to the temperature and salinity of the tanks (approximately 24 hours), the test species were removed from their substrates and transplanted onto rearing plates. Here, colonies were handled as briefly as possible to avoid stress. All transplantation and attachment occurred by hand with the test species remaining submerged in sampling trays filled with seawater. To begin, each colony was reviewed under a microscope (stereo microscope: Nikon C-Ws10 x B/22 SM21000) to give a brief esti0mate of zooid health and life. They were then segmented into smaller sizes ranging between 1-10 cm with a scalpel, taking care to avoid common atrial siphons and individual oral siphons.

Specimens were then placed gently onto a 14x14 cm projector film strip which was then glued to a 14x14 cm Polyvinyl Chloride plate (PVC). Colonies were attached to the projector film and not directly to the experimental plates as the film allowed for colony removal from the plate without disturbing the specimen, when deployed in the experimental containers. The projector film had been sanded (700 grit) to imitate marine substrate and provide a more desirable surface to settle on. Each specimen was individually glued to their plate using approximately 0.5 mL of reef glue (Seachem Reef Glue Cyanoacrylate Adhesive), making sure that the teste and growing edge of the animal lay flat against the projector film to promote attachment and further growth.

Each experimental plate and its specimen were labelled according to collection date and location, they were then placed into prepared glass tanks containing 30L of FSW and 500mL *R.salina* feed. Rearing plates were placed flat to allow time for the colony fragments to start active attachment without the added pressures of gravity when placed upright (Rinkevich & Fidler, 2014). After 24 hours of lying flat, the rearing plates were then slotted into a plate holder (figure

2.3). This previously prepared plate holder allowed the colonies to sit at 130° angle to prevent algae, faecal matter and other organic matter build up and promote water flow across the specimens (figure 2.3 and 2.6).

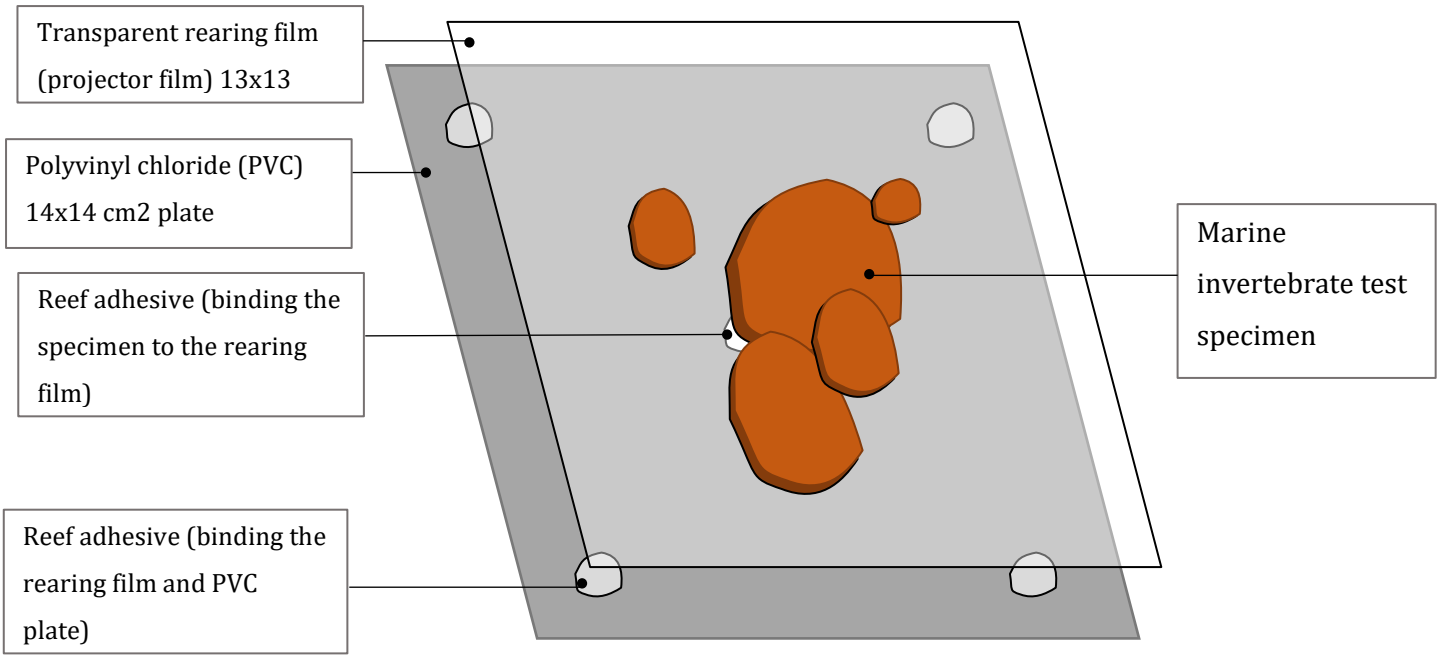

Figure 2.6: Schematic explosion diagram of rearing plate organisation with a test specimen.

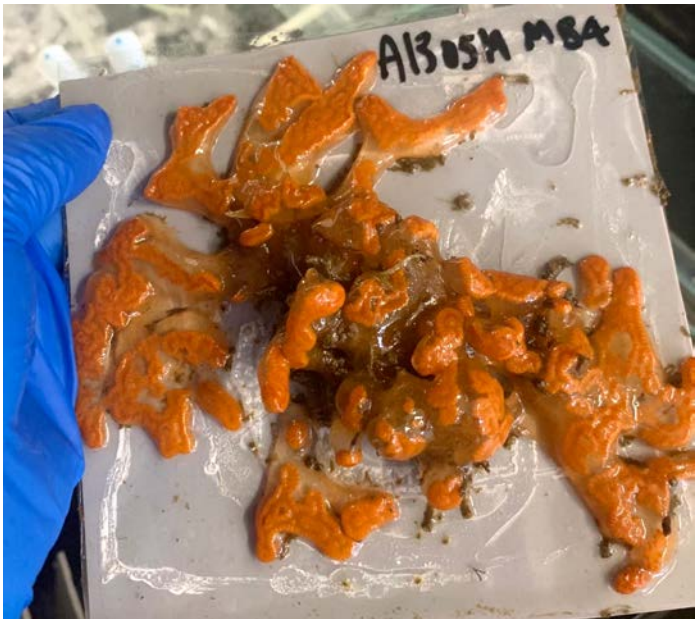

Figure 2.7: A *Botrylloides violaceus* test species, showing natural attachment to the rearing plate.

CHAPTER 3.

OBSERVATIONAL AND MANIPULATIVE EXPERIMENTS

3.1 Introduction

3.1.1 Habitat preference

The global increase in urbanization and increase in availability of artificial substrates in marine environments can expand the habitat range of NIS (Johnston et al, 2017). Colonial ascidians prefer cryptic habitats and submerged substrates, with this preference for shaded habitat, photonegative ascidian larvae will ultimately determine the distributions of their communities when settling on substrate (Feng et al, 2010; Forwars et al, 2000, Sorokin, 1995). Thus, having a strong influence over the community composition and structure of marine hard bottom communities (Svane & Young, 1989).

As benthic systems are often space limited, submerged substrates are under high demand with many taxa competing over the same habitat (Quinn, 1982) (see figure 1.3). Therefore, substrate and resource availability are key factors that can determine settlement success of sessile species (Davis et al. 2000). It is widely recognized that submerged anthropogenic substrates (ropes, boat hulls, pylons, wharves, waste surfaces, and concretes) act as a desirable habitat for ascidians (Chase et al, 2016). Artificial substrates in harbours around the globe can facilitate invasion success by selection for NIS over native species, as the spread of NIS from artificial substrate to nearby natural ecosystems is often rare (Lambert, 2001).

The magnitude of NIS ascidian impacts on an introduced region, is frequently determined by the recipient environments biogeographic make-up (Lins et al, 2018). For example, locations with large numbers of ascidians are often linked to warmer climates, in accordance with high rates of primary production and high food availability (Lambert, 2007). Species richness is also found to be the greatest in warm tropical regions, such as the temperate latitudes of the Northern Hemisphere and Temperate Australasia, areas that are often dominated by colonial ascidians (Shenkar & Swalla, 2011).

The sensitivity of benthic organisms to suspended sediments is apparent, yet NIS ascidians are known to thrive in eutrophic environments (Shekar & Swalla, 2011). The increased loading of sediment to catchment river systems and coastal environments supplies nutrient rich loads to suspension feeding communities (Zhan et al, 2015), the eutrophication of coastline waters due to terrestrial urbanization has been connected increases in planktonic blooms and therefore some NIS ascidian populations (Lambert, 2005). For example, an increase in the eutrophication of the Israeli coasts has formed a desirable habitat for NIS ascidians, providing them with advantages to compete for space with native corals and other natives in the region (Shenkar et al, 2008).

3.1.2 Propagule pressure

Propagule pressure is a common concept in invasion ecology that is often referred to as fundamental to invasion success (Johnston et al, 2009). The concept is defined here as the measure of the number of individuals released into an area in which they are non-indigenous (Carlton, 1996; Clark & Johnston, 2009). Propagule pressure is also defined as an event-level characteristic, differing for each introduced population (Blackburn & Duncan, 2001).

The arrival of propagules to an environment must coincide with resource availability, specific to the requirements of the invading species in order to be a successful invasion (Davis et al. 2000), as well as the spatial scale of the receiving environment (Lockwood et al, 2005). It is key to note that NIS propagules may be released yet fail to join local populations (Johnston et al, 2009), emphasizing the importance of resource availability.

The recruitment of a species is often limited by the propagule supply, therefore pressures exerted by NIS propagules will determine their invasion process (Locke et al, 2007, Johnston et al, 2009). The intensity of exposure (an increase in organisms released into an area) and anthropogenic vectors such as vessel frequency and movement will also largely determine the invasion success of a NIS, as the abundance of NIS propagules are often greater in anthropogenic (Clark & Johnston, 2009, Johnston et al, 2009, Locke et al, 2007). More specifically, the Tauranga Harbour is an ideal experimental site for this research as it is frequently exposed to high intensities of vessel movement and therefore greater propagule pressures (Clark & Johnston, 2009).

3.2 Hypotheses

The lines of evidence provided by these experiments will address the previously established hypotheses (H); (1) There will be greater settlement pressures exerted by NIS recruits than natives, (2) NIS recruits will settle more frequently as epibionts than native recruits, (3) Larvae will recruit at greater densities on experimental plates with native test species present than on experimental plates with NIS, (4) NIS recruits will settle closer to both native and NIS test species, (5) NIS recruits will settle at similar densities on experimental plates where NIS or native test species are present, (6) native recruits will settle closer to native test species.

Table 3.1: Table displaying ascidian test species, family, status, and number of experimental plates with the test species. A total of 19 test species and their plates were used in the deployment experiment.

Species	Family	Status	Number of plates
Didemnum incanum	Didemnidae	Native	6
Botrylloides sp.	Styelidae	Non-indigenous	3
Botrylloides leachi	Styelidae	Non-indigenous	5
Didemnum vexillum	Didemnidae	Non-indigenous	5

3.3 Methodology

3.3.1 Experimental design

Epibiont recruits are here defined as individuals settling on the surface of another, and recruits that settle next to or on the bare space of the experimental plate will be here on referred to as bare space recruits. *Ex-situ* manipulative experiments were used to examine potential competition (epibiotic or bare space settlement) recruiting species may have on NIS and native ascidians. Simultaneously, we examined if the status (NIS or native) test species impacted the settlement of the NIS and native recruiting species.

