Biofouling patterns and local dispersal in an aquaculture system in the Marlborough Sounds, New Zealand

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A thesis submitted in partial fulfilment
of the requirements for the degree of
Master of Science in Ecology
University of Canterbury
New Zealand
2014

"The journey is difficult, immense. We will travel as far as we can, but we cannot in one lifetime see all that we would like to see or to learn all that we hunger to know"

— Loren Eiseley

Marlborough Sounds, New Zealand

This thesis is dedicated in loving memory of my late father, Brian Watts, who taught me to love and respect the marine environment. I learnt from him that even the most challenging task can be accomplished with dedication and commitment by taking one step at a time.

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Acknowledgements

While this thesis represents a personal achievement, there are many people who provided support and advice along the way. First and foremost I would like to thank my fantastic supervisory team, Dr. Sharyn Goldstien (University of Canterbury) and Dr. Grant Hopkins (Cawthron Institute), for their patient guidance, enthusiastic encouragement and constructive criticism of this thesis. Thank you for sponsoring my attendance at an international conference and for providing me with ongoing intellectual stimulation. I could not have imagined better advisors and mentors for my master's thesis and hope I have done you proud.

Before I leave anyone out, I would like to extend my gratitude to the many staff members at the Cawthron Institute and the University of Canterbury who helped in one way or another with this thesis. More specifically, I would like to thank Fiona Gower and Rod Asher (Cawthron Institute) for assisting in taxonomic identification. To Dr. Lauren Fletcher (Cawthron Institute), thank you for sharing your insight into the ecology and impacts of *Didemnum* biofouling and for your enthusiastic assistance with laboratory and field work. I express my gratitude to Dr. Mike Page (NIWA), for assistance with ascidian identifications and for providing sound advice on species preservation. To my university field assistant, Dan Gregory, thank you for your hard work and commitment. I am extremely grateful for the support I received during my time in the genetics laboratory, particularly to Craig Galilee from the University of Canterbury Sequencing Service, to my fellow students (Rachel van Heugten and Sarah Redlich), and to the fabulous technician Maggie Tisch. I have learnt so much from you all.

Special thanks to Dr. Elena Moltchanova (University of Canterbury), Dr. Javier Atalah and Eric Goodwin (Cawthron Institute), for valuable discussions about multivariate statistics and the marvellous wonders of various statistics programs. To Eric Goodwin and Ben Knight (Cawthron Institute), thank you for assisting with the connectivity matrix used in this thesis. I am also indebted to Aaron Quarterman (Cawthron Institute) for constructing and entrusting me with valuable field equipment.

This project would not have been possible without considerable logistic support provided by Sanford Ltd. I am particularly grateful to Phil Hawke, Dick Jones and Les McClung for letting me accompany them on trips to the mussel farms, sharing industry knowledge on the impacts of biofouling and for making this part of my thesis so enjoyable. A very special thank you to James Brodie (Marlborough Commercial Diving Services Ltd) for enabling the collection of sea squirt samples and environmental profiles, for supplying historical knowledge of *Didemnum vexillum* and for the exchange of many funny moments shared with Javier Atalah, who assisted with these collections. Thanks are also due to the Marine Farming Association for industry information and marine farm guides.

I am extremely grateful to the Cawthron Institute for the scholarship and funding provided through the Shellfish Aquaculture Programme (Ministry of Business Innovation and Employment contract CAWX0802), to Dr. Andrew Fidler (Cawthron Institute) for genetics support and to the University of Canterbury for financial assistance provided by the UC Master's Scholarship.

Finally, to my dear family and friends, thank you for your unconditional and unwavering love and support throughout this entire process. I am continually amazed and humbled by your selflessness and patience. Especially Carl Johnston, thank you for showing me more adoration and understanding than I could ever ask for. To my mum, a special thank you for encouraging me and supporting all of my endeavours. I am very grateful to those who let me stay with them during my time away from home. I am also indebted to those who took the time to read through drafts of this thesis, providing comments and criticisms, and so many thanks are given to Jan McKenzie, Stacie Lilley and Kate Schimanski (University of Canterbury), Dr. Kirsty Smith, Dr. Andrew Fidler and Cherie Johansson (Cawthron Institute), William and Elizabeth Smyth, Kylie Watts and Sarah Redlich.

Abstract

Biofouling pests, including non-indigenous species, can have significant impacts on anthropogenic activities. This is particularly true for aquaculture industries, where biofouling communities grow on crop species and infrastructure, potentially reducing revenue and increasing processing and production costs. It is of interest to marine farmers and scientists to gain a better understanding of the processes facilitating the regional proliferation and spread of biofouling pests. The structure of biofouling communities associated with marine farms in New Zealand's main mussel growing region, Pelorus Sound, are characterised in this thesis. The patterns of connectivity and gene flow among biofouling populations are also investigated. Images and video footage of biofouling on mussel farms (*Perna canaliculus*) indicate strong spatial variation in the structure of biofouling communities, with a dominance of known problematic taxa and high wave energy tolerant species, such as the brown alga *Undaria pinnatifida* and the calcareous tubeworm *Pomatoceros* sp., near the entrance of Pelorus Sound. Genetic analyses and simple GIS-based modelling of a case study biofouling organism, *Didemnum vexillum*, revealed genetic differentiation among populations with extreme outcrossing and low levels of connectivity. Genetic analyses also suggest that anthropogenic-assisted dispersal may be vital for connecting certain *D. vexillum* populations compared to natural spread. The present study illustrates how multidisciplinary research approaches can be used to identify geographical areas that are less prone to biofouling and to inform the management of biofouling pests and invasive species in aquaculture environments.

Chapter I
General Introduction

1.1. Biofouling in the marine environment

The single most extensive habitat on the Earth's surface is the marine environment (Wimpenny 1996). It is within this habitat that artificial structures, such as recreational buoys and floats, oil and gas platforms, marina pontoons, ship hulls and aquaculture structures provide a potential refuge for and are vulnerable to the attachment and subsequent growth of unwanted biological organisms; a phenomenon termed biofouling (Southgate & Myers 1985; Abdul Azis *et al.* 2003; Dürr & Thomason 2010; Gittenberger & Van der Stelt 2011; Hopkins *et al.* 2011; Fitridge *et al.* 2012; Dobretsov *et al.* 2013).

Records of marine biofouling date back as far as the 5th Century BC, though mostly in connection to shipping activities (Benson *et al.* 1973; Kerr *et al.* 1998; Abdul Azis *et al.* 2003). For example, historical reports indicate that old wooden sailing ships during the 1750's could have carried nearly 120 marine organisms fouling, boring into, or nesting on the vessel's hull (Bax *et al.* 2003). Despite historical awareness, problems associated with biofouling have only received specific scientific attention over the last 60 years (Vedaprakash *et al.* 2013).

Marine biofouling often develops as a succession of organisms, progressing from an initial conditioning or slime layer of absorbed organic and inorganic matter, through multi-species microbial film formation dominated by bacteria, fungi and other microscopic organisms, to a community of macroscopic plants and animals (Kerr *et al.* 1998; Abdul Azis *et al.* 2003; Briand 2009). Over 5,000 different biological species worldwide have been described in the biofouling of structures exposed to or immersed in water (Abdul Azis *et al.* 2003; Braithwaite & McEvoy 2004; Dürr & Thomason 2010). However, marine biofouling communities are characterised by the presence of a dominant suite of sessile marine invertebrates including ascidians, sponges, bryozoans, barnacles, macroalgae, mussels and tube-building polychaetes, although spatial and temporal variation exists in the overall composition and biomass of these communities (Lesser *et al.* 1992; Minchin & Gollasch 2003; Canning-Clode & Wahl 2010).

Many dominant biofouling species are non-indigenous (NIS) to affected areas (i.e., species introduced to areas outside their native range) and share a common array of 'invasive' characteristics. These include a cosmopolitan distribution, strong spatial competitive attributes with high growth rates and multiple reproductive strategies (i.e., asexual and sexual reproduction), a limited number of natural predators and diseases and the ability to thrive in a variety of environments (Ehrlich 1989; Sakai *et al.* 2001). For example, the cosmopolitan brown alga *Undaria pinnatifida* is a well-known biofouling species that is highly adaptable, tolerant of a broad range of habitats, capable of attachment on most natural and artificial substrata and able to form dense monospecific algal stands, as evident within some southern areas of New Zealand (Forrest *et al.* 2000; Russell *et al.* 2008).

The dominance of NIS in biofouling communities has been attributed to the location of artificial structures in areas that experience high levels of disturbance or traffic flow, such as aquaculture regions and commercial and recreation shipping areas (Airoldi *et al.* 2005; Bulleri & Airoldi 2005; Floerl *et al.* 2009; Bulleri & Chapman 2010; Fitridge *et al.* 2012; Simkanin *et al.* 2012). For example, Pettengill *et al.* (2007) attributed close genetic relatedness between world-wide

populations of the ubiquitous biofouling tubeworm *Hydroides elegans* to regular and consistent shipping transport movements. The physical attributes of artificial structures also make them favourable for colonisation by NIS (Bulleri & Airoldi 2005; Vaselli *et al.* 2008; Bulleri & Chapman 2010). For instance, the sheltered, landward-sides of breakwaters along the coasts of Italy provide shelter for two non-indigenous green macroalgae, *Codium fragile* and *Caulerpa racemosa*, consequently contributing to their spread (Bulleri & Airoldi 2005; Vaselli *et al.* 2008).

Biotic interactions, including the indirect effects associated with the presence of biofouling organisms may also facilitate the establishment of other marine pests[1] (Heiman & Micheli 2010; Green *et al.* 2011). This is evident within the southwestern Atlantic, where reefs created by the non-indigenous polychaete *Ficopomatus enigmaticus* provide refuge for the predatory crab *Cytograpsus angulatus* (Schwindt *et al.* 2001). Similarly, shells of the invasive Asian hornsnail *Batillaria attramentaria* were found to generate habitat for two NIS invertebrates, the Atlantic slipper shell *Crepidula convexa*, and the Asian anemone *Diadumene lineata* on the northwest Pacific coast of the United States (Wonham *et al.* 2005).

1.2. The impacts of biofouling

The accumulation of biofouling is one of the most important factors affecting the safety and service lifetime of artificial structures. For commercial industries, including the global shipping trade, offshore oil and gas production, power stations and aquaculture operations, biofouling can cause serious operational and maintenance problems (Figure 1.1a) (Foster & Willan 1979; Southgate & Myers 1985; Kerr *et al.* 1998; Hopkins & Forrest 2010). In heat exchangers, heavy biofouling can result in blocked process pipes and enhanced corrosion (Figure 1.1b) (Melo & Pinheiro 1992; Railkin 2003), whereas on ships biofouling increases frictional drag, decreases manoeuvrability and promotes fuel consumption and the emission of greenhouse gases (Figure 1.1c) (Railkin 2003; Terlizzi & Faimali 2010). Other oceanic equipment, such as buoys and beacons, can be smothered, causing reduced efficiency, increased hydrodynamic loading stress

[1] The term 'marine pest' refers to native and non-indigenous species, whose growth can be problematic for artificial substrate.

and structural deterioration through enhanced surface corrosion (Figure 1.1d) (Whomersley & Picken 2003; Chambers *et al.* 2006; Phang *et al.* 2009; Hopkins & Forrest 2010; Dafforn *et al.* 2011). Biofouling can also impair visual inspection and at times, the maintenance of underwater structures, such as the underwater parts of oil and gas platforms (Yan & Yan 2003).