Ex-situ experiments took place at The Tauranga Bridge Marina wharf (Latitude: 37° 40.25 'S, and longitude: 176° 10.5 'E). 20 test species of colonial ascidian (6 native species and 14 NIS) and their experimental plates underwent manipulative *ex-situ* experiments from a wharf of The Tauranga Bridge Marina (see table 3.1). To record the settlement of recruit species, the experimental plates and the test species were left in the water at a depth of 1-2m below surface, for a total of 15 days to allow for recruit larvae to establish successfully on the experimental plates (easily identifiable and measured). Test species and their fouled experimental plates were then returned to the biosecure laboratory. Experimental plates were photographed, and the data

of each plate (recruit counts, sizes, species, status, and distance metrics) were analysed using ImageJ (version 1.53a) (Rasband, 2006) and Microsoft Excel.

Environmental factors such as weather and season, determined the timing and longevity of this experiment. In addition, ideal tidal and weather conditions (calm, flat, high tide, no storm events, or large amount of fresh-water inputs) were required to ensure deployed containers were not damaged or lost. In order to meet the water temperatures similar to that of the biosecure laboratory (18°C ± 1°C), deployments had to take place in spring or summer (November-December in New Zealand), this also coincided with optimal larval spawning and algae blooms in the harbour that would enable the test species to have the best chances of growth and survival.

3.3.2 Experimental construction methods

To manipulate and control the settlements of new recruitments, settlement reduction containers were constructed. The experimental containers had to hold the test species and their plates in the water column securely, while also allowing water and nutrients to pass through. The containers also had to exclude larvae or allow recruits to settle depending on the lid position. Experimental containers were needed as test colonies and recruits needed time to grow once attached to the experimental plates. Colonies needed to grow past the ancestrula stage to where they had the morphological characteristics to be identified.

Experimental containers were modified from 20 1L ice-cream containers (13 x 13 x 8.5cm), to hold specimens and control exposure to recruiting species (figure 3.4). Two rectangular holes were cut in opposing sides of the container, creating a flow through effect. 100 µm mesh was glued to cover these holes, using marine adhesives (marine grade sika flex silicone and AzkoNobel International epiglue). This mesh allowed for water, light, and nutrients to flow through the container yet prevented larvae larger than 100 µm to enter the container.

Four settlement plate arrays were created using 80mm polyvinyl chloride (PVC) pipes. Each pipe had two large holes drilled on each end of the pipe to allow for 5m rope to be tied to the pipe and hold bricks at the end for stability in the water column. A series of 5 smaller holes approximately 1 cm in diameter were drilled along the centre of each of the four pipes (20cm apart) so that cable ties could secure the experimental containers (see figure 3.1 and 3.4). Once fully assembled, PVC pipes were labelled with a standard marker to better identify them from the wharf.

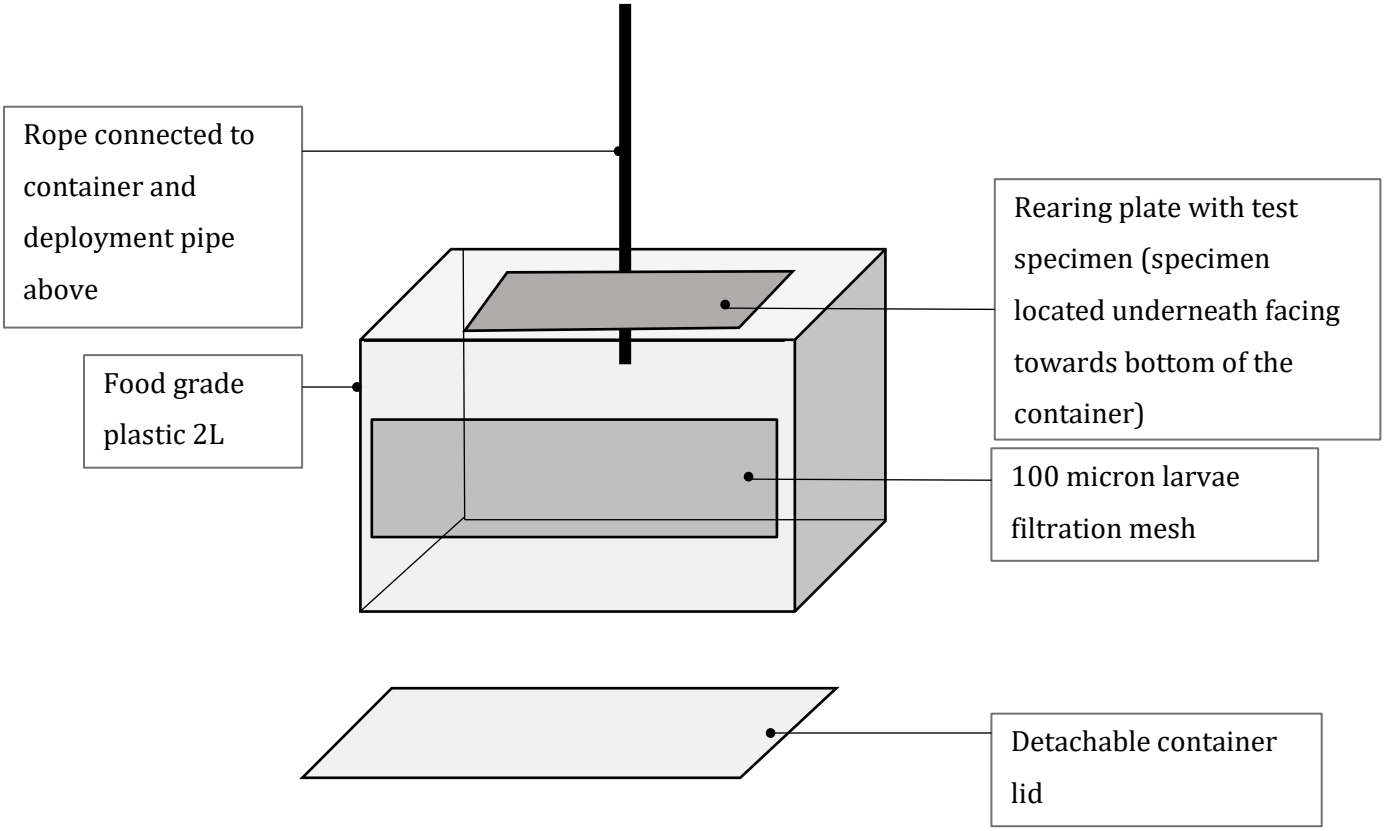

Figure 3.1: Diagram of the experimental container design. Used to hold colonies in the water column and allow water flow through the container.

3.3.3 Harbour deployments

Colonies attached to their projector films were randomly assigned positions on settlement arrays to ensure no bias was introduced to their assortment and position on the wharf when deployed. Photographs of each of the 20 specimens were taken on a Canon EOS 1500D camera from a height of 30 cm (scale=20 mm). Projector films containing the test colonies were then gently removed from their rearing plates and ordered into their pipe code groups in a bucket of FSW. The buckets of FSW with the test species on their projector films contained two aerator stones and 500 mL of *R. salina*, to oxygenate the water and reduce stress during transport to the deployment site at the Tauranga Bridge Marina (figure 3.2).

At the Marina on Pier A, the PVC array and experimental containers pipes were placed next to the deployment location (figure 3.3). These locations were selected along the pier due to their access to cleat horns, availability of space due to docked vessels and their location in correlation to the high flow of the channel. The tide flows through this channel. A snorkeler entered the water, and the pipes were lowered one at a time into the water until they were submerged just

below the surface. The pipes were held upright by the snorkeler with the experimental containers facing upright and open, to ensure the person on the pier could access the containers and begin allocating and gluing test species (attached to their projector film) to their experimental containers.

The colonies were then glued with reef glue (Seachem Reef Glue Cyanoacrylate Adhesive) onto their corresponding container plate underwater. The glue was left to set for 10 seconds, after which the pipe was gently lowered to a depth of approximately 1.5 m below the water surface without the lids attached. Each end of the rope was then tied off on the cleat horns of the pier and an attention sign was placed at each individual site, to reduce public interference. This process was repeated a further three times until all four pipes and their containers with test species inside were in place and secure.

Figure 3.2: The Tauranga Harbour, with the Port of Tauranga on the left and the Tauranga Bridge Marina on the right. Image from Ship technology.

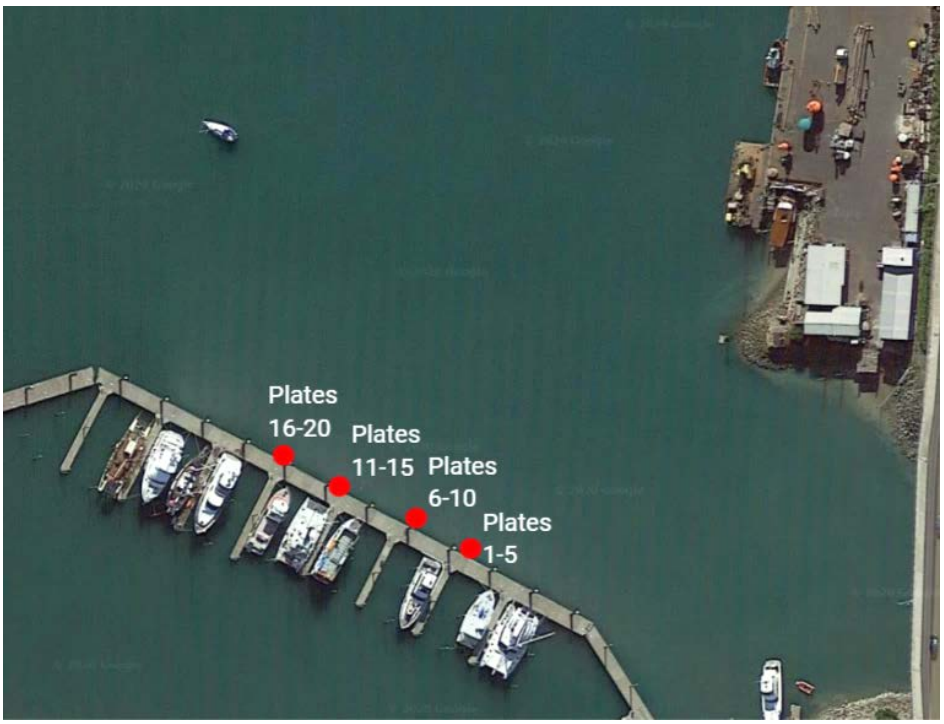

Figure 3.3: The experimental container deployment location. Tauranga Bridge Marina Wharf A. In the Stella passage channel. Image retrieved from Google Maps (created with BioRender.com)

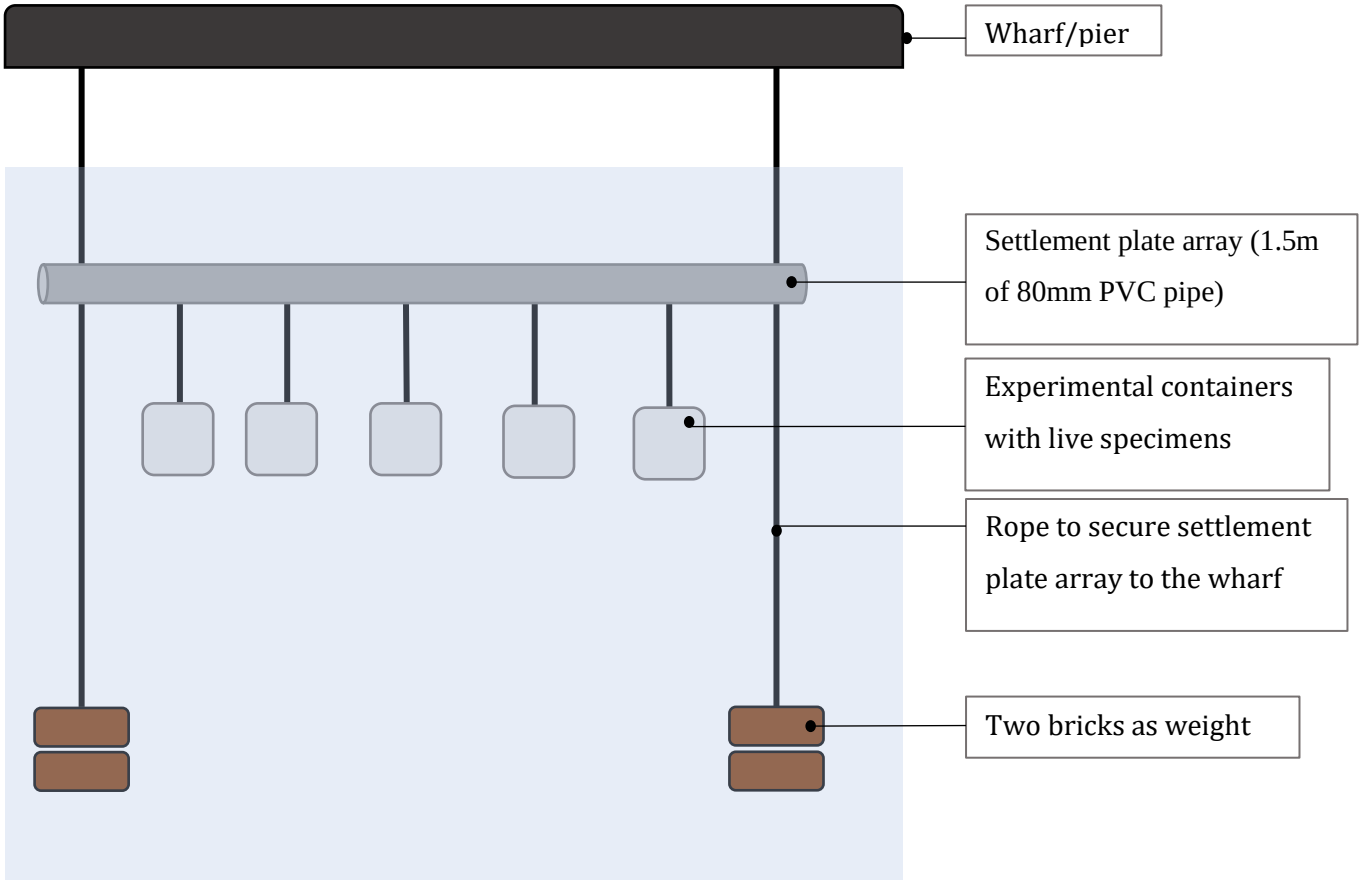

Figure 3.4: Concept diagram of the settlement plate array and its equipment, used to deploy and hold containers 1.5 m below the water surface

Containers were left in the water for a total of 15 days to allow natural settlement of benthic sessile invertebrates. Settlement arrays and test colonies were monitored initially after 2 days to ensure the success of the pipe and container deployment design. Any loose film edges were glued down, and preliminary observations were made with notes as quickly as possible to avoid specimen stress. Following the initial check, arrays were then monitored every third day, to remove debris such as sea lettuce (*Ulva* sp.), sea grass (*Zostera muelleri*) and algae build up were gently removed from container mesh surfaces.

After the 7-day recruitment period, with the lids off, the lids were attached to restrict/prevent natural settlement for an additional 7 days. The additional time allowed for recruiting species to metamorphosize and grow to aid in later identification. Replacing lids was achieved by hand from the wharf, pulling the pipes, being careful to not expose them to the air, and quickly attaching lids.

On day 15, containers and their pipes were removed entirely from the water. The ropes and pipes were gently pulled up again, revealing the containers yet ensuring they remained underwater. Experimental plates were removed very quickly from their containers and placed upright (PVC rack) (figure 2.3) into a large cool box containing harbour seawater, 500 mL *R. salina* feed and three aerator stones. The lid on the cool box was kept closed between the additions of test species to maintain temperature (20 °C) to avoid temperature and light stress.

Once all plates and equipment were collected, the specimens were brought back to the CMFS laboratory. The cool box with the test species was placed immediately in the biosecure container and left to acclimate for a further 72 hours. Once fully acclimated to laboratory conditions, the colonies were fed with 500 mL *R. salina*. Each plate was then processed individually; placed into a 10 cm deep tray containing filtered seawater under a camera stand, individual photos of each experimental plates were taken with a Canon EOS 1500D camera from a height of 30 cm (scale= 20mm), to capture the new recruits and any test species growth or death.

3.3.4 Image analysis

19 experimental plates with 19 test species (5 *B. leachi*, 3 *Botrylloides* sp., 5 *D. vexillum* and 6 *D. incanum*) were analysed in this study (a total of 20 plates were deployed but one plate was lost during the experiment). Data collected from all 19 experimental plates was analysed to establish recruit species, recruit status, recruit counts, epibiont and bare space recruit frequency and recruit location on the plate (mm).

To gather multiple lines of evidence from the experimental plates, photographs were taken of all 19 plates before they were deployed and when they returned after deployment. Photos were taken with a Canon EOS 1500D camera from a height of 30 cm (scale= 20mm).

Images of the plate assemblages were analysed using the program ImageJ (Rasband, 2006). The size of the test colonies pre and post deployment were measured. Larval recruits of one or more zooids were considered, they were first identified as either epibionts (settling on the surface of the test species) or as bare space recruits (settling adjacent to the test species). Bare space was defined as any inanimate substrate and occupied space (epibiont space) was defined as the exterior surfaces of any organism. The entire plate space was measured and referred to as total plate space (19600 mm^2).

Larval recruits were counted and identified by species and status. Specimens were identified at a species level, where this was not applicable, they were assigned a name that closely matched their morphological description and phylum. All specimens were identified under a compound microscope (10x and 40x). Once a specimen was identified to a species level, it was then classed as a status (either native or nonindigenous). Specimens that had an unknown status, were classed as native as they did not meet the morphological characteristics of known NIS in the Tauranga Harbour, and therefore were assumed as native (specifically the recruits Arborescent bryozoan and Yellow sponge). The distance of each recruit from the test species was then measured using ImageJ (2cm=20mm).

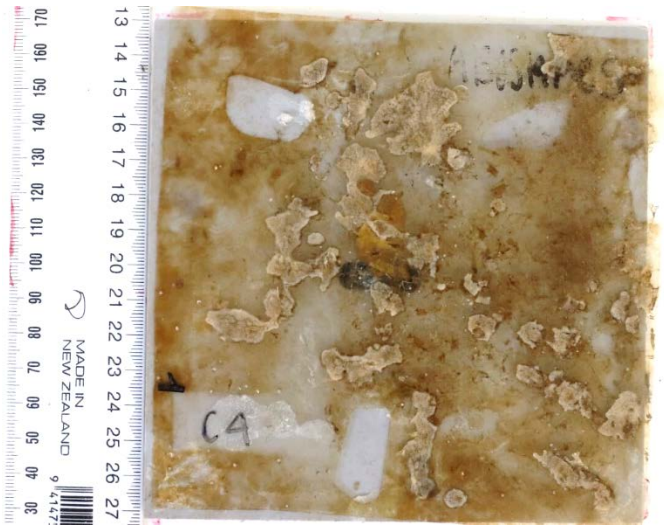

Figure 3.5: An example of plate image analysis post-deployment, a ruler for scale.

3.4 Data analysis

The larval settlement data on or adjacent to test species (mm^2) was not normally distributed, and thus violated the assumptions of parametric testing, therefore, non-parametric testing was used. To determine the difference in settlement prevalence of NIS and native species , the non-parametric Kruskal-Wallis test was used when more than two groups existed or the Mann-Whitney U testing was used when the dependent variable only considered two groups.

A chi-square test for independence (correlation for association) ($X^2=\Sigma(O-E)^2/E$) was conducted to determine if the association between substrate preferences and NIS or native test species was significant.

As the data was not normally distributed, the data was transformed using log10 to meet the assumptions of the parametric test. The distances of the recruits could not be compared statistically at a species level as there were not enough densities to calculate a mean, for example only one individual from one species settled on the experimental plate, therefore the data could not meet the assumptions of a comparison of means and a One-Sample T-Test was used. Differences in settlement distance (mm) were analysed using a One-Way ANOVA. The general differences across test species and recruit species settlement distance (mm) were analysed using a Factorial ANOVA.

The independent variables for distance analyses were test species status and recruit status (NIS or native), and the dependent variables for this analysis were distance (mm and mm^2).

3.5 Results

A total of 2325 individual recruits were recorded in this study, NIS were the most prevalent (table 3.2). Of the total 2325 recruits, 82.9% were non-indigenous species and only 17% were native species. 89.7% of the total recruits settled on the bare substrate adjacent to the test species and 9.7% settled as epibionts on the surfaces of the test species.

Table 3.2: Counts of native and non-indigenous recruits across the four test species (*B. leachi*, *Botrylloides sp*, *D. vexillum* and *D. incanum* from a total of 19 experimental plates).

Plate number	Test species	Native recruits	Non-indigenous recruits
Plate 1	*Botrylloides* sp.	6	207
Plate 2	*B. leachi*	22	44
Plate 3	*D. incanum*	115	128
Plate 4	*D. incanum*	11	204
Plate 5	*B. leachi*	2	112
Plate 6	*Botrylloides* sp.	0	173
Plate 7	*D. vexillum*	53	57
Plate 8	*B. leachi*	7	70
Plate 9	*D. incanum*	47	255
Plate 10	*D. vexillum*	7	72
Plate 12	*B. leachi*	11	286
Plate 13	*D. vexillum*	22	101
Plate 14	*D. vexillum*	21	77
Plate 15	*B. leachi*	15	22
Plate 16	*D. incanum*	17	65
Plate 17	*D. incanum*	16	20
Plate 18	*D. vexillum*	6	1
Plate 19	*Botrylloides* sp.	11	21
Plate 20	*D. incanum*	7	14

3.6 Epibiotic and settlement on bare space adjacent to test species

More larvae settled per mm^2 as epibiont on the test species than settled on bare space adjacent to the test species. More larvae were recorded as settling as epibionts (M= mean) (M= 0.531 mm^2) on NIS test species than on native test species (M= 0.287 mm^2) (figure 3.6). Moreover, a greater number of larvae settled next to native test species (M= 0.009 mm^2) than NIS test species (M= 0.006 mm^2) (figure 3.7).

Mann-Whitney U testing revealed no statistical significance between test species status and epibionts per mm^2 (Mann-Whitney U = 37.50, p = .895, figure 3.6), and no statistically significant relationship between test species status recruits settling adjacent to the to test species per mm^2 (Mann-Whitney U = 32.50, P = .566, figure 3.7).

The presence of native and NIS recruits was dependent on the status of the test species. Chi-square analysis revealed strong associations between the frequency of recruits as epibionts or bare space recruits on test species (NIS or native) $X2(1, N = 2325) = 88.57$, p = < .001, figure 3.8).

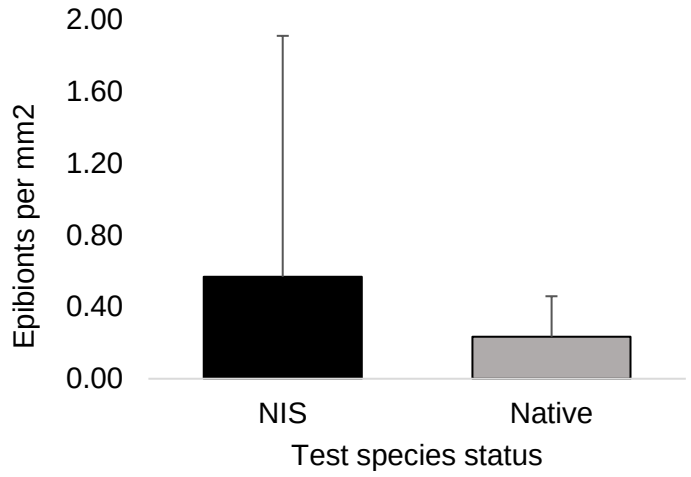

Figure 3.6: Mean epibiont recruits per mm2 on native and NIS test species. With Standard Error of the mean (SEM). Grey represents native species and black represents NIS.