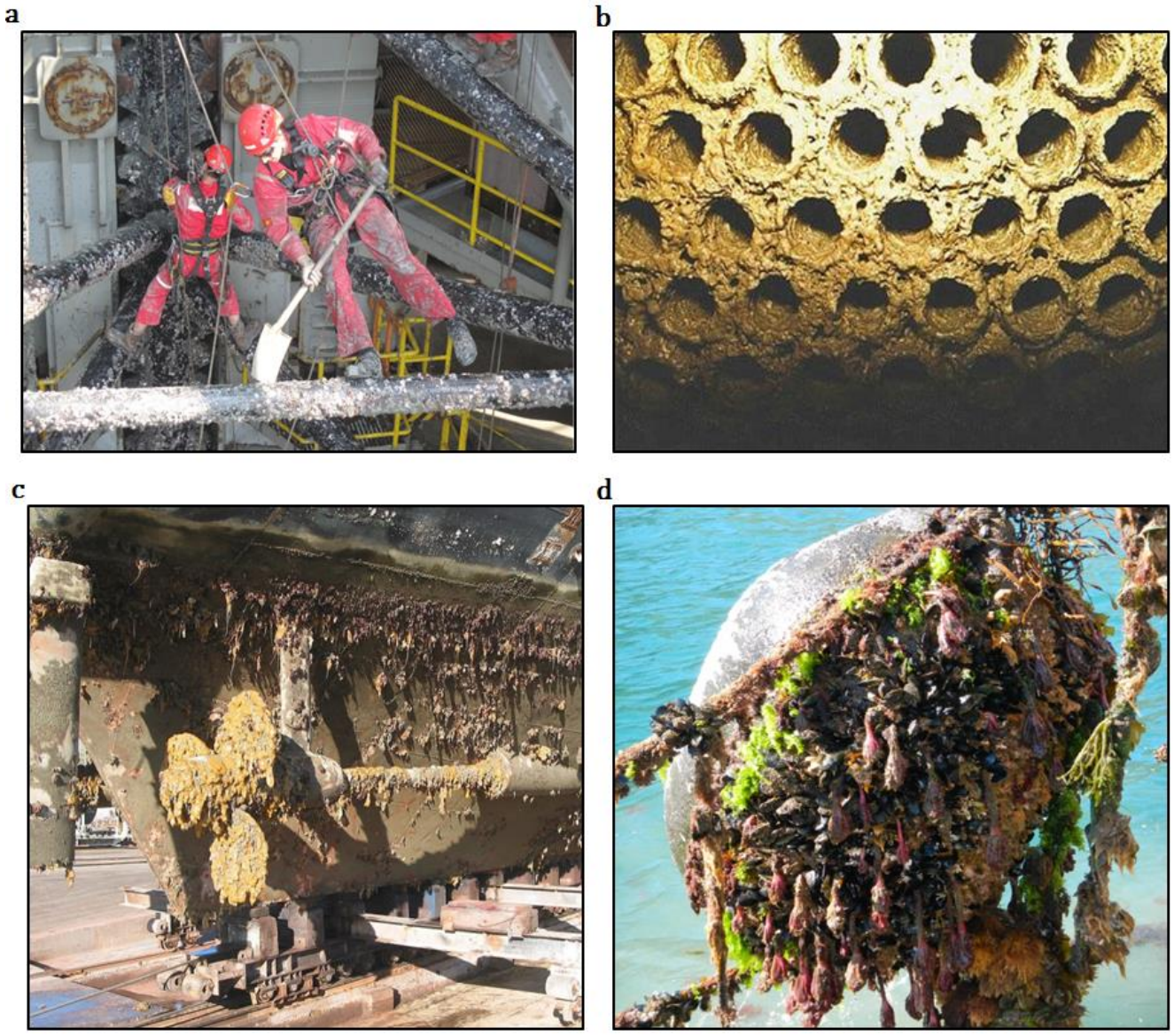

Figure 1.1. (a) Workers removing biofouling from a jack-up drilling rig. (b) Biofouling accumulation on underwater condensing tubing, photo credit: K.A. Selby (http://www.powermag.com/biofouling-control-options-for-cooling-systems). (c) Biofouling on the hull of a dry-docked commercial vessel. (d) Biofouling smothering the under-side of a mussel farm buoy. Remaining photo credits: Cawthron Institute.

Aquaculture operations frequently battle with biofouling and predatory organisms, although the consequences of their presence varies (Dalby *et al.* 1993; Ross *et al.* 2002b; Antoniadou *et al.* 2013; Lacoste & Gaertner-Mazouni 2014). For example, the growth of sponges on farmed bivalves is considered a positive influence, through the provision of increased protection for shellfish from predators and harmful epibionts via the secretion of bioactive compounds or camouflaging (Ross *et al.* 2004; Antoniadou *et al.* 2013). In contrast, the growth and accumulated biomass of ascidians is typically damaging, reaching a point where the byssal threads of mussels are unable to sustain the combined weight, resulting in mussel detachment and stock loss (Witman & Suchanek 1984; Ramsay *et al.* 2008; Woods *et al.* 2012; Antoniadou *et al.* 2013; Sievers *et al.* 2013). Although the settlement of biofouling organisms has been documented as potentially beneficial (Dalby *et al.* 1993; Ross *et al.* 2002b; LeBlanc *et al.* 2003), these and other examples are thought to be the "exceptions" (Fitridge *et al.* 2012) as most biofouling organisms are considered a nuisance with primarily detrimental impacts (Fitridge *et al.* 2012; Sievers *et al.* 2014). For instance, the clogging of nets and cages by biofouling organisms can reduce water circulation, oxygen and food availability to cultured species (Switzer *et al.* 2011; Rosa *et al.* 2013; Sievers *et al.* 2013; Aldred & Clare 2014).

1.3. The dispersal of biofoulers and influence of artificial structures

The adult life stage of most biofouling species is sedentary (Marshall *et al.* 2009). Consequently, recruitment to and migration among populations is restricted to dispersive larval stages. The scale of larval dispersal dictates connectivity between marine populations, which governs a population's resilience against catastrophe or disease, influences their potential as a source of new individuals to other populations and dictates community structure and genetic mixing (Bradbury *et al.* 2008; Thomas & Bell 2013).

The average scale of dispersal is influenced by a range of ecological and environmental factors, and therefore varies among taxa and across geographical, temporal and spatial scales (Cowen *et al.* 2003; Kinlan *et al.* 2005; Cowen *et al.* 2006). Generally, algal sporelings and ascidians have

short larval competency periods, with poor dispersal capabilities (Svane 1989; Santelices 1990), whereas oceanic molluscs (Crooks 1996), tubeworms (Bochert 1997) and sea stars (Kasyanov 1984; Kasyanov 1988; Kasyanov *et al.* 2001) have greater dispersal capabilities, with longer larval competency periods. Some species are also able to cover the spectrum of dispersal possibilities, enabling the potential for population replenishment and the possibility of distant dispersal. For example, the northern Pacific seastar *Asterias amurensis* is capable of asexual reproduction through fission and sexual reproduction, with the development of long-lived planktonic larvae (Hatanaka & Kosaka 1959; Kasyanov 1984; Kasyanov 1988; Byrne *et al.* 1997; Kasyanov *et al.* 2001). However, hydrodynamic variability and behavioural adaptations can cause dispersal scales to vary between larvae from the same species, but different populations (Jones & Babb 1968; Lau & Qian 2001; Shanks *et al.* 2003; Mace & Morgan 2006; Shanks 2009).

The deployment of artificial structures, resultant removal of isolation barriers and increase in habitat heterogeneity can alter the dispersive potential of biofouling species. This is because structures can function as corridors or stepping-stones within the marine environment, connecting otherwise separated populations (Figure 1.2) (Glasby & Connell 1999; Thuiller *et al.* 2007). For example, the deployment of oil and gas platforms has been found to enhance the dispersal of coral populations in the Gulf of Mexico (Sammarco *et al.* 2004). Increased dispersal via stepping-stone movements can also enhance gene flow within a system (Airoldi *et al.* 2005), ultimately leading to reduced genetic diversity through reducing local adaptation within a species and, overtime, reducing the evolution of new species (Airoldi *et al.* 2005). This is evident along the Belgian Coast, where man-made structures have enabled the dispersal of the rough periwinkle *Littorina saxatilis*, which lacks a planktonic larval stage, resulting in reduced genetic variability in populations associated with artificial structures compared to those on natural coastlines (Johannesson & Warmoes 1990).

In addition, wave propagation and tidal currents can be modified by the introduction of artificial structures, limiting propagule dispersal and altering connectivity between marine populations

(Waite 1989; McNeill *et al.* 1992; Floerl & Inglis 2003; Koehl 2007; Bulleri & Chapman 2010). These alterations can enhance the entrainment of water within artificial environments such as enclosed marinas, dictating local propagule pressure (i.e., the abundance, quality and rate of arrival of larvae or juveniles into a new area), and altering the recruitment of species to surrounding habitats (McNeill *et al.* 1992; Floerl & Inglis 2003). Floerl and Inglis (2003) found that circulation patterns, altered by permanent breakwalls within marina basins, limit the dispersal of planktonic propagules, effectively increasing propagule pressure to available surfaces and enhancing the opportunity for the entrainment and subsequent spread of NIS larvae. Similarly, along-shore currents may be distrupted by the introduction of groynes, resulting in the accumulation of larvae and propagules along coastlines (Burcharth *et al.* 2007).

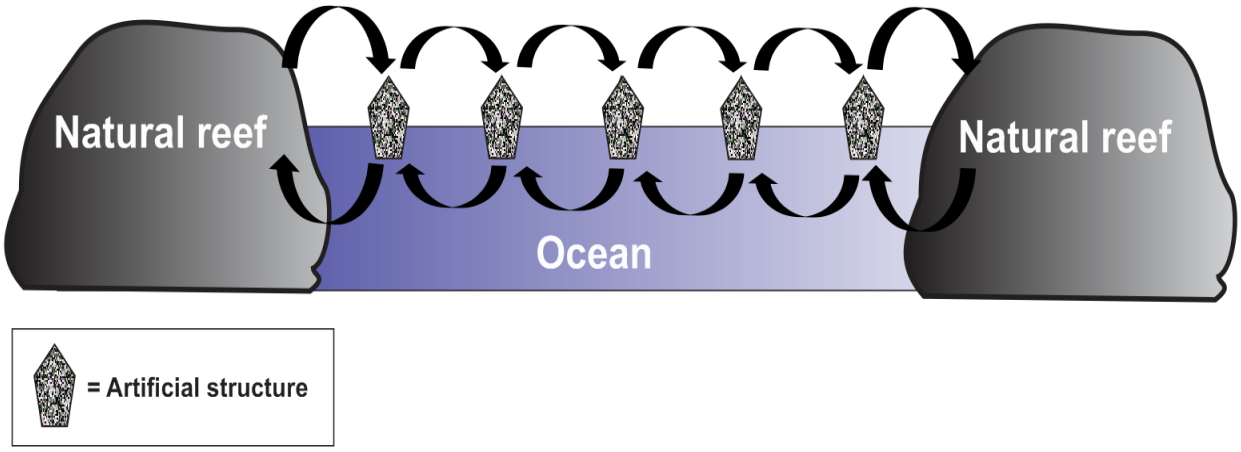

Figure 1.2. Schematic illustration showing how implementing artificial structures in areas with few natural substrates can favour dispersal of species outside their natural ranges, at a regional scale, thus increasing connectivity between habitats (in this case reef habitats) that would be naturally isolated. Figure adapted from Airoldi et al. (2005).