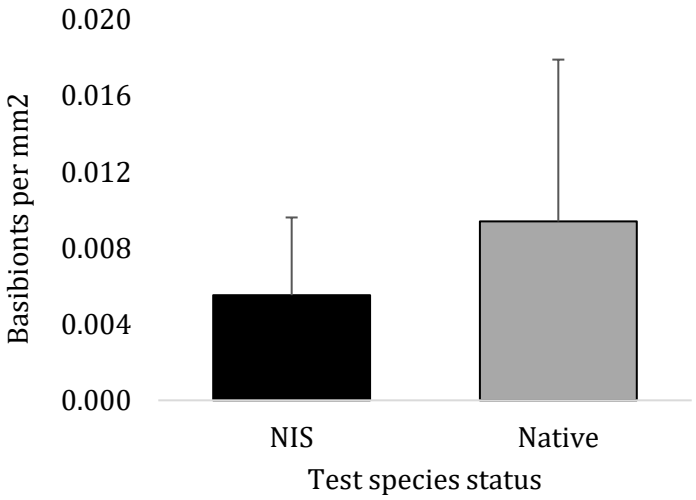

Figure 3.7: Mean recruits per mm^2 adjacent to NIS and native test species. With Standard error of the mean (SEM). Grey represents native species and black represents NIS.

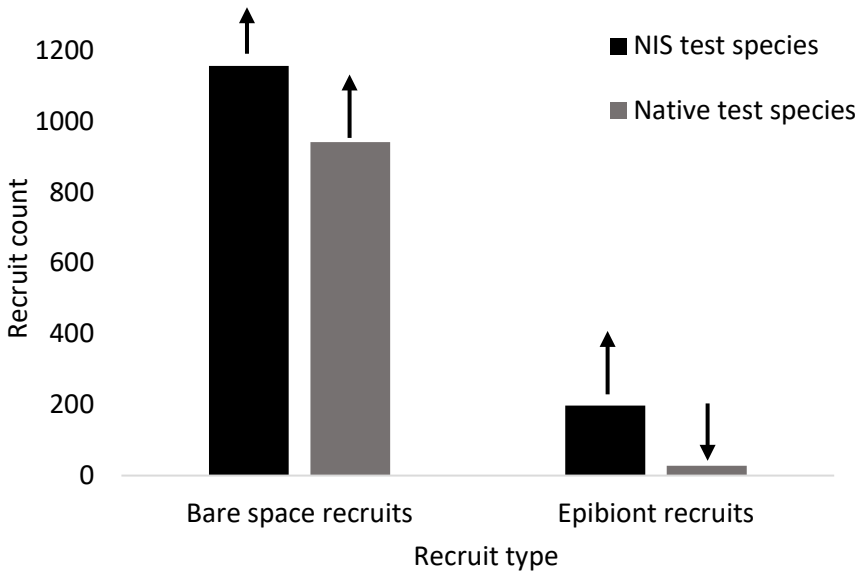

Figure 3.8: Frequency (observed) of occurrences of epibionts/bare space recruits settling on plates with NIS and native test species. Arrows indicate where there were counts of epibionts or bare space recruits than statistically expected, determined by Chi-Square test for association. Grey represents native species and black represents NIS.

Analysis of recruits per mm^2 revealed no statistical significance between epibiont recruits per mm^2 across the four test species (H= Kruskal Wallis H-test) (H = 1.648, p = .649 figure 3.6), or settlement next to test species per mm^2 (H = .687, p = .876; figure 3.17).

Across the four test species, more larvae settled as epibionts than on bare space per mm^2 (figure 3.9 and 3.10). The highest epibiotic recruitment was recorded on *Botrylloides sp* (M = 1.70 mm^2), and the least on *D. vexillum* (M= 0.08 mm^2) (figure 3.9). Species settled adjacent to *D. incanum* in the highest numbers (M = 0.008 mm^2), and the least next to *D. vexillum* test species (M = 0.0034 mm^2) (figure 3.10).

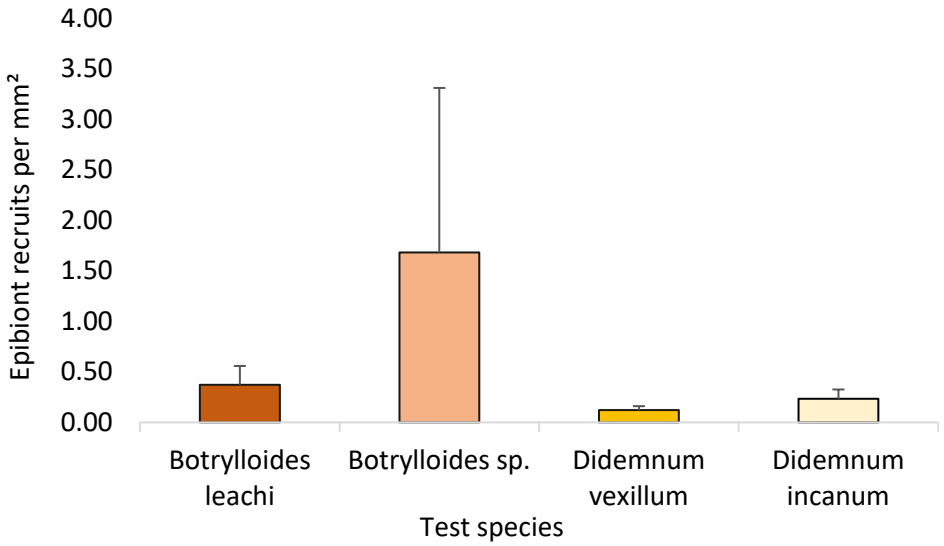

Figure 3.9: Mean epibiont recruits per mm^2 settling on the surfaces of the test species (*B. leachi*, *Botrylloides sp*, *D. vexillum* and *D. incanum*). With Standard error of the mean (SEM).

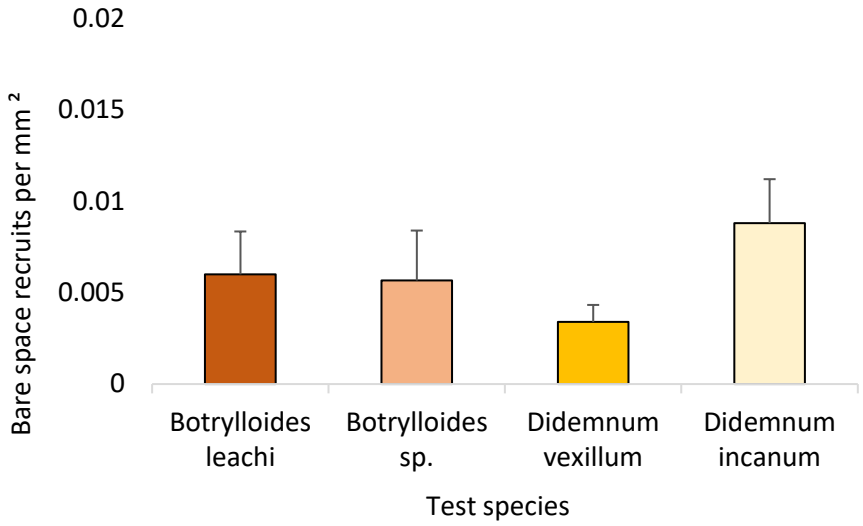

Figure 3.10: Mean bare space recruits settling per mm2 adjacent to the four test species (*B. leachi*, *Botrylloides sp*, *D. vexillum* and *D. incanum*). With Standard error of the mean (SEM).

3.7 Larval recruitment as epibionts or adjacent to test species

NIS larvae settled at greater densities per mm^2 than native larvae (figure 3.11 and 3.12). NIS settled as epibionts on test species (M = 0.4 mm^2) at greater densities than they settled on native test species (M= 0.002 mm^2) (figure 3.11). NIS larvae were also observed to settle at greater densities (M = 0.002 mm^2) on bare space than native larvae (< .001 mm^2) (figure 3.12).

Kruskal-Wallis testing revealed that there was no statistically significant relationship between epibionts per mm^2 and epibiont status (NIS or native) (H= 118.0, p = .1334, figure 3.10). However, Mann-Whitney U tests confirmed a statistically significant relationship between bare space recruits per mm^2 and bare space recruit status (NIS or native) (Mann-Whitney U= 361.5, p = .0021, figure 3.11).

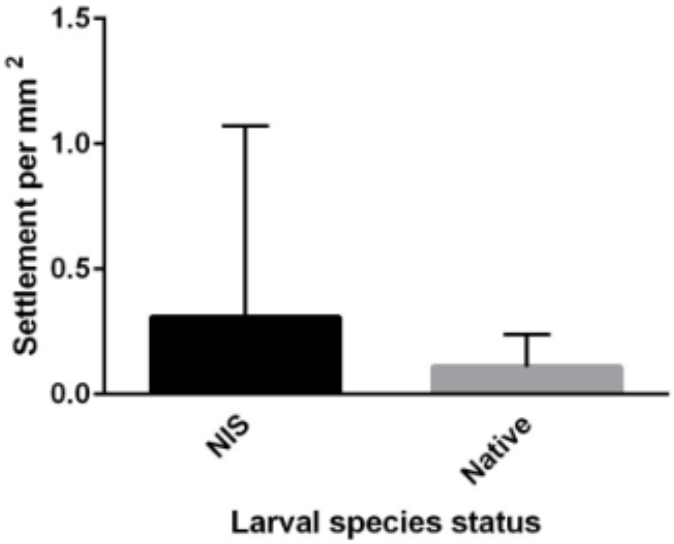

Figure 3.11: The larval species status (NIS or native) of mean epibiont recruits per mm^2. With Standard deviation (S.D). Grey represents native species and black represents NIS.

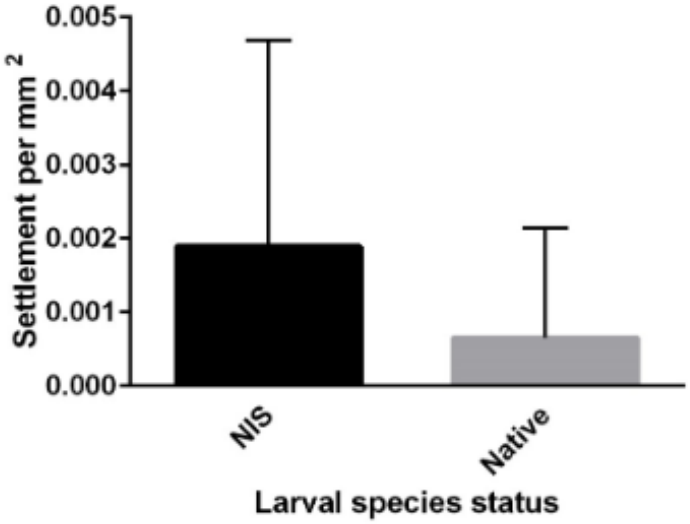

Figure 3.12: The larval species status (NIS or native) of mean bare space recruits per mm^2. With Standard Deviation (S.D). Grey represents native species and black represents NIS.

3.8 Larval settlement distances from test species

NIS recruits settled further from test species than native recruits (M= 22.54 mm and M= 20.81 mm respectively). Generally, NIS recruits settled further away from native test species (M= 34.17 mm) than native recruits, who settled closer to native test species M= 22.99 mm). NIS recruits settled closer to NIS test species than native recruits (NIS M= 18.66 mm, Native M= 20.08 mm).

A factorial ANOVA was conducted to compare the main effects of test species status and larvae species status on the interaction effect between test species status and larvae species status on the distance settled from the test species (mm). The main effect for test species status was significant $F(1, 2080) = 85.48$, $p = <.001$), post hoc analysis between test species status and distance settled revealed (H= 10.852, $p = .001$). The main effect for larvae status was insignificant $F(1, 2080) = 1.23$, $p = .266$).