The movement of artificial structures including, but not limited to, aquaculture transfers, commercial and recreational vessel movements and dry-docking activities, also influence the potential dispersal of marine larvae (Lambert 2002; Floerl & Inglis 2003; Minchin *et al.* 2006). For example, pleasure crafts have been implicated in the introduction of invasive algal species, such as *U. pinnatifida* and *C. fragile,* along with unwanted mussel species such as the black stripped mussel *Mytilopsis sallei* (Hay 1990; Chapman 1998; Power *et al.* 2004). The movement of aquaculture equipment (especially rope) and transfer of stock between main aquaculture regions has contributed to the spread of problematic biofouling species, including colonial and solitary ascidians, such as the 'carpet ascidian' *Didemnum vexillum* and 'sea vase' *Ciona intestinalis*, respectively (Forrest & Blakemore 2006; Denny 2008; Therriault & Herborg 2008).

1.4. Aquaculture as an artificial habitat

Substratum properties, specifically roughness, wettability, surface tension and polarisation, influence biofouling patterns by regulating the early stages of biofilm development (Terlizzi & Faimali 2010). The attributes of aquaculture infrastructure make them particularly suitable as an artificial habitat for biofouling organisms. For instance, the rough fibrous and porous texture of shellfish ropes is favourable for larval settlement (Anderson & Underwood 1994). Similarly, finfish cages, which use nylon netting, have a rough micro-surface that enhances colonisation by increasing the potential surface area and providing depressions that protect colonising species (Costa-Pierce & Bridger 2002).

Along with the structure of marine farms, their location is often conducive to biofouling growth and accumulation (Blum *et al.* 2007; Fitridge *et al.* 2012; Antoniadou *et al.* 2013; Sievers *et al.* 2013). Farms are predominantly located in sheltered areas, where natural substrates are of limited availability, and in long-line shellfish cultures, biofoulers are protected from benthic predators due to the physical separation of the culture from the benthic habitat (Jory *et al.* 1984; Inglis & Gust 2003; LeBlanc *et al.* 2007; McKindsey *et al.* 2007; Woods *et al.* 2012; Fletcher *et al.* 2013b). Furthermore, some aquaculture facilities (e.g., finfish farms) supply

additional nutrients to the surrounding area, potentially increasing the quantity of otherwise limiting nutrients and enhancing the overall growth and structure of biofouling communities (Costa-Pierce & Bridger 2002).

Biofouling accumulation is predominantly detrimental for aquaculture industries, increasing processing and production costs, reducing revenue and, in some instances, impacting the growth of crop species (Fitridge *et al.* 2012; Lacoste & Gaertner-Mazouni 2014). For example, heavy fouling by solitary ascidians, such as *C. intestinalis* and *Styela clava*, has been associated with higher mussel mortality and lower overall stock size and condition (Howes *et al.* 2007; LeBlanc *et al.* 2007; Sievers *et al.* 2013); fouling by the pink-hearted hydroid *Ectopleura crocea* can reduce the availability of wild mussel larvae in commercial cultures of the Mediterranean mussel *Mytilus galloprovincialis* (Fitridge & Keough 2013); and shell-boring polychaete worms, such as *Polydora* species, reduce hinge stability, disrupt shell-formation and increase the vulnerability of shellfish to predation by generating blisters within the nacreous layer of shellfish (Che *et al.* 1996; Lleonart *et al.* 2003; Silina 2006; Simon *et al.* 2006). The growth of calcareous biofoulers also reduces shellfish appearance, devaluing aquaculture products (Fitridge *et al.* 2012; Sievers *et al.* 2014).

The biomass of these communities can be substantial (Grant *et al.* 1998; McKindsey *et al.* 2009), with fouling organisms reaching 15% of the total biomass of mussel long-lines in New Zealand (Fletcher *et al.* 2013b), and 20% of the biomass of the Pacific oyster *Crassostrea gigas* in the Atlantic (Royer *et al.* 2006). This additional weight has implications for the mechanical handling of equipment. For example, dominant growth by the ringed tubularian *Ectopleura larynx* in the Norwegian fish farming industry increases the duration of infrastructure cleaning during the peak of fouling seasons (Guenther *et al.* 2009; Guenther *et al.* 2010). Excess weight also contributes to structural damage in aquaculture and increases costs associated with buoyancy and anchoring systems (Claereboudt *et al.* 1994).

Aquaculture crop also suffers from competitive interactions and predation associated with biofoulers (Ramsay *et al.* 2008). For example, an increased biomass of the invasive colonial ascidian *D. vexillum* was found to displace small, cultured New Zealand green-lipped mussels *Perna canaliculus* (Fletcher *et al.* 2013b). Biofouling organisms can prey directly on stock (Ross *et al.* 2002a; Fitridge & Keough 2013) or attract their own suite of predators that subsequently consume cultured species (O'Connor & Newman 2003). Similarities in the clearance rates of biofouling species and crop organisms can generate competition for food resources, potentially inhibiting crop growth (Arakawa 1990; Riisgård *et al.* 1996; LeBlanc *et al.* 2003; Petersen 2007; Daigle & Herbinger 2009; Sievers *et al.* 2013). For example, *C. intestinalis* has an overlapping preference in the size range of particles consumed by cultured blue mussels *M. edulis* (Daigle & Herbinger 2009). Consequently, after inoculating experimental mussel ropes with different densities of *C. intestinalis,* Sievers *et al.* (2013) found fouled blue mussels had a shorter shell length and reduced flesh weight compared to unfouled mussels. Interestingly, there are documented examples where farmed species have 'adapted' to the presence of biofouling organisms, including mobile species such as crabs. For example, reduced spat retention and increased byssal thread production has been detected in *P. canaliculus* in response to predation by the decorator crab *Notomithrax minor* (Van de Ven 2007).

1.5. Management strategies, current and future

Owing to the significant negative impacts associated with biofouling in the marine environment, a number of techniques have been trialled and employed to avoid, mitigate or prevent biofouling (Fitridge *et al.* 2012). However, avoidance of biofouling can be challenging, leading to approaches predominantly focused upon treatments, which can be effective, but also time and labour intensive, rather than preventative (Fitridge *et al.* 2012; Sievers *et al.* 2014). Depending upon the intensity and composition of the biofouling communities, common techniques used for biofouling removal include exposing artificial infrastructure to periods of air-drying, antifouling or protective coatings, power washing, chemical dips (e.g., acetic acid) and fresh or hot water baths (Chambers *et al.* 2006; Forrest & Blakemore 2006; López-Galindo

et al. 2010; Carl *et al.* 2012; Fitridge *et al.* 2012). While these techniques may be successful for the removal of soft-bodied biofoulers, they fail to remove several species of barnacles and calcareous tubeworms (Carver *et al.* 2003; Forrest & Blakemore 2006; LeBlanc *et al.* 2007). In addition, their implementation can have undesirable effects, such as the fragmentation of colonial organisms, which may contribute to their localised spread (Hopkins & Forrest 2010; Paetzold & Davidson 2011). For example, the *in-situ* removal of colonial ascidians, such as *D. vexillum*, *Botrylloides schlosseri* and *Eudistoma elongatum* from mussel long-lines and intertidal oyster racks could cause fragmentation and enhance species spread (Page *et al.* 2011; Switzer *et al.* 2011; Morris & Carman 2012). In aquaculture industries, biofouling treatments can also increase the intensity of stock stress and mortality (LeBlanc *et al.* 2007; Antoniadou *et al.* 2013).

Sievers *et al.* (2014) suggested that an alternative strategy to using treatments in isolation for biofouling management, could be to incorporate a more information-based approach. Such an approach would involve linking knowledge about site-specific patterns of biofouling development, or predictions of their occurrence, with strategies to avoid specific locations during times of heavy biofouling or within areas where detrimental species are dominant. This approach has also been advocated by Fitridge *et al.* (2012) to provide aquaculture industries with cost effective opportunities for the successful application of removal strategies.

Another important aspect in the management of biofouling populations is that these populations are connected by the exchange of individuals, predominantly through pelagic larval transport (Bradbury *et al.* 2008). This has important implications for managing populations within heterogeneous environments, where the distance between "stepping-stone" habitats and the source habitat have been found to be imperative in the process of species spread (Söndgerath & Schröder 2002). Connectivity has therefore been widely implemented in invasive species management, marine reserve design and species conservation projects (Söndgerath & Schröder 2002; Shanks *et al.* 2003; Levin 2006; Treml *et al.* 2008).

To enhance management strategies, site-specific knowledge of biofouling patterns could be incorporated with an understanding of the scales at which local biofouling 'subpopulations' are connected by dispersal (larvae, recruits, juveniles or adults), also described as connectivity between metapopulations (Cowen & Sponaugle 2009). This can be achieved through the collaborative use of ecological, genetic and oceanographic-modelling tools (Selkoe *et al.* 2008). These multidisciplinary approaches have already been used to provide new insights into the spatial ecology of marine populations, guiding new approaches in fisheries management and marine reserve networks (Baums *et al.* 2006; Galindo *et al.* 2006; Selkoe & Toonen 2006; Selkoe *et al.* 2008; Treml *et al.* 2008; Galindo *et al.* 2010; Piñones *et al.* 2013). For example, Stenseth *et al.* (2006) combined long-term ecological monitoring data, oceanographic modelling, and genetic analyses to evaluate the ecological and genetic impact of larval drift on Atlantic cod (*Gadus morhua*) population dynamics in the North Sea-Skagerrak area.

Marine artificial structures, such as aquaculture farms, can generate a spatially heterogeneous environment as they are fragmented in their arrangements, generating patchy habitats for biofouling organisms and promoting metapopulation dynamics. Incorporating an understanding of sourcing dynamics, dispersal abilities and population connectivity with the knowledge of local population dynamics, may therefore be an important and currently underused step forward for commercial industry management. This integrative approach may also have important implications for the ongoing management and ecological understanding of pest species world-wide, whether they are present in marine, freshwater or terrestrial systems.