Average settlement distance of *B. leachi* was further away from the *B. leachi* test substrates than any other recruiting larvae (M= 58.86 mm) (figure 3.13). A T-test revealed a strong statistical association between NIS and native recruits settling on *B. leachi* test species $F(8, 732) = 6.271$, $p = < .001$). *B. leachi* recruits have a greater propensity to settle near *B. leachi* test species (M= 37.16).

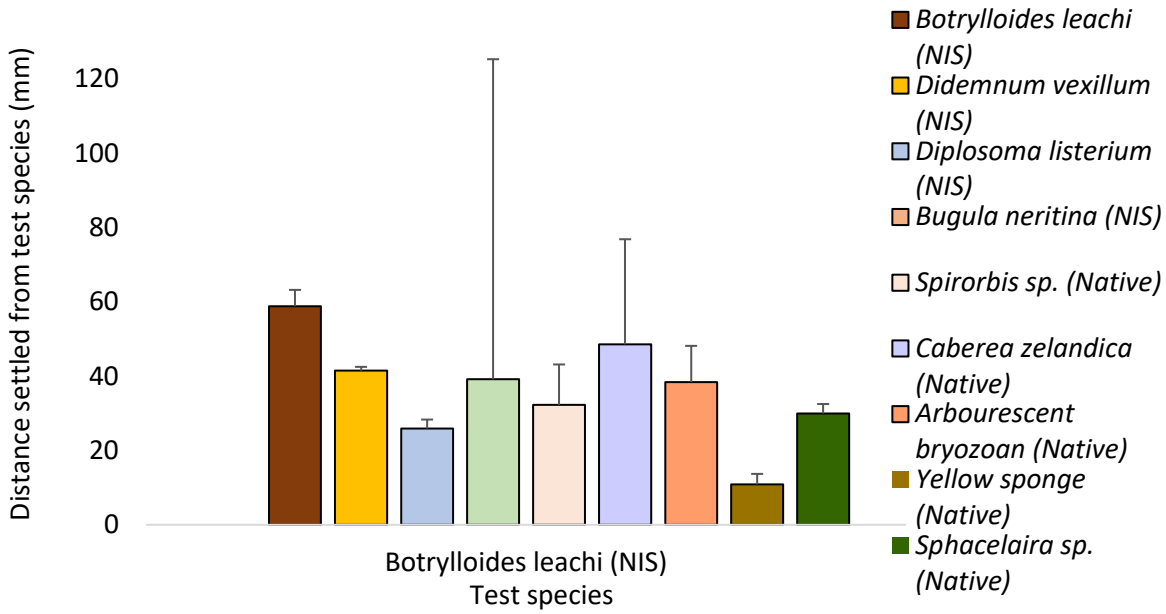

Figure 3.13: The mean settlement distances (mm) of all recruits observed across all experimental plates of *B. leachi* test species. With Standard Error of the mean (SEM).

A One-Way ANOVA revealed no statistical association between NIS and native recruits settling on *Botrylloides* sp. test species F(1, 383) = 1.572, p = < .211). NIS recruits have a greater propensity to settle near *Botrylloides* sp. test species

(M= 1.36). Plates with *Botrylloides* sp. test species had the least recruits, with *Bugula neritina* recruits settling the closest (M= 10.1 mm) and *D. vex* and *Spirorbis sp.* recruits settled the furthest away (M= 26.3 mm, M= 35.1 mm) (figure 3.14).

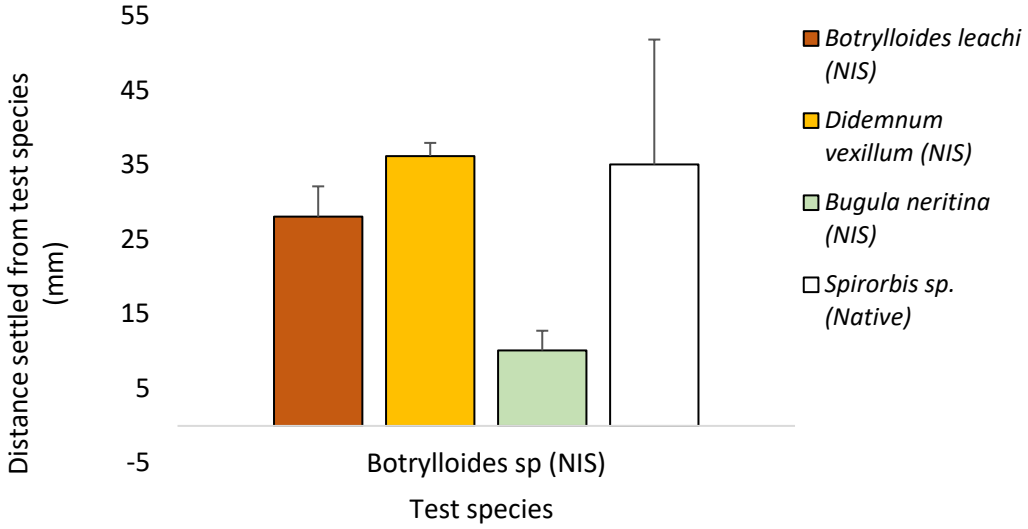

Figure 3.14: The mean settlement distances (mm) of all recruits observed across all experimental plates of *Botrylloides* sp. test species. With Standard Error of the mean (SEM).

D. vexillum and *D. incanum* experimental plates had the greatest number of settled recruiting species (9 species) (figure 3.15 and 3.16). Native recruits *B. neritina* and *Spirorbis sp.* settled the closest on *D. vexillum* experimental plates (between 8-10 mm), and *Mesophyllum sp*. and Yellow sponge recruits settled furthest away (M = 44.5 mm, M= 43.4 mm) (figure 3.15). A One-Way ANOVA revealed strong statistical association between NIS and native recruits settling on *D. vexillum* test species $F(1, 59) = 10.53$, $p = .002$). NIS recruits have a greater propensity to settle near *D. vexillum* test species (M= 1.25).

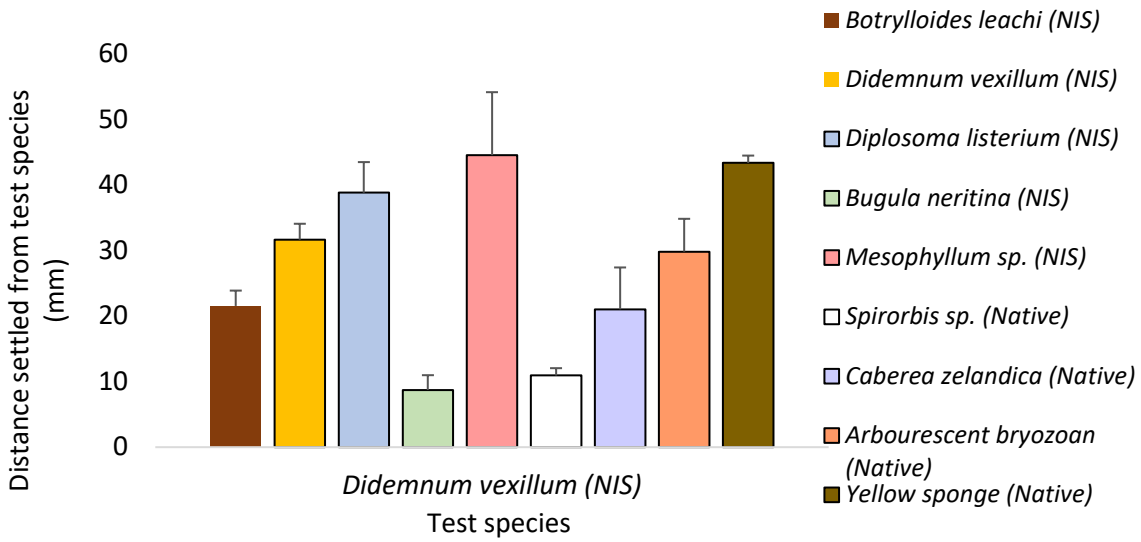

Figure 3.15: The mean settlement distances (mm) of all recruits observed across all experimental plates of *D. vexillum* test species. With Standard Error of the mean (SEM).

On native experimental plates (*D. incanum*) (figure 3.16), settlement distances across the recruiting species were generally similar (between 30-50 mm), native recruits *Spirorbis sp.* and *Caberea zelandia* settled at the greatest distance (M = 53. 4 mm, M= 53 mm), and the NIS bryozoan *Watersipora subtorquata* settled the closest (M= 30.4 mm). A One-Way ANOVA revealed strong statistical association between NIS and native recruits settling on *D. incanum* test species $F(1, 660) = 50.623$, $p = <.001$). Native recruits have a greater propensity to settle further from *D. incanum* test species (M= 1.61 m).

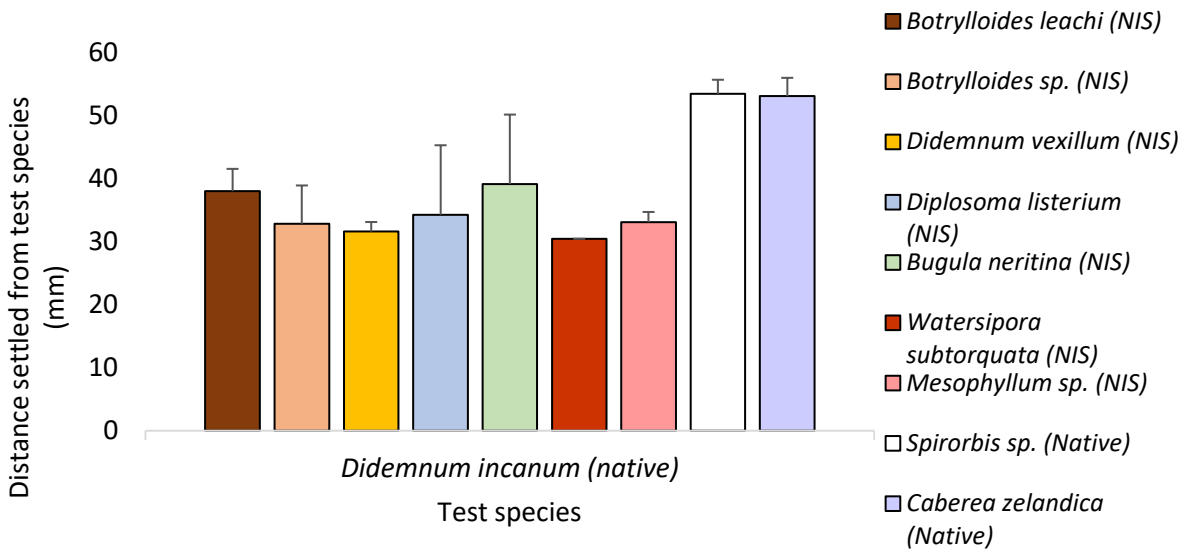

Figure 3.16: The mean settlement distances (mm) of all recruits observed across all experimental plates of *D. incanum* test species. With Standard Error of the mean (SEM).

3.9 Discussion

The strong competitive abilities of NIS are illustrated in this study. This chapter was composed with the overarching assumption that NIS ascidians are successful due to their settlement abilities. This chapter sought to illustrate the recruiting tendencies of NIS and native species as epibionts or as settlers on bare space, on or in the presence of NIS and native colonial ascidian test species. Additionally, settlement distance of recruiting species near NIS and native test species was examined.

Nineteen experimental plates with 19 test species (5 *B. leachi*, 3 *Botrylloides* sp., 5 *D. vexillum* and 6 *D. incanum*) were analysed in this study. The experimental plates in this study illustrated heavy fouling with a combination of algae and sessile organisms drawn to cryptic environments such as sponges, ascidians, and bryozoans (Stoecker, 1980). Generally, NIS were shown to exert stronger settlement pressures across all test species and occupy space in greater volumes through increased propagule pressure compared to natives.

3.9.1 Recruit settlement and substrate preference

Results detailed that epibiotic settlement was relatively low, accounting for 9.7% of all recruits. Test species were shown to experience more settlement pressures next to them (by bare space recruits) than on their surfaces (by epibiont recruits). This implies that the recruits in this study

preferred to settle on bare available substrate, supported by the high frequency of bare space recruits (89.7% of all recruits). In explanation of the results, the fitness of a settlement site (substrate) is crucial to sessile organisms, as once attached relocation is impossible (Chase et al, 2016), therefore it is evident that settling on bare space would be advantageous as the individual would exist temporarily free of harmful competitive interaction from neighbours (until others settle). Other ascidians; *Ciona intestinalis* and *Botrylloides violaceus* were also observed to illustrate substrate settlement preferences, establishing more on concrete plates than other test substrates (granite and PVC) (Chase et al, 2016).

Upon comparing the frequencies of recruits within the plate area (per mm^2), no statistical association could be made between test species status (NIS or native) and recruit frequencies per mm^2. Additionally, when comparing epibiont and bare space recruits per mm^2 on the four test species experimental plates (1) *B. leachi* (NIS), 2) *Botrylloides* sp. (NIS), 3) *D. vexillum* (NIS) and 4) *D. incanum* (Native), results failed to form a statistical association between the type of recruit (epibiont or bare space recruits) and the frequency per mm^2. These insignificant results are indicative of a requirement for more replicates as they did not fulfil the requirements to be statistically significant (see chapter 4.2). Yet, trends were still observed from these comparisons and will be discussed in this section.

When comparing the counts of recruits on bare substrate and occupied space (surfaces of the test species, strong statistical evidence suggested that bare substrate was the preferred settlement site for recruits. Therefore, the observed habitat selectivity by recruits in this study supported by the high densities of recruits on bare available space across the experimental plates (Morris, 2003). In support, colonial ascidians have illustrated such preferences for bare substrate in other studies, like that of the substrate preferences observed in this study.

Ascidians have been cited to show increased survivability when settled on primary space (bare substrate) compared to secondary space (epibiont space) (Johnston et al, 2009). Test colonies of *Ciona intestinalis* and *Botrylloides violaceus* have illustrated in other research to prefer bare concrete plates over naturally occurring granite and the surfaces of other species (Chase et al, 2016). Ultimately, habitat and substrate selection drive the fitness and distributions of these crowded assemblages, by selecting for unoccupied space and thus improving resource availability and reducing overgrowth interactions, recruits can increase their fitness (Price, 2010).

The preference for bare substrate exhibited by recruits in this study is supported by the concept that an increase in density (recruits per mm^2) decreases individual fitness (Morris, 2003). The

relationship between density and assemblage fitness has made it evident that bare space is often exempt of competitive interaction, and therefore a more desirable substrate for recruiting organisms. Bare space created by anthropogenic disturbance, however, is of concern. The success of a NIS is often driven by its propagule pressures and the susceptibility of the habitat to invasion (more available substrate and space) (Colautti et al, 2006). Within the theoretical framework of this study, bare space would become heavily targeted and colonised by NIS, just as the bare space was recorded with high settlement frequencies in this study (chapter 3.8).

3.9.2 Settlement pressure

In this chapter I hypothesized that there would be greater settlement pressures exerted by NIS recruits than natives (H2), and that NIS recruits would settle the most per mm^2 than native recruits (H3). Results found native species settlement was generally low (17% of all recruits). NIS comprised of 82.9% of all recruits, leading to the notion that NIS exert more settlement pressures and have higher propagule pressures than native species. As the Port of Tauranga is a site of high intensity exposure and high levels of arrival events (Johnston et al, 2009), the high percentages of NIS recruits (82.9%) observed in this study are indicative of intense propagule pressures as the recruitment of a species is limited by the propagule supply (Johnston et al, 2009).

Established species have been found to be introduced more often and in greater numbers than introduced species who fail to establish; therefore, propagule pressures are associated with the establishment stage of a NIS (Colautti et al, 2006). Our results confirmed the statements of Colautti et al (2006). *D. vexillum* is a known established invader in New Zealand and recruits of the NIS were recorded in high abundances across all experimental plates in this study, with larvae heavily fouling the plates. Of the total 2325 recruits, 54.88% were identified as *D. vexillum* larvae. This illustrates the competitive power of established NIS, with more propagules in the water and therefore higher settlement rates.

Though the colonizing success of NIS can be unpredictable (Holle & Simberloff, 2005), the large volume of *D. vexillum* recruits in this study infers that the species is a successful colonizer. In addition, high propagule determines recruit settlement, and as *D. vexillum* were observed to heavily foul the experimental plates it is indicated that *D. vexillum* has high propagule pressure in the Tauranga Harbour (Johnston et al, 2009). Of the total *D. vexillum* recruits, 88% settled on bare space and 12% settled on the surfaces of the test species, like the settlement preferences of the other recruits in this study, *D. vexillum* also illustrated a tendency to settle on bare space.

However, introduced encrusting species have been cited to settle opportunistically on any available space (bare, native, or introduced) (Leonard et al, 2017). As *D. vexillum* recruits accounted for over half of all recruits, it is suggested that due to their high propagule pressure and strong competitive power, they were the most successful recruiter in this study.

New Zealand is not the only location subject to the invasion and dominance of *D. vexillum*, reports have been observed worldwide; the non-indigenous colonial ascidian was cited to cover an area of approximately 230 km^2 in Georges Bank (USA) (Lengeyl et al, 2009), and was even added to the Atlantic Canada watch list in British Columbia in 2009 (Moore et al, 2014).

3.9.3 Differential settlement distance

In my findings, distance and settlement observations show that there is a strong relationship between the status of the test species and the distance that the recruits settle from the test species supported by post hoc analysis. There is strong statistical association between native recruits and the distance they settle to native test species, this indicates that NIS recruits have a greater propensity to settle near native test species.

Epibiont (both NIS and native) recruits were generally more densely aggregated (per mm^2) than bare space recruits (figure 3.6 and 3.7), this may be explained by their limitations to the surface area of the test species as epibionts. Specifically, there were recorded epibionts per mm^2 on NIS test species. Bare space recruits were observed to settle the most per mm^2 on plates with native test species (*D. incanum*). This suggests that recruits may have been avoiding the surfaces of *D. incanum* test colonies. To reiterate, larval recruits were selective when settling in response to either native or non-indigenous origins of the settled test species.

I hypothesized that native test species would have greater numbers of recruits (both NIS and native) than NIS test species (H3). Results found NIS experimental plates to have relatively low species diversity, yet more recruit counts were recorded. On native experimental plates, species diversity was high but individual recruit counts were low. Therefore, the hypothesis (H3) was rejected, which contradicts previous literature, such as Leonard, 2015 who found native epibionts to prefer to settle on native test species which suggested co-evolved recognition between natives (Leonard, 2015).

I hypothesized in this chapter that native recruits would settle closer to native test species (H6), as other studies have found native species to settle more frequently as epibionts and bare space recruits near other native test species, compared to NIS test species (Leonard et al, 2017).

However, this was not supported in the results. Experimental plates showed native recruits to settle further away from test species than NIS recruits, for example two native species *Spirorbis sp*. and *Caberea zelandica* that settled on *D. incanum* experimental plates settled at a greater distance than the NIS recruits (figure 3.16). If native recruits had showed a strong preference to settle adjacent to native test species, it may have suggested that there was some inherent reason why native recruits have a greater propensity to settle near native recruits, it may have inferred that NIS have some sort of allelochemical release that is deterring the settlement of natives and forcing them to settle further away, however such result was not observed in this particular study (figure 3.16).

I hypothesized that NIS recruits will settle closer to both native and NIS test species (H4). This may suggest that NIS are opportunistic when settling and are not driven or influenced by competitive interactions, if a recruit settled on bare space it could be hypothesized that it would experience less competion and be given more time for growth and attachment, yet NIS do not show this. Therefore, they are excellent competitors and consumers of space, as they are not ruled by such concepts.

Results found NIS recruits to indeed settle much closer to the test species than native recruits, accepting the hypothesis (H4). In addition, NIS recruits settled closer to NIS test species than native recruits, with NIS recruiting on average much further away from native test species in general than native recruits. These findings reiterate the concept that NIS are the better invaders as they tend to opportunistic settlers (settling where they are able).

3.9.4 Fouling defence

Allelochemistry can aid in the maintenance of a successful position amongst crowded hard bottom communities (Braekman et al, 1978). The three mechanisms cited to relieve epibiont pressure (1) tolerance, (2) avoidance and (3) defence (Wahl, 89), were exhibited in test colonies in my study. Colonies of *B. leachi* were observed to tolerate epibionts, with high recruit counts per mm^2 surface area as well as heavy pressures from bare space recruits (figure 3.13). *Botrylloides* sp. and *D. vexillum* colonies had few epibionts, suggesting the two species may be releasing allelochemicals as a defence. *D. vexillum* has also been observed in other studies to

exist epibiont free (Coutts & Forrest, 2007). *D. incanum* colonies had high counts of bare space recruits per mm^2 but few epibionts, indicating that recruits were actively avoiding settling on *D. incanum* surfaces.

Results found *D. vexillum* test species to also have the least epibiont and bare space settlement in comparison to the other test species in this trial (figure 3.9 and 3.10). This suggests that *D. vexillum* exists as a notorious NIS in the Tauranga Harbour with reduced competition by epibionts and bare space recruits. The lack of recruits settling on the surfaces of *D. vex* test species in this study suggests that despite the surfaces of many organisms being suitable substrate for biofouling recruits, some species can deter the settlement of epibionts (Price, 2010).

D. vexillum test species in this study appeared to exist free of epibionts to a certain degree, *D. vexillum* colonies in other studies such as Coutts and Forrest (2007) and Auker & Oviatt (2007) have also illustrated surfaces free of epibionts. Results suggest that there may be more complex elements to larval substrate preference that may explain why NIS test species tend to experience less epibiosis, such as the possible release of secondary metabolites as an epibiont defence mechanism (Watters, 2018) and (Blunt et al, 2013), mucus production and epidermal sloughing (Davis, 1991) or the surface texture and properties of the test species (Hirose & Sensui, 2019).

The employment of chemical defence mechanisms to maintain positions in highly competitive hard bottom communities by colonial ascidians is evident in previous research (see chapter 1.1) yet understudied and arguably inadequate in evidence. Extracts of the colonial ascidian *Eudistoma olivaceum* indicated antiviral, antimicrobial and cytotoxic activity with test species subject to only light fouling under experimental conditions, *E. olivaceum* have been proven to inhibit larval settlement and dose-dependent growth of *B. neritina* recruits (Davis & Wright, 1989). There have even been reports made on five species of Antarctic ascidians that may prevent predation of starfish through their acidities: *Corella eumycota, Distaplia cylindrica , D. colligans , Sycozoa gaimardi* and *Trididemnum* sp. (Koplovitz et al, 2009)

Perhaps the complexity of allelochemical release is not the only plausible epibiont deterrent, but the surface microtopography and properties of test species themselves. Surface microtopography known to influence the settlement of sessile organisms (Lapointe & Bourget, 1999). Epibiont sponges on the surfaces of ascidian tunics have been known to show patterns of distribution on the different zones of the tunic surface, in response to their surface topography, 87.8% of the species were settled on the posterior ascidian zones whereas anterior zones were noted to have less settled recruits, consisting of 9.8% of all recruits, (Voultsiadou et al (2010).