1.6. Significance for New Zealand aquaculture and general aims

Aquaculture is a significant component of New Zealand's economic wealth, which is dominated by the long-line culture of the endemic green-lipped mussel *Perna canaliculus*. Green-lipped mussel cultivation produces around 95,000 tonnes per annum worth over $200 million (NZD) in export and domestic supply (Fisheries. 2011). In New Zealand, Pelorus Sound (located in the Marlborough Sounds) is the major growing region for the mussel farming industry, with

approximately 645 farms spread across 5,000 ha of farming area (Woods *et al.* 2012). Despite the value and extent of this area, aside from a few site-specific studies (Woods *et al.* 2012), our knowledge about the spatial and temporal variation of biofouling organisms associated with these aquaculture farms is limited. Furthermore, while studies have shown that the dispersal of planktonic propagules and connectivity between suitable habitats, such as artificial structures, play an important role in the persistence and localised spread of marine populations (Kinlan *et al.* 2005; Gaines *et al.* 2007; Cowen & Sponaugle 2009), investigations into the genetic and demographic connectivity of NIS biofouling taxa have not been undertaken wholly within New Zealand aquaculture environments.

Through a combination of ecological, genetic and mathematical modelling tools, using field and laboratory-based investigations, the overall aims of this thesis were:

1. To characterise the spatial structure of biofouling assemblages associated with marine farms in Pelorus Sound.
2. To elucidate patterns of connectivity and gene flow trajectories among biofouling populations within the Marlborough Sounds region, using the invasive, colonial ascidian *Didemnum vexillum* as a case study organism.

This thesis has been divided into two data chapters. Chapter II is a characterisation of the structure of biofouling communities, whereas Chapter III is an investigation into the patterns of genetic connectivity between *D. vexillum* populations within the study region. Chapter IV expands on the main findings of this research and discusses these in the context of biofouling and invasive species literature. The implications of this research within an aquaculture setting and the validity of the results for use by managers and industry members alike are also discussed.

Chapter II
Variable Structure in Communities Fouling Mussel Long-Lines in New Zealand

2.1. Introduction

Marine farm infrastructure comprises a diverse range of anthropogenic (or artificial) components, including ropes, floats, anchors, cages, nets and rafts (Fitridge *et al.* 2012). These surfaces, which intercept water flow and consequently larvae in the water column, provide extensive habitat for the colonisation of biofouling organisms (Metri *et al.* 2002; McKindsey *et al.* 2007; Dürr & Watson 2010; Adams *et al.* 2011; Fitridge *et al.* 2012; Antoniadou *et al.* 2013; Sievers *et al.* 2013). For shellfish farms (e.g., mussels, oysters and scallops), the crop also provides a large three–dimensional habitat for colonisation. Numerous studies have shown that a diverse range of biofouling organisms can accumulate on farm structures and crop, with suspension-feeders (e.g., bivalves, hydroids, bryozoans, and ascidians) among the most abundant (Dean 1981; Cronin *et al.* 1999; Railkin 2003; Fitridge *et al.* 2012; Lacoste & Gaertner-Mazouni 2014). For example, Woods et al. (2012) found nearly 88% of biofouling biomass on mussel ropes in New Zealand comprised suspension-feeding organisms. Similarly, 97% of the biofouling biomass on Norwegian salmon farm nets comprised sessile filter-feeders including the blue mussel *Mytilus edulis* and the hydroid *Ectopleura larynx* (Bloecher *et al.* 2013).

Marine biofouling communities are often dominated by introduced species (i.e., non-indigenous species, or NIS), especially in areas where human-mediated colonisation is frequent, such as in ports and marinas (Lambert & Lambert 1998; Tyrrell & Byers 2007). For example, annual dock surveys in Bodega Harbour, California, revealed that NIS represented 71% of biofouling cover in this area (Sorte *et al.* 2010). Artificial structures such as marine farms can act as reservoirs for NIS, where species biomass can accumulate and secondary spread can be facilitated (Foster & Willan 1979; Bulleri & Airoldi 2005; Glasby *et al.* 2007; Goldstien *et al.* 2010; Fitridge *et al.* 2012). For example, the introduction and subsequent spread of the solitary ascidian *Eudistoma elongatum* among oyster farms in Northland, New Zealand has been attributed to the movement of oyster farm equipment and stock (Morrisey *et al.* 2009). Internationally, the role of shellfish cultivation as a pathway for the spread of marine pests and NIS is well documented, linking crop and equipment transfers to the regional spread of toxic or noxious microalgae, invasive ascidians, parasites and diseases (Naylor *et al.* 2000; Wasson *et al.* 2001; Hewitt *et al.* 2004; Ruesink *et al.* 2005; Keeley *et al.* 2009; Antoniadou *et al.* 2011; Fletcher *et al.* 2013b). Estimates by Ruesink *et al.* (2005) revealed that oyster aquaculture alone contributed to the introduction of 40% of exotic marine species across Europe, the western United States and the North Sea.

The occurrence of biofouling in aquaculture is a significant issue which impacts cultured species directly and indirectly, promotes operational interference, shortens the window available for harvesting and can substantially reduce economic revenue (Fitridge *et al.* 2012; Lacoste & Gaertner-Mazouni 2014). Dominance of specific biofouling species and their associated impacts can vary across locations (Ceccherelli & Campo 2002; Thomsen *et al.* 2006), within geographical regions (Bulleri *et al.* 2010; Heiman & Micheli 2010) and over time (Stæhr *et al.* 2000; Forrest & Taylor 2002). Some biofouling populations proliferate rapidly, and gradually retreat. This is especially true for taxa such as ascidians, including *Didemnum vexillum, Ciona intestinalis* and *Styela clava*, and filamentous green algae belonging to the genus *Cladophora* in New Zealand. For example, a population 'explosion' of *C. intestinalis* in the New Zealand aquaculture industry

in the late 1990's was followed by gradual decline and variability in the impacts associated with this species (Forrest *et al.* 2011). Furthermore, 'boom and bust' population cycles have been documented for *D. vexillum* colonies in the Marlborough Sounds, with seasonal regression recorded during colder winter months (Valentine *et al.* 2007b; Fletcher *et al.* 2013a). It has been postulated that this may be a result of resource exploitation, but the underlying mechanisms are poorly understood and are currently under scientific investigation (Cawthron Institute, unpublished data).

For many years, processes determining patterns in the number and composition of co-occurring species have been central to community ecology. According to niche-based theories, random patterns in community assemblages may be driven by deterministic process, such as environmental filtering and biotic interactions (Chesson & Case 1986; Chesson 2000; Condit *et al.* 2002). In contrast, neutral theories assume species are equivalent to one another in all important ecological respects, emphasising the role of stochastic processes, such as chance colonisation, dispersal limitations, random extinction and ecological drift (Bell 2001; Hubbell 2001; Chave 2004; Leibold & McPeek 2006). These perspectives, which date back to the earliest days of ecology, continue to fuel contemporary debates (McGill 2003; Volkov *et al.* 2003; Gilbert & Lechowicz 2004; Wootton 2005; Gravel *et al.* 2006). However, an increasing number of studies have emphasised the importance of both processes in structuring ecological communities (Thompson & Townsend 2006; Cadotte 2007; Chase & Myers 2011; Stegen *et al.* 2012; Kitching *et al.* 2013). For example, Caruso *et al.* (2011) demonstrated that the structure of desert microbial communities depends upon a balance between deterministic and stochastic forces. Similarly, investigations of moth fauna composition across primary and post-logging forests in Bornean rainforests found niche and neutral concepts explained species turnover. (Kitching *et al.* 2013). Additionally, a recent meta-analysis of metacommunitites found nearly 50% of the variation in community composition was explained by environmental and spatial variables (Cottenie 2005).

Temporal variation in marine invertebrate populations is often driven by seasonality, with the arrival of recruits, growth intensity and times of dormancy and regression impacting community development (Mazouni *et al.* 2001; Howes *et al.* 2007). However, spatial variability is thought to be primarily driven by specific planktonic events, larval choice during settlement and metamorphosis, as well as mortality, which are predominantly correlated with environmental conditions and habitat structure (heterogeneous or homogeneous) (Fitridge *et al.* 2012). For example, a higher abundance of *C. intestinalis* within shaded areas on the lower surface of mussel rafts (Rius *et al.* 2011) and collection plates (Howes *et al.* 2007) has been attributed to the settlement behaviour of larvae, which show a preference towards shaded and sheltered places (Tsuda *et al.* 2003; Howes *et al.* 2007; Rius *et al.* 2010; Rius *et al.* 2011). Area-specific variation in biofouling distribution is also influenced by biotic interactions, light availability, water flow, depth and the orientation of infrastructure (Cronin *et al.* 1999; Howes *et al.* 2007; Zintzen *et al.* 2011; Fitridge *et al.* 2012). For example, Woods *et al.* (2012) and Cronin *et al.* (1999) found reduced biofouling biomass and less diversity in biofouling communities with increased depth on marine farm structures (mussel long-lines and tuna sea cages, respectively).

The accumulation and dynamics of biofouling assemblages on artificial structures have been studied for over several decades (Bailey-Brock 1989; Lesser *et al.* 1992; Claereboudt *et al.* 1994; Terlizzi & Faimali 2010). However, recent literature has highlighted the need for more quantitative studies investigating the spatial and temporal variation in the larval recruitment and settlement biology of biofouling species, as well as their associated development on marine farming infrastructure (Fletcher *et al.* 2013b; Sievers *et al.* 2014). Furthermore, as biofouling communities are inherently complex, information on the entire community is needed to maximise biofouling management strategies (Fitridge *et al.* 2012; Antoniadou *et al.* 2013; Sievers *et al.* 2014). Biofouling patterns have been investigated on artificial structures overseas, including biofouling accumulation on mussel farm long-lines in the eastern Mediterranean

(Antoniadou *et al.* 2013) and on PVC plates suspended within mussel farm areas in Australia (Sievers *et al.* 2014). Nevertheless, knowledge around the spatial and temporal variation of biofouling organisms associated with aquaculture infrastructure in New Zealand is limited, although a few site-specific studies (e.g., Woods *et al.* (2012) have recently been conducted.

In the current study, there were three specific aims: (1) to characterise biofouling on mussel farm long-lines in the Pelorus Sound, New Zealand, with the expectation that community structure and the relative abundance of pest species would vary across the study region, (2) to investigate the influence of depth on biofouling community structure and species abundance, with the expectation that biofouling cover would decrease with increasing long-line depth, based on the findings of previous research (Dharmaraj *et al.* 1987; Cronin *et al.* 1999; Woods *et al.* 2012), and (3) to assess the potential mechanisms contributing to biofouling structure. The relationship between biofouling community similarity and the distance between marine farms was assessed. In accordance with the known environmental differences in Pelorus Sound and the spatial heterogeneity of artificial substrata, the expectation was that community similarity would decrease as the geographical distance between farms increased.