The posterior zone of the tunic are reportedly much harder and more stable, and therefore more suitable for settlement, the anterior zones are often more contractile and the youngest areas of the animal where growth is occurring, thus less desirable for settlement (Voultsiadou et al (2010) (see figure 1.1). Of relevance to this study, is the concept that recruits are settling only on the surfaces of test species who have more desirable surface topography. Our results found epibiont larvae to settle in much greater densities per mm^2 than bare space larvae, more specifically, NIS epibiont larvae recruited in much greater densities on test species than native epibiont larvae (figure 3.10). Despite the lack of statistical evidence to support the relationship between epibionts an their aggregation per mm^2 at a status level, parallels could still be drawn to stipulate that epibionts are not only restricted by the surface area of the test species but also by the surface topography of the test species, settling in greater densities on the posterior zones of the tunic where there is less disturbance through growth and more surface stability (Voultsiadou et al (2010) (see figure 1.2).

What is more intriguing, is that the surfaces of some metazoan species can be partially or entirely (species dependent) covered with an array of nipples approximately 100 nm in height (Hirose & Sensui, 2019). Recent research conducted by Hirose & Sensui, 2019 found the nipple array structures to prevent the settlement of ascidian epibionts, acting as a form of anti-fouling (Hirose & Sensui, 2019). Large numbers of ascidian larvae were observed to settle on flat and bare surfaces more than on the nipple array simulated surface (MOSMITE™) (Hirose & Sensui, 2019), like the observations made in our study; where there were more bare space recruits settling on the bare plate substrate than on the surfaces of the test species (figure 3.6 and 3.7).

Hirose & Sensui, 2019 found their test ascidians attached to the nipple array less tightly than on the flat surface, indicating that nipple array surfaces combined with chemical defence releases may achieve epibiont free surfaces (Hirose & Sensui, 2019). Other marine organisms such as mussels have been observed to employ microtopography to deter settling epibionts (Wahl, 2009). Yet little research has been conducted on the microtopography of colonial ascidian surfaces.

A similar study found three species of ascidian (*Corella antarctica, Ascidia challengeri* and *Synoicum sp.)* to show marked differences in their tunic structure which in turn caused a variation of the percent cover of macro-epibiont species (Rimondino, et al 2015). The tunic morphology of an ascidian can determine the epibionts that settle on its surface, smooth surfaces have been cited to gain higher epibiont percent cover (Rimondino, et al 2015).

Other sessile organisms have also exhibited active settlement preferences in relation to surface texture, often choosing rougher substrate (bare space) to as a more secure space in fluid

environments (Lapointe & Bourget, 1999). Even at a species level, our results support the notion that surface topography may play a role in settlement; native bare space larvae settled in greater densities per mm^2 of available space than NIS bare space larvae (figure 3.11), supported by strong statistical evidence to illustrate a relationship between bare space recruit status and bare space larvae per mm^2.

It is acknowledged that due to the number of replicates, some aspects of the data presented in this study demonstrated statistical insignificance. However, when taking in to account the limitations of this study (see chapter 4.2), the results (observed and statistical) show that a combination of test species and larval status does in fact have an influence on the settlement behaviours of recruiting organisms amongst these highly competitive assemblages. This work is evidence of a clear difference between the competitive abilities of native and NIS sessile species.

CHAPTER 4.

SYNTHESIS

4.1 Summary

This thesis illustrates that the dynamics of native sessile marine communities are largely determined by the presence of NIS. The core of this dissertation is centralised around the introductions of NIS to native hard bottom communities and the possible competitive advantages they are equipped with in lieu of co-evolution, that in turn may explain their global invasion success. Results illustrate that yes; NIS are indeed equipped with competitive advantages shown through their settlement abilities observed in this study.

The introductions of NIS ascidians are to a large extent related to the global increase in interoceanic travel (Lambert, 2007). The facilitation if NIS to new areas can be enhanced in areas of increased anthropogenic activity, where bare space is frequently opened through disturbance (Johnston et al, 2009). A previous study on the non-indigenous bryozoan *W. subtorquata*, found recruiting numbers to double when disturbances were high, as well as a noted increase in survivability per recruit when new bare substrate was provided (Johnston et al, 2009). The results of this study support this concept, that the rate in which environments are accumulating NIS coincides with such anthropogenic activities (Lockwood et al, 2005) and (Johnston et al, 2009), as the site for this experiment was the Tauranga Harbour (a busy shipping channel) which indicated high NIS propagule pressures with large amounts of NIS recruits settling on the experimental plates.

Understanding a species selectivity for substrate and settlement behaviours is a key motif in this work. Preferences for substratum and location either on (epibiont) or near (basibiont) neighbouring species can contribute to our understanding of competition and its consequences on native marine communities (Moore et al, 2014). The competitive dynamics of marine hard bottom communities are not as simple as previously thought. Species phenotype, genotype and aggression were once attributed to a life free of epibionts, however such traits are simply not enough to explain the dominance that NIS can exert on natives (McClintock & Baker, 1997). Previous research suggests that settlement is not completely random or based on chance (Lapointe & Bourget, 1999), with organisms actively seeking substrate that is more desirable to carry out their life processes, in this case, substrate that is less densely occupied is a preferred settlement surface for recruits.

The results of this study saw NIS settle more frequently as opportunistic epibionts with first pickings of space on the experimental plate. Despite their preference for cryptic and rough substrate (Sorokin, 1995), our results show they are highly adaptable and versatile with enduring survivability, which is supportive of my hypotheses. NIS ascidians are often cited to tolerate wide ranges of environmental conditions (fluctuations in temperature, wide range of salinities and toleration of pollutants and heavy metals) (Lambert, 2005). Within this framework, also exists mechanisms used to alleviate settlement pressures of epibiont and basibiont neighbours (Wahl, 1989). Such mechanisms of defence and preferences for selective substrate type are more commonly witnessed in NIS (Shenkar et al, 2008), which supports the nature of my findings.

Although my study did not test to produce secondary metabolites in ascidians, my results still strongly suggested that some species (*D. vexillum* and possibly *D. incanum*) in particular may utilise allelochemicals to deter epibionts. Mechanisms to relieve epibiont settlement such as tolerance, avoidance and defence (Wahl, 1989) were observed in the test colonies of this study; *B. leachi* appeared to tolerate heavy basibiont and epibiont pressures, *Botrylloides* sp. and *D. vexillum* demonstrated defence, as less recruits settled on their plates, and *D. incanum* colonies had few epibionts, indicating possible avoidance as well as defence. Such mechanisms can contribute to the invasion successes of NIS and their abilities to dominate entire native hard bottom communities.

This study suggests that allelochemical release may also not be the single sufficient mechanism used by ascidians to deter epibiont settlement, but tunic morphology too. Nipple structures (100 nm in height) on the surfaces of some ascidians have been found to be undesirable settlement surfaces for epibionts (Hirose & Sensui, 2019). Test species *D. vexillum* and *D. incanum* had the least recorded epibionts in this study, indicating that such structures may exist on these species, but may be species dependent, as other test species (*B. leachi*) had high epibiont recruit densities.

The surface microtopography of an organism can influence recruiting settlement (Lapointe & Bourget, 1999). Different surface zones of the tunic offer different surface topographies. Anterior zones are more contractile, high growth and less stable, and posterior zones are much harder and stable and therefore more desirable to settle on (Voultsiadou et al, 2010). Here I report that test species in my study experienced more settlement pressures from basibiont recruits settling adjacent to them than epibiont recruits.

NIS opportunistically settled across the two substrate types (bare space and occupied epibiont space) with no obvious pattern. NIS recruits (epibionts and basibionts) were more prominent settlers than native recruits, with greater numbers of NIS settling per mm^2 of the experimental

plates. In addition, NIS recruits settled much closer in proximity to test species than native recruits, with strong statistical associations existing between the status of the test species and frequencies of recruits. This infers again that NIS are excellent occupiers of space and competitors. They appear to settle where they can and are not driven by possible basibiont defence mechanisms such as allelochemistry or sloughing.

The ability of NIS to exert intense settlement pressures on existing sessile organisms is unparalleled. Introduced species' ability to settle heavily as both basibionts and epibionts allows them to litter submerged substrates with larvae, illustrative of high propagule pressures. This work is evidence of a clear difference between the competitive abilities of native and NIS sessile species. The main findings of this research represent the competitive power of NIS.

4.2 Future directions

The notion that this study is the first of its kind is accommodated by the apparent gap in the understanding on NIS settlement on marine hard bottom communities. The settlement pressures exerted by NIS on native assemblages are highlighted in this study, findings demand for further investigation to better understand and prepare for the implications of NIS. Within this ambitious study: time, resources, and the need to remain within the requirements of a Master of Research qualification, refrained the research from advancing to promising pathways. Yet, from the results and learnings of this work there are many recommendations that can be put forward to those who continue similar studies after this publication. This section of this dissertation will investigate the study limitations experienced, direction for future research and concluding statements.

4.2.1 Study limitations

The rearing of ascidian cultures *ex-situ* is a novel process, one that in my case required extensive research and time. The study limitations of this research are duly recognised, any further research should utilise the learnings of this thesis and apply their future directions in accordance:

1. Indeed, it is advantageous to conduct all experiments *in-situ*, the question may arise as to why I cultured ascidians and re-deployed them back into the harbour. To elucidate, a sufficient surface area across all colonies was required (5cm or larger), I required a uniform collection of live and healthy NIS and native colonies, to do this I needed to grow and monitor them in tanks before conducting any further experiments, a surface

clear of any other organisms was also required so that the recruitment numbers, distance metrics and colony cover could be more accurately measured.

2. It is desirable to have even numbers of NIS and native test species across the experimental plates as well as an increase in replicates. However due to the complexity of rearing ascidians *ex-situ*, such evenness was unattainable. Growing and keeping healthy test species alive proved very difficult, test species experienced mortalities and native species were found to be particularly sensitive to tank conditions (chapter 2).

3. It is more suitable to collect and grow ascidians within a short time frame. This research endured 12 months of developing a robust culture for ascidians. It was significant for colonies to grow and attach naturally to the experimental plates, I required time to overcome colony mortalities and to research and experiment with different rearing techniques.

4. Indeed, it is desirable to rear the test species in flow through seawater tanks which may have improved growth and survivability, as seen in the work of Moody et al (1999) and Mackie et al (2006). Yet availability and access to seawater in this study was limited. The laboratory lacked the resources to construct large flow through seawater tanks, in addition, funding such construction would exceed the research allowance.

4.2.2 Future research

In recognition of the study limitations listed above (Chapter 4.2.1), the following will address proposed methodology amendments recommended by the findings, future studies on ascidians in aquaria should:

1. Use unfiltered seawater as an additional food source alongside microalgae, previous work found *R. salina* and its size of >10 μm to be less suitable for juvenile ascidians who preferred smaller algae (Berrill, 1947), although *R. salina* was an appropriate feed for adult colonies, it may have slowed the growth of smaller juveniles in this study,

2. Use flow through water tank systems; opting for flow through systems as used in the work of Moody et al (1999) and Mackie et al (2006) for example, may reduce environmental and oxidative stress of test species (Tasselli et al, 2017), using this method in place of air stone bubbles may reduce mortality rates as the likes seen in this study.

3. Reduce the stocking densities of tanks (Keough & Marshall, 2003), and allow for only 10 specimens (5cm in size approximately) per 20L of seawater, too many specimens in one tank may drive stress responses such as reduction in their metabolism (Joly et al, 2007).

4. Deploy the test species experimental plates in the harbour for a longer period, perhaps 20 days or more, to allow for settled recruits to grow onto the substrate and provide easier identification in the laboratory.

I propose the following directions for research should take place in the future, to contribute further to our understanding of non-indigenous settlement success; (1) define and develop an accurate taxonomic record of non-indigenous ascidians and their distribution in the harbour, this database would prove incredibly beneficial to both managers and researchers, (2) develop a robust methodology of ascidians in aquaria, to better define desirable environmental conditions in order to rear successful and healthy ascidian colonies *ex-situ*, similar to the system developed by Rinkevich & Fidler (2014), (3) analyse the growth rates between ascidian status and species (*in-situ* and *ex-situ*) to provide managers with a timeline and better information on which species pose greater threats when incursions occur, (4) better define larval substrate preferences, and test the possibilities of utilising undesirable substrates in marinas and ports to deter the settlement of NIS ascidians, similar to the study of Chase et al (2015) (5) explore the avenues of ascidian eradication and control and implement them in New Zealand marinas and ports, notable

examples of ascidian control are the use of antifouling biocides (Bellas, 2006; De Nys et al, 2009) such as the use of bleach (0.2% solution) to kill off *D. vexillum* from mussels in aquaculture (Denny, 2008), and (6) develop and test better surveillance and monitoring systems within the Tauranga Harbour, such as the use of remotely underwater operated vehicles (ROVs), future studies could collaborate with Bay Dynamics New Zealand, a company engineering and operating marine robotic systems.

4.3 Concluding statements

I reared over 100 colonies of NIS and native ascidians *ex-situ*, under environmentally monitored conditions, in a biosecure laboratory. 20 experimental plates with 20 test colonies attached were deployed into the Tauranga Harbour for 15 days to allow for natural settlement. I then examined and analysed the settlement pressures (epibionts and basibionts) of the recruiting species, and compared the settlement pressures that each status and species may be exerting on the test colonies, to illustrate a difference in the settlement patters between NIS and native species.

The main findings of this research were:

- NIS recruits were the most prevalent across all experimental plates (accounting for 89.7% of all recruits) and exerted the greatest settlement pressures on the test species, indicative of high propagule pressures.
- Bare substrate was the preferred settlement surface by all recruit taxa and status, sorted by strong statistical associations
- The settlement preferences and abilities observed suggest possible use of defence mechanisms to deter epibionts (Allelochemistry, sloughing, surface microtopography)
- Epibiont recruits exerted more settlement pressures on the test species than basibionts, as they were more densely associated per mm^2 on the experimental plates
- No associations could be made between test species status and recruits per mm^2, indicative of a need for more replicates
- Interestingly, NIS larvae settled further away from test species than natives which contradicts other studies, however not by a significant amount.
- Native recruits did not settle more on native test species, which rejects the hypothesis (H6)

- There were positive associations between the status of the test species and the distance that larvae (and their status) settled from test species

While this research cannot provide evidence of allelochemical defence or the presence of surface nipples on some species, it can certainly provide better understanding of the settlement abilities of NIS colonial ascidians, and can demonstrate the threat that NIS colonial ascidians can have on native hard bottom communities. Such findings will hopefully drive a change in New Zealand's current marine biosecurity system, pushing managers and researchers to develop more efficient monitoring and surveillance of marine NIS hot spots (wharves, marinas, and ports).

The findings of this dissertation will improve preparedness and readiness by informing managers on the settlement abilities and impacts of NIS colonial ascidians. In addition, it will allow for the more efficient use of manager resources when dealing with NIS incursions in the marine environment, by understanding the overgrowth interactions and settlement abilities of these infamous invaders.

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APPENDIX

Table A.1 All test species and the recruited phyla and their subphylum, species, status and number of recruits on each experimental plates

Plate number	Test species	Recruit phylum	Recruit subphylum	Recruit species	Recruit status	Number of recruits
Plate 1	***Botrylloides* sp.**	Chordata	Ascidiacea	*B. leachi*	NIS	47
		Chordata	Ascidiacea	*D. vexillum*	NIS	160
		Annelida	Polychaeta	*Spirorbis sp.*	Native	6
Plate 2	***B. leachi***	Chordata	Ascidiacea	*Diplosoma listerianum*	NIS	4
		Chordata	Ascidiacea	*D. vexillum*	NIS	40
		Ochhrophyta	Phaeophyta	*Sphacelaira sp.*	Native	17
		Annelida	Polychaeta	*Spirorbis sp.*	Native	5
Plate 3	***D. incanum***	Bryozoa	Gymnolaemata	*Watersipora subtorquata*	NIS	1
		Chordata	Ascidiacea	*B. leachi*	NIS	7
		Chordata	Ascidiacea	*D. vexillum*	NIS	120
		Annelida	Polychaeta	*Spirorbis sp.*	Native	115
Plate 4	***D. incanum***	Chordata	Ascidiacea	*B. leachi*	NIS	3
		Chordata	Ascidiacea	*D. vexillum*	NIS	48
		Annelida	Polychaeta	*Spirorbis sp.*	Native	11
		Rhodophyta	Hapalidiaceae	*Mesophyllum sp.*	NIS	151
		Chordata	Ascidiacea	*Diplosoma listerianum*	NIS	2
Plate 5	***B. leachi***	Chordata	Ascidiacea	*D. vexillum*	NIS	110
		Annelida	Polychaeta	*Spirorbis sp.*	Native	2
		Chordata	Ascidiacea	*Diplosoma listerianum*	NIS	2
Plate 6	***Botrylloides* sp.**	Chordata	Ascidiacea	*B. leachi*	NIS	6
		Chordata	Ascidiacea	*D. vexillum*	NIS	130
		Bryozoa	Cheilostomata	*Bugula neritina*	NIS	15
		Chordata	Ascidiacea	*B. leachi*	NIS	22
Plate 7	***D. vexillum***	Chordata	*Ascidiacea*	*D. vexillum*	NIS	29
		Annelida	Polychaeta	*Spirorbis sp.*	Native	44
		Chordata	Ascidiacea	*B. leachi*	NIS	22
		Bryozoa	Native	*Arborescent bryozoan*	Native	9
		Bryozoa	Cheilostomata	*Bugula neritina*	NIS	6
Plate 8	***B. leachi***	Chordata	Ascidiacea	*B. leachi*	NIS	18
		Chordata	Ascidiacea	*D. vexillum*	NIS	52
		Annelida	Polychaeta	*Spirorbis sp.*	Native	5
		Bryozoa	Native	*Arborescent bryozoan*	Native	2
Plate 9	***D. incanum***	Chordata	Ascidiacea	*B. leachi*	NIS	19
		Chordata	Ascidiacea	*D. vexillum*	NIS	69
		Rhodophyta	Mesophyllum/Hapalidiaceae	*Mesophyllum sp.*	NIS	164

		Bryozoa	Cheilostomatida	*Caberea zelandica*	Native	2
		Annelida	Polychaeta	*Spirorbis sp.*	Native	45
		Bryozoa	Cheilostomata	*Bugula neritina*	NIS	3
Plate 10	***D. vexillum***	Chordata	Ascidiacea	*B. leachi*	NIS	20
		Chordata	Ascidiacea	*D. vexillum*	NIS	41
		Chordata	Ascidiacea	*Diplosoma listerianum*	NIS	7
		Bryozoa	Cheilostomatida	*Caberea zelandica*	Native	3
		Bryozoa	Cheilostomata	*Bugula neritina*	NIS	3
		Rhodophyta	Mesophyllum/Hapalidiaceae	*Mesophyllum. Sp*	NIS	1
		Annelida	Polychaeta	*Spirorbis sp.*	Native	4
Plate 12	***B. leachi***	Chordata	Ascidiacea	*B. leachi*	NIS	25
		Chordata	Ascidiacea	*D. vexillum*	NIS	260
		Ochhrophyta	Phaeophyta	*Sphacelaira sp.*	Native	3
		Bryozoa	Cheilostomatida	*Caberea zelandica*	Native	11
		Porifera	Native	*Yellow Porifera*	Native	2
		Bryozoa	Native	*Arborescent bryozoan 3*	Native	2
		Bryozoa	Cheilostomata	*Bugula neritina*	NIS	1
Plate 13	***D. vexillum***	Chordata	Ascidiacea	*B. leachi*	NIS	12
		Chordata	Ascidiacea	*D. vexillum*	NIS	77
		Chordata	Ascidiacea	*Diplosoma listerianum*	NIS	1
		Porifera	Native	*Yellow Porifera*	Native	1
		Rhodophyta	Hapalidiaceae	*Mesophyllum sp.*	NIS	11
		Bryozoa	Native	*Arborescent bryozoan*	Native	4
		Annelida	Polychaeta	*Spirorbis sp.*	Native	15
Plate 14	***D. vexillum***	Chordata	Ascidiacea	*B. leachi*	NIS	9
		Chordata	Ascidiacea	*D. vexillum*	NIS	68
		Porifera	Native	*Yellow Porifera*	Native	1
		Bryozoa	Native	*Arborescent bryozoan*	Native	2
		Bryozoa	Cheilostomatida	*Caberea zelandica*	Native	4
		Annelida	Polychaeta	*Spirorbis sp.*	Native	12
Plate 15	***B. leachi***	Chordata	Ascidiacea	*B. leachi*	NIS	15
		Chordata	Ascidiacea	*D. vexillum*	NIS	7
		Bryozoa	Cheilostomatida	*Caberea zelandica*	Native	12
Plate 16	***D. incanum***	Chordata	Ascidiacea	*B. leachi*	NIS	9
		Chordata	Ascidiacea	*D. vexillum*	NIS	55
		Chordata	Ascidiacea	*Diplosoma listerianum*	NIS	1
		Annelida	Polychaeta	*Spirorbis sp.*	Native	5
		Bryozoa	Cheilostomatida	*Caberea zelandica*	Native	12
Plate 17	***D. incanum***	Chordata	Ascidiacea	*B. leachi*	NIS	10
		Chordata	Ascidiacea	*D. vexillum*	NIS	9
		Chordata	Ascidiacea	*Diplosoma listerianum*	NIS	1
		Bryozoa	Cheilostomatida	*Caberea zelandica*	Native	10
		Annelida	Polychaeta	*Spirorbis sp.*	Native	2
		Bryozoa	Native	*Arbourescent bryozoan 2*	Native	2
Plate 18	***D. vexillum***	Annelida	Polychaeta	*Spirorbis sp.*	Native	1
		Chordata	Ascidiacea	*Dilposoma listerium*	NIS	1
		Bryozoa	Native	*Arborescent bryozoan*	Native	2

		Bryozoa	Cheilostomatida	*Caberea zelandica*	Native	3
Plate 19	***Botrylloides sp.***	Chordata	Ascidiacea	*B. leachi*	NIS	6
		Chordata	Ascidiacea	*D. vexillum*	NIS	1
		Chordata	Ascidiacea	*Diplosoma listerianum*	NIS	14
		Bryozoa	Cheilostomatida	*Caberea zelandica*	Native	6
		Bryozoa	Native	*Arbourescent bryozoan 3*	Native	5
		Bryozoa	Cheilostomata	*Bugula neritina*	NIS	2
Plate 20	**D. incanum**	Chordata	Ascidiacea	*D. vexillum*	NIS	7
		Bryozoa	Cheilostomatida	*Caberea zelandica*	Native	7
		Chordata	Ascidiacea	*Diplosoma listerianum*	NIS	1
		Chordata	Ascidiacea	*Botrylloides sp.*	NIS	6

Figure A.1: Stella passage, Tauranga Bridge Marina. The experimental container deployment site.

Figure A.2: Collecting and removing colonies from wharf substrate (Sulphur Point Marina, Tauranga).

Figure A.3: The biosecure temperature-monitored laboratory and its tank organisation

Figure A.4: *R. salina* culture organisation and equipment

Figure A.5: 250mL flasks containing *R. salina* next to a UV lamp in the biosecure container

Figure A.6: The transplanting process, gluing a new ascidian colony onto a settlement plate.

Figure A.7: A colony of *Botryllus schlosseri*, (10x10 magnification, on a compound microscope). The zooids, atrial siphon and new zooids forming along the animals growing edge be seen here.

Figure A.8: A colony of *Botrylloides* sp. showing natural attachment to the settlement plate

Figure A.9: Rearing and their specimen's placed flat on the tank bottom to promote attachment.

F**igure A.10:** In tank organisation of plate holders, aerator stone aeration and experimental plate organisation.

Figure A.11: *Ex-situ* deployment of experimental containers, the experimental plates array can be seen here facing upwards before being deployed to approximately 2 metres deep.