2.2. Materials and Methods

2.2.1 Study region

The study region, Pelorus Sound, is situated within the Marlborough Sounds, located at the northern end of New Zealand's South Island (Figure 2.1). It is a 56 km long, relatively deep (average depth 40 m) and highly indented estuarine system with variable freshwater input from the Pelorus and Kaituna rivers, as well as oceanic exchange from upwelling waters in Cook Strait (Heath 1974; Woods *et al.* 2012). Records from Gibbs *et al.* (1991) and Heath (1974) describe significant density stratification across the Pelorus Sound associated with salinity gradients. The strongest density stratification was recorded within the side arms and embayments near the head of Pelorus Sound (Heath 1974; Gibbs *et al.* 1991), with a gradual decrease in mean salinity moving towards the confluence with Kenepuru Sound (Heath 1982). In this study I used a 'Conductivity/Temperature/Depth' profiler (CTD) to conduct a coarse-level survey of the environmental parameters during a pre-rain (following a drought period) and post-rain event (following heavy flooding). Despite the limitations of one-off or infrequent water column monitoring (Comin *et al.* 2004), it was hoped that these surveys would help identify environmental gradients that could be used to assist in interpreting patterns in biofouling distribution data. Interestingly, there was no evidence of a salinity or temperature gradient from the two CTD surveys conducted, even immediately after a flood event (Appendix I, Figures A1.1-A1.2). However, in areas near the head of Pelorus Sound higher levels of chlorophyll-*a* and turbidity were recorded (Appendix I, Figures A1.3-A1.4). PAR irradiance, on the other hand, was recorded at higher levels near the entrance of Pelorus Sound (Appendix I, Figure A1.5). A detailed investigation into the drivers of biofouling biomass and diversity in Pelorus Sound is currently being conducted (Cawthron Institute, unpublished data) and consequently a larger scale investigation of environmental variables was not undertaken in this study.

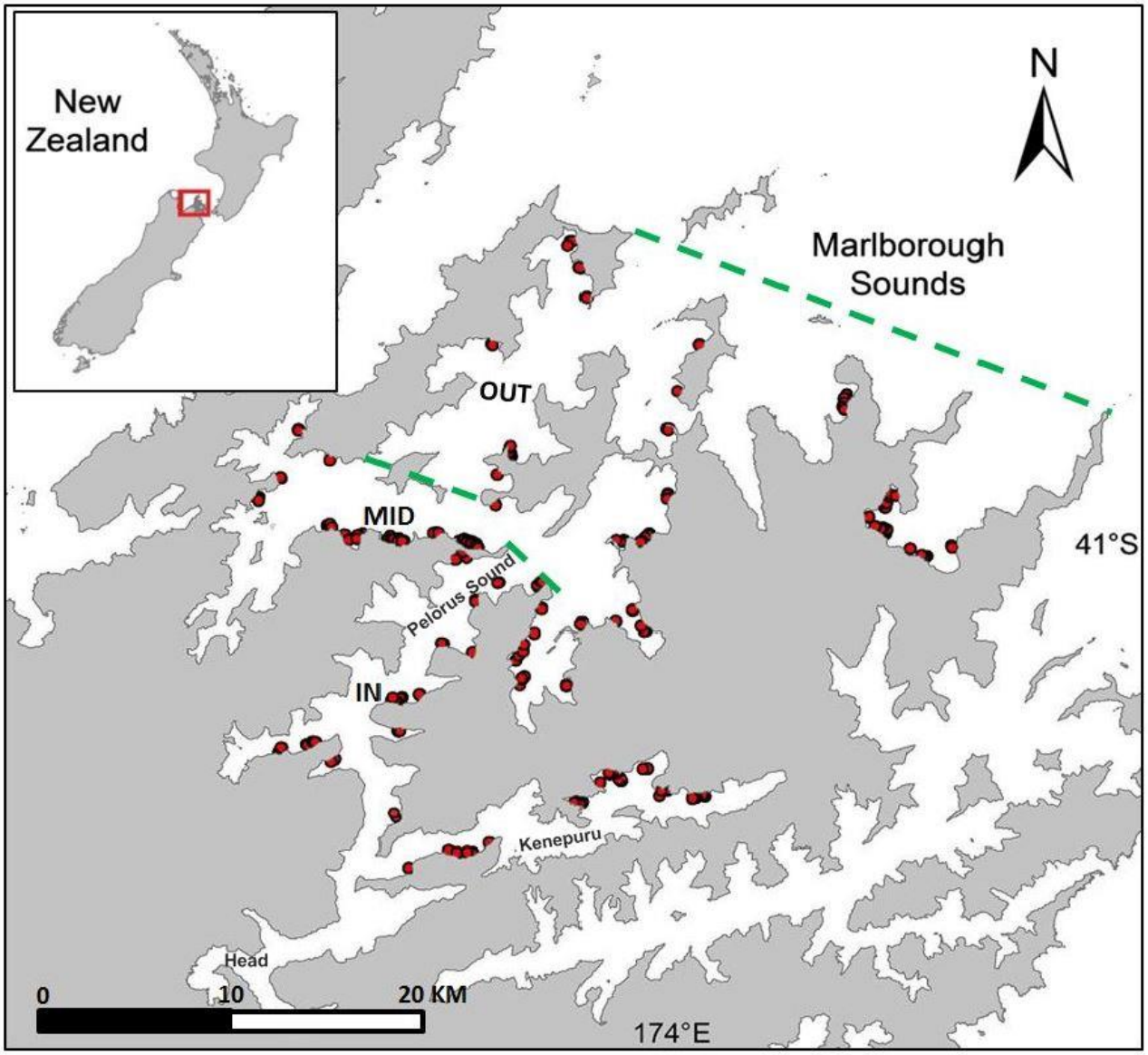

Figure 2.1. Map of the Marlborough Sounds region, showing the location of green-lipped (*Perna canaliculus*) mussel farms sampled in Pelorus Sound (filled red circles), from the inner = IN, middle = MID, and outer = OUT areas. Dotted green lines (- - -) indicate the delineation of the three areas. The 'head' of the Pelorus Sound and the Kenepuru Sound are indicated. Inset shows the location of the Marlborough Sounds at the northern tip of New Zealand's South Island.

2.2.2 Field methods

In January 2013, 74 green-lipped (*Perna canaliculus*) mussel farms were sampled within the Pelorus Sound, from the inner (N=30), middle, (N= 27) and outer (N=17) areas (Figure 2.1). Area boundaries were set in accordance with previously conducted dispersal studies (Knight *et al.* 2010), and the known environmental gradients (Heath 1974; Gibbs *et al.* 1991) in Pelorus Sound. At each mussel farm a random selection of long-line droppers (≥ 2 per farm) were lifted from the water column using a winch on-board the Sanford mussel-sourcing vessel and were systematically sampled using photographs (Nikon Coolpix AW100), video footage (Sony HDR-XR350VE), visual assessments and by collecting a subset of biomass samples. Visual assessments involved observing and recording characteristics of each long-line dropper (i.e., the entire length), including the presence of dominant biofouling species, biofouling extent (low ≤ 30%, medium 31 – 70%, or high ≥ 71% cover), age of the crop, spat type, percent cover of bare rope space and the morphology (i.e., 2-D versus 3-D) of the invasive colonial ascidian *Didemnum vexillum* (Figure 2.2a, b). In addition, biofouling development on the backbone ropes and floating buoys of all sampled farms was quantified. Photographs and voucher specimens of most taxa were collected for identification and future reference.

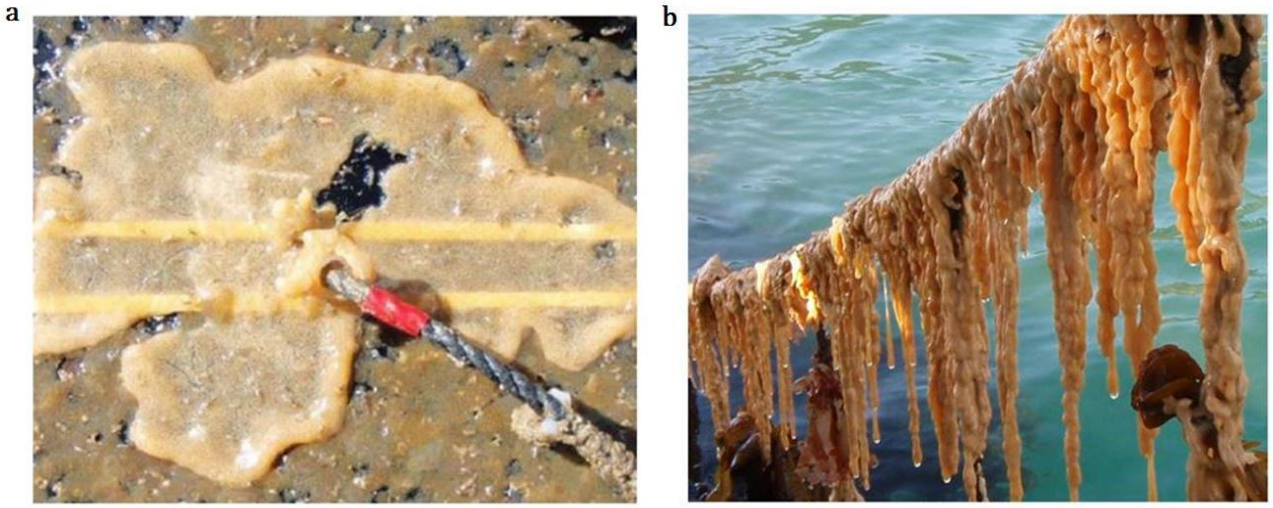

Figure 2.2. Morphology of *Didemnum vexillum* colonies: (a) encrusting or 2-D, and (b) long tendril-like 3-D lobes. Photo credits: L. Fletcher, Cawthron Institute.

Photoquadrats (40 x 20 cm) were taken from each long-line dropper at two depths. Three photoquadrats were positioned within the surface 3 m of the dropper, and three photoquadrats within the bottom 3 m (Figure 2.3). Due to the topography of Pelorus Sound, the water depth of the three bottom images varied across some samples, with mussel farms near the head of Pelorus Sound generally in shallower water than those near the entrance (approximately 12 m and 24 m water depth, respectively). Video footage covered the areas where photoquadrats were taken as well as the remaining length of long-line droppers. Footage was only used as a back-up resource for confirmation of visual assessments or images.

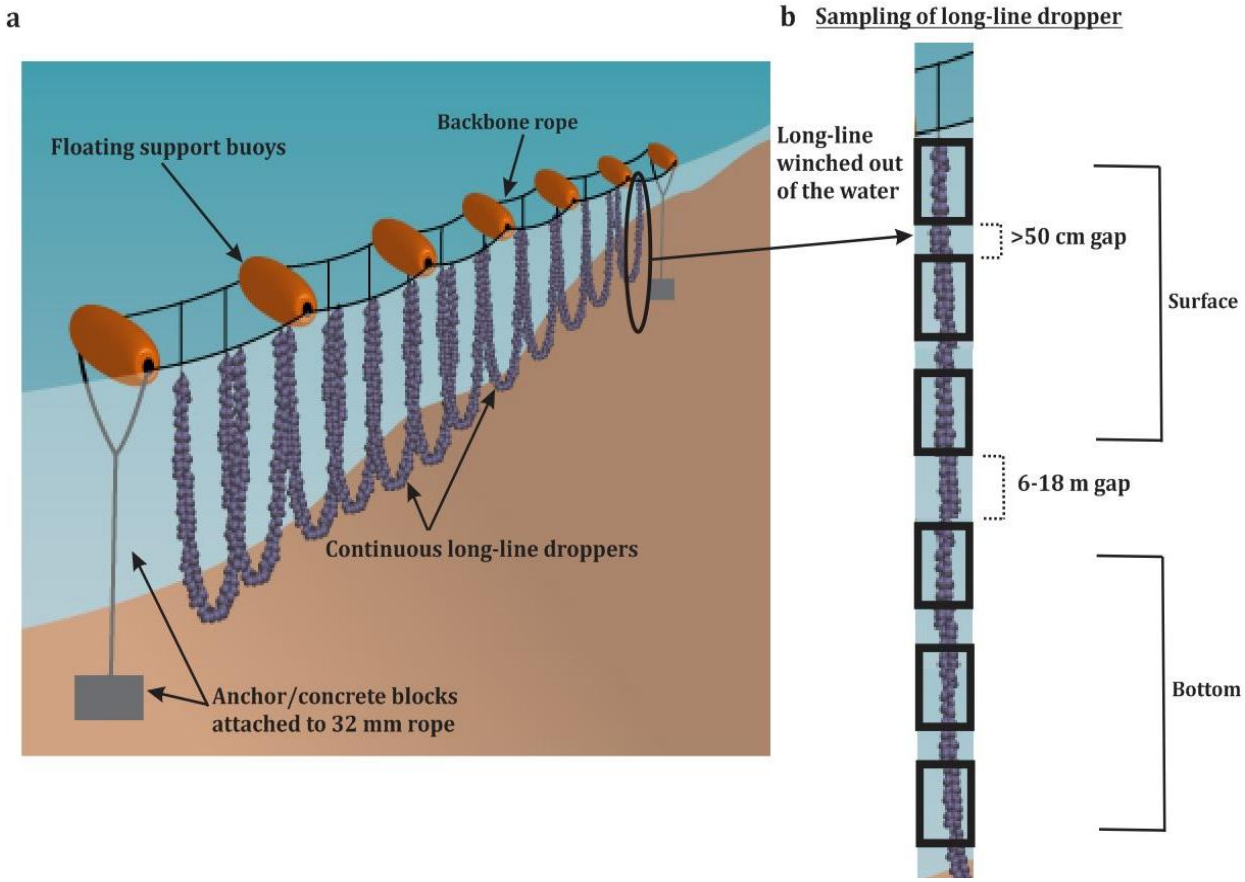

Figure 2.3. Schematic diagram of (a) a surface long-line mussel farm, and (b) the sampling design used on mussel long-line droppers after they were lifted from the water. Three photoquadrats were taken at random within the surface 3 m of long-line droppers and three within the bottom 3 m. A gap of at least 50 cm was left between images within each depth bracket, and 6-18 m gaps were left between the surface and bottom photoquadrats, depending on the depth of the long-line droppers (12-24 m).

During crop-condition assessments, staff on-board the mussel-sourcing boat, which services over 200 mussel farms in Pelorus Sound (Sanford Ltd, pers.comm.), often estimate and record the presence and extent of biofouling biomass on green-lipped mussels. Following discussion with these staff members, biomass categories (low, medium and high) were selected and assessed to determine how to reliably estimate biomass visual assessments and to investigate how these related to excess line weight. Vertical sections (0.4 m) of mussels and associated biofouling were therefore collected from a total of 15 long-lines, with five long-lines per biomass category. Samples were photographed prior to removal from the dropper, labelled, bagged and transported chilled to the Cawthron Institute laboratory, Nelson. In the laboratory, the total wet weight of bivalves with biofouling was measured for each section. Also, the wet and dry weights (after drying to a constant weight at 80°C for 48 hours) of 20 New Zealand green-lipped mussels and their associated biofouling were recorded for each section, along with separate measurements of the weights of biofouling.

2.2.3 Image analyses

Images from photoquadrat sampling were cropped and enhanced in Google Picasa v.3.9 to improve image quality. Poor quality images were removed from analyses. The identity and percent cover of biofouling organisms were determined by importing images into Coral Point Count with excel extension (CPCe) for point count analysis and superimposing randomly stratified points over each image (Kohler & Gill 2006). A random stratification design was employed for overlaying points to reduce the potential for bias and occurrence of point over-clustering, while still generating a random measure (Kohler & Gill 2006).

Prior to processing the images from the main study, the accuracy and precision of point count analysis methods and the optimal number of points per image were verified during preliminary analyses (Appendix II, A2.1 Methods) and from the results, 75 points per image were selected (Appendix II, A2.2 Results, Tables A2.1-A2.3 and Figures A2.1-A2.3a, b). Biofouling organisms were identified to the highest taxonomic resolution possible from photographs. For certain

groups, such as filamentous algae and ascidians, identification was restricted to higher level descriptions (e.g., unidentified colonial ascidian 1). Where available, voucher species were used to assist with identification, and in some cases verification was provided by expert advice.

2.2.4 Statistical methods

2.2.4.1 Photoquadrat data verification

At the completion of photoquadrat data collection, biofouling measures were verified through comparisons with richness estimates generated from two non-parametric species richness estimators, the Chao1 and Chao2 indices, using PRIMER 6 with PERMANOVA add-on (Anderson 2001a; Clarke & Gorley 2006; Anderson *et al.* 2007). Both indices were employed because the Chao1 index is an abundance-based estimator, whereas the Chao2 index is an incidence-based approach which accounts for the number of species in the community that were not seen, rather than not present (Chao 1984, 1987; Chao & Shen 2003; Foggo *et al.* 2003).

2.2.4.2 Comparing photoquadrat sampling to visual assessments

To determine the efficiency of using photographs to capture biofouling taxa compared to visual estimates of presence/absence data for dominant biofouling taxa, the two datasets generated from these methods were compared. Percent cover data from photoquadrats were first transformed into presence/absence data using PRIMER 6 with PERMANOVA add-on (Anderson 2001a; Clarke & Gorley 2006; Anderson *et al.* 2007). The percent of records where photoquadrats recorded taxa as present were then compared to the percent of records where visual estimates did not observe these taxa, and vice versa, using R v.3.0.2 (R Core Team 2013).

2.2.4.3 Univariate analyses

Taxonomic richness in Pelorus Sound was measured using sample-based rarefaction. Rarefaction methods were employed to account for the unbalanced experimental design used in this study (i.e., a different number of mussel farms and long-lines were sampled across areas in Pelorus Sound) (Sanders 1968; Gotelli & Colwell 2001), and were based on the lowest

photoquadrat sample size (3 surface and 3 bottom images, N=6). Richness was compared across areas (fixed with three levels; inner, middle and outer) and with depth (fixed with two levels; surface and bottom) using a two-way univariate permutational analysis of variance (ANOVA) (Anderson 2001b). A univariate permutational analysis of variance is preferable over traditional ANOVA tests because *P*-values are attained using permutations, rather than relying on tabled *P*-values, which assume normality (Anderson *et al.* 2007).

Each term in the analysis was tested using 4999 non-restricted permutations of the appropriate units under a reduced model, and a Type I SS (sums of squares) was employed, given that the experimental design was inherently unbalanced (Anderson 2001b; Anderson *et al.* 2007). A Type I SS is a sequential approach and analyses are sensitive to the order in which factors enter the model (Shaw & Mitchell-Olds 1993; Langsrud 2003). Therefore, all analyses using a Type I SS were re-analysed, switching the order of factors in the ANOVA part of the model to check for any effects on result interpretations. Pielou's evenness was also calculated and compared across areas, and with depth, using univariate permutational analysis of variance (ANOVAs) as described above (Anderson 2001b). In addition, changes in dominance and diversity were evaluated by *k*-dominance species abundance curves, with a logistic transformation carried out on the y-axes to overcome difficulties of visual interpretation (Clarke 1990). Samples were averaged by area and depth. Richness and evenness variables and the *k*-dominance species abundance curves were measured and analysed using PRIMER 6 with PERMANOVA add-on (Anderson 2001a; Clarke & Gorley 2006; Anderson *et al.* 2007). Prior to analysing richness and evenness, the assumptions of homogeneity of variance and normality were checked using Levene's tests and by visual inspection of residual plots, respectively. To fulfil assumptions, taxonomic richness and evenness were power transformed.

2.2.4.4 Multivariate analyses

Distance-based permutational multivariate analysis of variance (Anderson 2001b) based on Bray-Curtis dissimilarities of the square root transformed data (Bray & Curtis 1957) were

conducted to investigate biofouling community structure across Pelorus Sound (from the inner, middle to outer areas), with depth. The Bray-Curtis coefficient was adopted as it is not affected by joint absences and is sufficiently robust for marine data (Field *et al.* 1982). A square root transformation was selected over a more severe transformation (e.g., fourth root or presence/absence transformation) where rare species contribute disproportionately more to analyses (Anderson 2001a). It is generally recommended that ecological species abundance data be transformed in multivariate analyses to reduce the influence of very abundant species in relation to less abundant species (Clark 1988; Anderson *et al.* 2007).

Mean squares calculated by PERMANOVA were used to estimate multivariate variance components for each term in the analysis, which were subsequently expressed as square root variance components (i.e., converting values to percentages of Bray-Curtis dissimilarity). These estimates are analogous to univariate estimators of variance components (Underwood & Petraitis 1993; Anderson *et al.* 2007). Each term in the analysis was tested using 4999 non-restricted permutations of the appropriate units under a reduced model (Anderson 2001b; Anderson & Braak 2003). Significant terms were then investigated using *a posteriori* pairwise comparisons with the PERMANOVA *t*-statistic and 999 permutations. To increase the power of analyses, non-significant interaction terms (i.e., $P>0.05$) were pooled (Winer 1962; Underwood 1997; Clarke & Gorley 2006). Differences in community structure among areas were visualised through principal coordinates analysis (PCO) (Gower 1966).

The PERMANOVA design comprised four factors and one covariate: (1) area (fixed with three levels; inner, middle and outer), (2) depth (fixed with two levels; surface and bottom), (3) farm (random, nested within area), and (4) long-lines (random, nested within farm), with crop age (i.e., the months since long-lines were first seeded) included as a covariate. Assumptions of normality and homogeneity of variance were assessed for the covariate and a square root transformation was performed to fulfil assumptions. The percent cover of bare rope space (average cover overall: 4%) and green-lipped mussels (average cover overall: 9%) were

removed from analyses to ensure similarity was not driven by substrate. Interactions between the covariate (age) and the fixed and random variables were excluded from analyses, as these were not of interest in this study. As the experimental design in this study was inherently unbalanced and a covariate was included in this analysis, a Type I SS was selected, as recommended by Anderson *et al.* (2007).

A one-way similarity percentage analysis (SIMPER, (Clarke 1993), based on the depth x area interaction, was used to identify the percentage contribution of each species (or taxon) to observed community dissimilarities (cut-off set to 80%). Taxa were considered important if their contribution to percentage dissimilarity was ≥ 6% (11% was the maximum contribution made by taxa to dissimilarity). The ratio of the average dissimilarity and standard deviation (Diss/SD) was used to indicate the consistency with which a given species contributed to dissimilarity (Clarke 1993; Clarke & Gorley 2006). Values ≥1.5 indicated a high degree of consistency. Taxa that consistently discriminated between areas and depths, and had a correlation >0.3 with the PCO axes were displayed as vectors in the PCO plot. Variability in the cover of contributing taxa were assessed using separate two-way univariate permutational analysis of variance tests (ANOVAs) on the square root transformed variables (Anderson 2001b). Each term in the analyses were tested using 4999 non-restricted permutations of the appropriate units under a reduced model (Anderson 2001b; Anderson & Braak 2003). The ANOVAs design comprised two factors: (1) area (fixed with three levels; inner, middle and outer), and (2) depth (fixed with two levels; surface and bottom). To account for multiple comparisons and control alpha inflation, a False Discovery Rate (FDR) correction was applied (N=8, P<0.02 represented significance) (Benjamini & Yekutieli 2001; Narum 2006). FDR corrections control the expected fraction of incorrectly rejected hypotheses out of the total number of hypotheses rejected (Benjamini & Yekutieli 2001).

PERMANOVA analyses are sensitive to differences in dispersion among groups (Anderson 2006; Anderson *et al.* 2007). Therefore, community dissimilarity may result from differences in the

areas location, relative dispersion, or both. Using PERMDISP analysis in combination with PERMANOVA can assist in identifying possible reasons for community dissimilarities. However, PERMANOVA is robust to many forms of heterogeneity, especially with large sample size and PERMDISP often detects differences in dispersion that are not substantial enough to inflate the error rates of PERMANOVA tests (Box 1953; Anderson 2006). A distance-based test for homogeneity of multivariate dispersions (PERMDISP), with pairwise comparisons, was used to uncover any differences in dispersion within areas and across depths using 4999 permutations.

2.2.4.5 Distance-decay assessment

Alongside information on the spatial structure of ecological communities, distance approaches provide an effective and informative approach for gauging the spatial turnover of communities, uniting several ecological phenomena, such as dispersal propensity and environmental structuring (Soininen *et al.* 2007; Morlon *et al.* 2008). Species spatial turnover, or beta diversity, often induces reduced community similarity with increasing geographic distance, known as the distance-decay relationship (Morlon *et al.* 2008). Distance-decay relationships were investigated in this study using Mantel tests calculated in R v.3.0.2 (R Core Team 2013) and the vegan community ecology package (Oksanen *et al.* 2011). Mantel tests investigate whether two different matrices show similar patterns of inter-site variation using a correlation between two dissimilarity matrices (Pearson correlation coefficient), and test the significance of the statistic using Monte Carlo techniques.

This study investigated two separate distance-decay relationships between Bray-Curtis dissimilarity matrices of (1) the taxonomic richness (rarefied richness), and (2) community structure (square root transformed) data, with a Euclidean matrix of the geographic distances between sampled farms within Pelorus Sound. Geographical distances were obtained from GPS coordinates (measured in decimal degrees). In addition, the Wisconsin double standardisation method, which improves the gradient detection of dissimilarity measures (Oksanen 2011), was used alongside Bray-Curtis dissimilarity to generate the community structure matrix. The use of

transformed community data reduces the influence of bias caused by sampling on distance-decay relationships, such as sampling the most abundant species by chance (Morlon *et al.* 2008). Several extensions of the basic Mantel randomisation test are sensitive to spatial autocorrelation, and permutation methods perform the most reliably in these situations (Poulin 2003; Green *et al.* 2004). As pairwise similarity values and distances were not truly independent in a statistical sense, partial Mantel statistics were estimated using the 'matrix permutation' method with 9999 permutations. Dissimilarity values were then regressed against geographical distance.

2.3. Results

2.3.1 Biomass characterisation

Biomass quantification revealed that, when a 0.4 m section of long-line was classified as having low levels of biofouling biomass, this equated to 57.8 ± 14.5 g (biofouling wet weight). For medium and high categories, biofouling biomass was on average 83.8 ± 15.5 g and 121.2 ± 20.3 g, respectively. In New Zealand, a typical three hectare mussel farm has nine backbone ropes, measuring 110 m each, and each rope supports a crop long-line of 3,750 metres (Marine Farming Association Inc., pers. comm.). Thus, if biofouling was categorised as low across the entire farm, this would extrapolate to approximately 4877 ± 1223 kg of biofouling biomass along crop long-lines. Similarly, biofouling biomass would be expected to reach 7071 ± 1307 kg and 10226 ± 1713 kg for farms categorised as having medium and high biomass, respectively.

2.3.2 Photoquadrat data verification

Richness estimates showed that photoquadrat collections in this study provided a good overall representation of the 'true' diversity of biofouling organisms associated with mussel long-lines in Pelorus Sound. On average, even at the widest point between photoquadrat sampling efforts and estimations by the Chao2 index (~400 samples), photoquadrat sampling in this study was within 80% of the 'true' number of species observed (Figure 2.4a, b).

2.3.3 Comparing photoquadrat sampling to visual assessments

When comparing species cover attained from photoquadrats with cover from visual estimates in R v.3.0.2 (R Core Team 2013), it was evident that visual estimation alone was not a reliable method for determining biofouling species on mussel long-line droppers. 50% of the records attained from photoquadrats detected taxa as being present when visual estimates recorded them as absent, while only 2% of the records gained by visual estimates recorded taxa as present when photoquadrats did not. Photoquadrats therefore provided a more reliable representation of biofouling cover on long-lines compared to visual estimates.

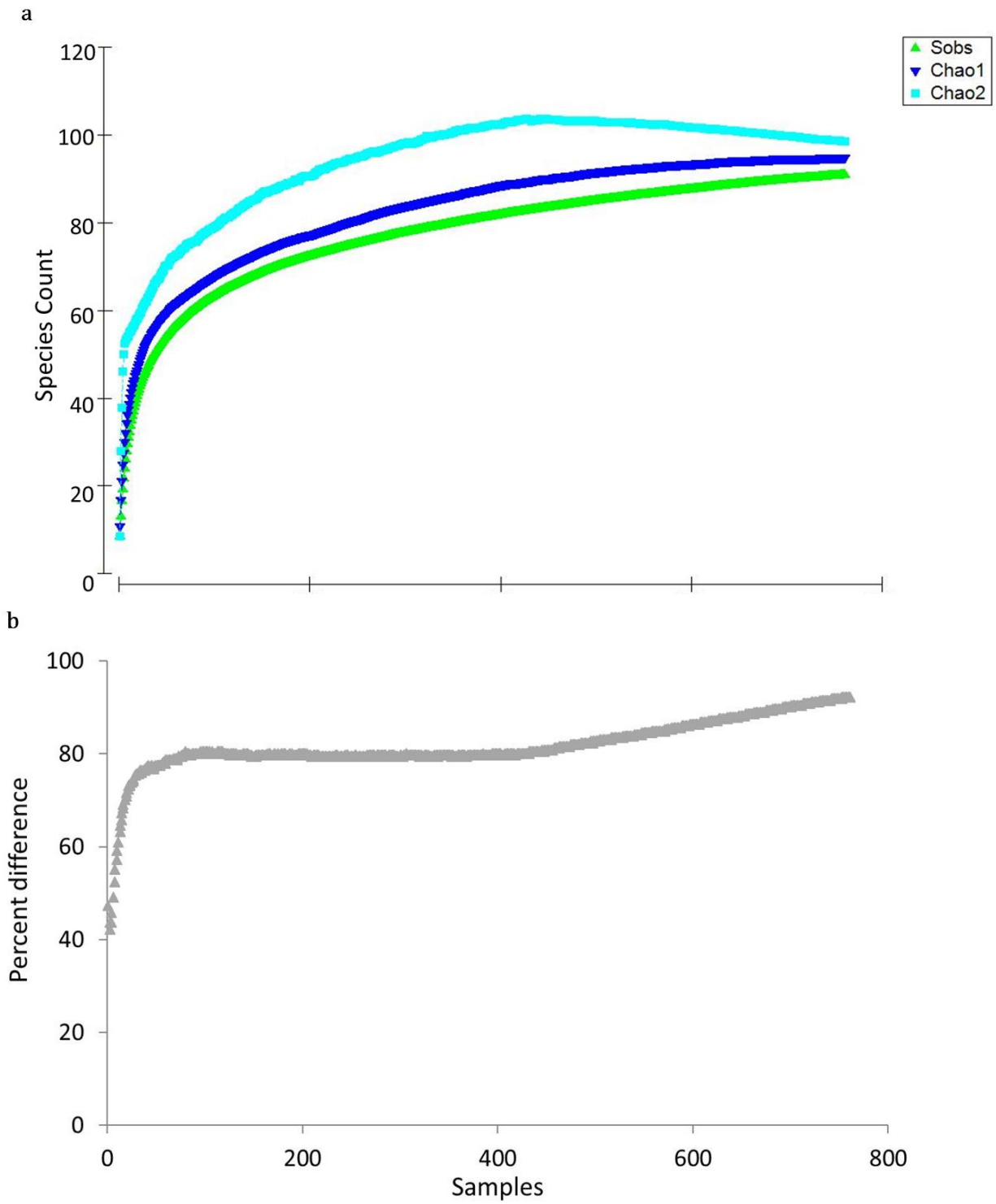

Figure 2.4. (a) Species accumulation curves demonstrating the increasing number of different species observed from photoquadrat collections, as samples were pooled (Sobs), compared to the predicted 'true' number of species that would be observed as the number of samples tended to infinity, represented by the Chao1 and Chao2 indices. (b) Percent difference between Sobs and the predicted 'true' total number of species that would be observed as the number of samples tended to infinity (Chao2 index).

2.3.4 Description of biofouling assemblages

In total, 81 biofouling taxa were identified occupying vacant space on crop long-lines, backbone ropes and culture buoys, and occurring epibiotically on mussel shells and other biofouling organisms (Table 2.1 and Figures 2.5a, b). Species assemblages comprised both sessile taxa (e.g., algae, sponges, hydroids, anemones, bivalves, bryozoans and ascidians) and mobile organisms such as amphipods, isopods, crabs, starfish and fish.

Across depths, biofouling cover on sampled mussel long-lines was dominated by red filamentous seaweed and *Undaria pinnatifida* (macroalgae), *Mytilus galloprovincialis* (bivalves), an unidentified hydroid, hereafter referred to as hydroid species-1, bryozoans and ascidians (Figure 2.6a). Taxa that represented a relatively minor proportion of the overall biofouling cover included sponges, anemones and mobile taxa, such as amphipods, isopods, sea cucumbers and crabs (Figure 2.6a). Biofouling cover was significantly greater near the surface of mussel long-lines (df=1, t=5.75, P<0.01), and this was dominated by macroalgae, hydroids and bivalves (Figure 2.6b).

The spatial distribution of some problematic biofouling species (i.e., those with previously documented impacts to aquaculture in New Zealand and overseas) varied across Pelorus Sound. There was a trend for high cover of hydroid species *Amphisbetia bispinosa,* and macroalgae including, *Cladophora* sp., *U. pinnatifida* and *Colpomenia* sp. near the entrance of Pelorus Sound, within the surface 3 m of sampled long-lines (Figure 2.7). There was also a trend for high cover of the calcareous tubeworm *Pomatoceros* sp. near the entrance of Pelorus Sound, but within the bottom of mussel long-lines. The cover of the blue mussel *M. galloprovincialis* tended to be highest in the middle of Pelorus Sound at the surface of mussel long-lines, and the colonial and solitary ascidians *Didemnum vexillum* and *Ciona intestinalis* (respectively) had a tendency for high cover near the head of Pelorus Sound, within the surface 3 m of sampled long-lines (Figure 2.7).

Table 2.1. List of taxa and species occurring in biofouling communities on green-lipped mussel (*Perna canaliculus*) long-lines across Pelorus Sound, New Zealand. Known problematic biofouling organisms for aquaculture industries in New Zealand and overseas are indicated with an asterix (*);(Page *et al.* 2007; Van de Ven 2007; Turcotte & Sainte-Marie 2009; Fitridge *et al.* 2012).

Taxon	Group	Genus and species
Macroalgae	Cladophora	*Cladophora* sp.*
		Ulva sp.
		Codium fragile *
		Unidentified green filamentous algae
	Phaeophyta	*Colpomenia* sp.*
		Undaria pinnatifida *
		Macrocystis pyrifera
		Spatoglossum sp.
	Rhodophyta	*Porphyra* sp.
		Ceramium sp.
		Asparagopsis sp.
		Unidentified red filamentous algae 1
		Unidentified red filamentous algae 2
		Unidentified red filamentous algae 3
		Unidentified red filamentous algae 4
Porifera		*Halichondria* sp.
		Sycon sp.
		Haliclona sp.
		Unidentified sponge
Cnidaria	Hydrozoa	*Amphisbetia bispinosa* *
		Sertularella sp.
		Unidentified hydroid sp. 1
		Unidentified hydroid sp. 3
	Anthozoa	*Cilicia rubeola* *
		Actinothoe albocincta
		Diadumenidae sp.
		Bunodeopsis sp.
Annelida	Sabellida	*Galeolaria hystrix* *
		Pomatoceros sp. *
		Spirorbidae sp. *
		Serpulidae sp. *
Crustacea	Amphipoda	*Caprella* sp. *
	Isopoda	*Paridotea ungulata*
	Brachyura	*Halicarcinus* sp.
		Hemigrapsus sp.
		Notomithrax sp. *
		Unidentified crab
Arthropoda	Sessilia	*Elminius modestus*
		Balanus trigonus
Mollusa	Bivalvia	*Mytilus galloprovincialis*

Table 2.1. continued.

Taxon	Group	Genus and species
Bryozoa	Cheilostomata	
	Erect	*Bugula* sp.
		Bugula stolonifera
		Bugula flabellata
		Bugula neritina
		Unidentified erect Bryozoa
	Encrusting	*Watersipora* sp.
		Unidentified encrusting Bryozoa
Echinodermata	Aspidochirotida	*Stichopus mollis*
	Ophiuroidea	*Ophionereis fasciata*
	Asteroidea	*Coscinasterias calamaria*
		Patiriella sp.
Teleostei	Tripterygiidae	*Fosterygion varium*
Ascidiacea	*Colonial*	*Didemnum* sp. *
		Didemnum incanum
		Didemnum vexillum *
		Diplosoma listerianum
		Diplosoma sp. *
		Leptoclinides sp.
		Lissoclinum notti
		Aplidium sp.
		Botrylloides leachi
		Botryllus schlosseri *
		Unidentified colonial ascidian sp. 1
		Unidentified colonial ascidian sp. 2
		Unidentified colonial ascidian sp. 3
		Unidentified colonial ascidian sp. 4
		Unidentified colonial ascidians
		Ascidiella aspersa *
	Solitary	*Asterocarpa humilis*
		Ciona intestinalis *
		Corella eumyota
		Pyura pachydermatina
		Cnemidocarpa sp.
		Unidentified solitary ascidian sp. 1
		Unidentified solitary ascidian sp. 2
		Unidentified solitary ascidian sp. 3
		Unidentified solitary ascidian sp. 4
		Unidentified solitary ascidian sp. 5
		Unidentified solitary ascidian sp. 6
		Unidentified solitary ascidian sp. 7
		Unidentified solitary ascidians

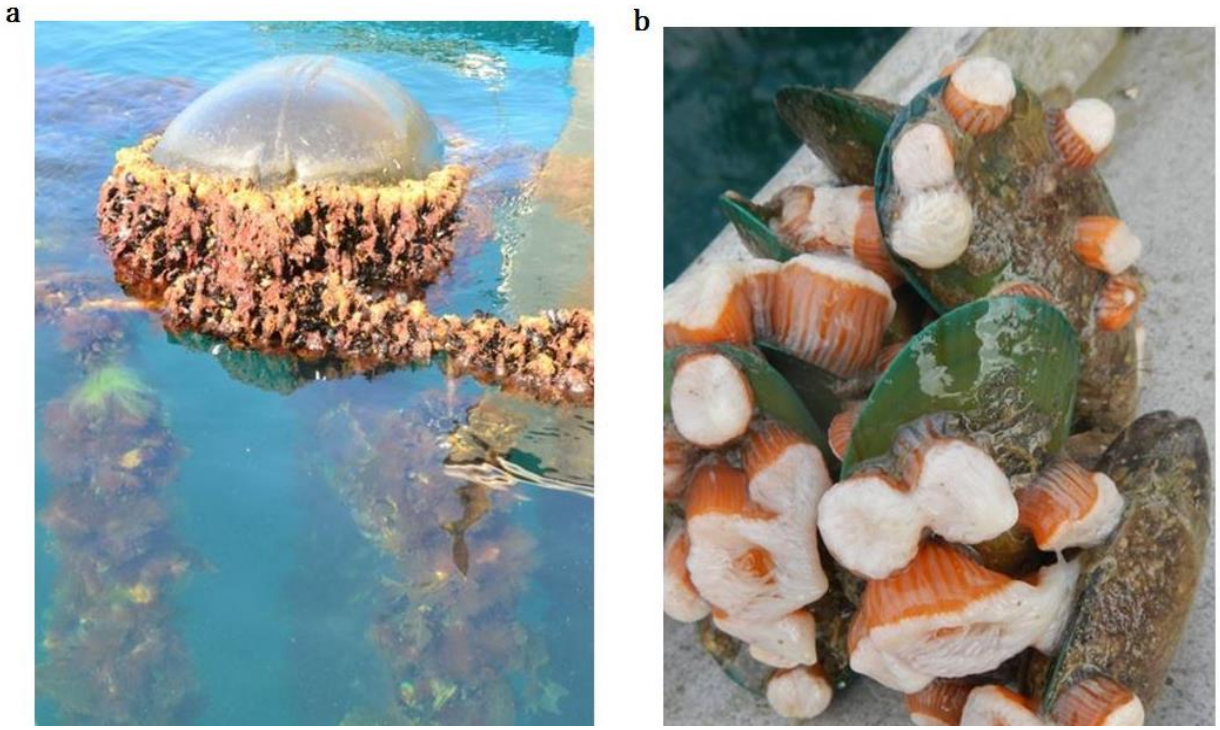

Figure 2.5. Images displaying variable cover of biofouling organisms on green-lipped mussel ropes and shells; (a) a range of biofouling species smothering floating buoys of a mussel farm; (b) anemones growing on green-lipped mussel shells.

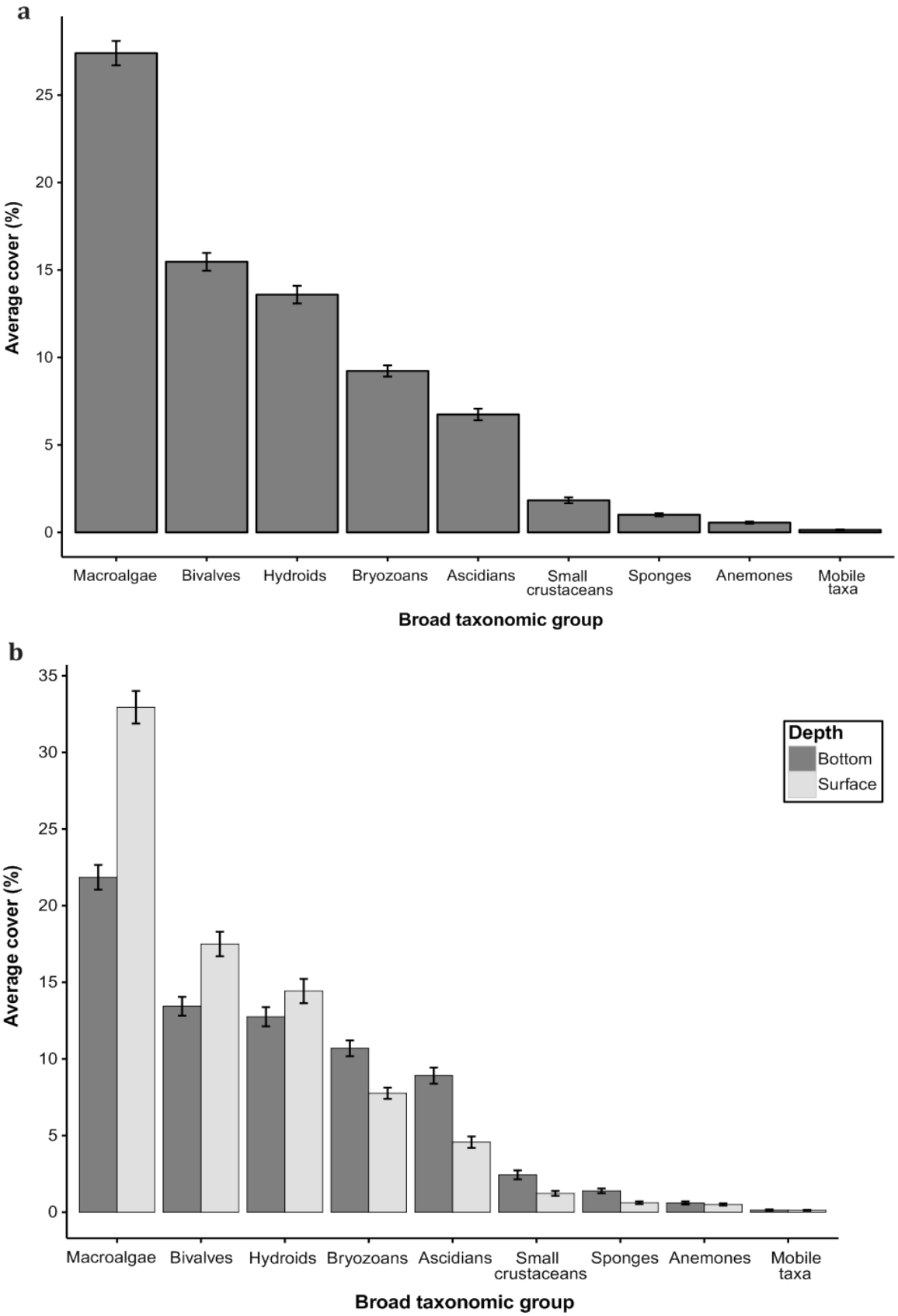

Figure 2.6. Average percent cover (±SE) of broad taxonomic groups of biofouling organisms to total biofouling cover on green-lipped mussel (*Perna canaliculus*) long-lines. Results are shown across**:** (a) all areas and depths sampled in Pelorus Sound, and (b) across all areas, in accordance with depth.

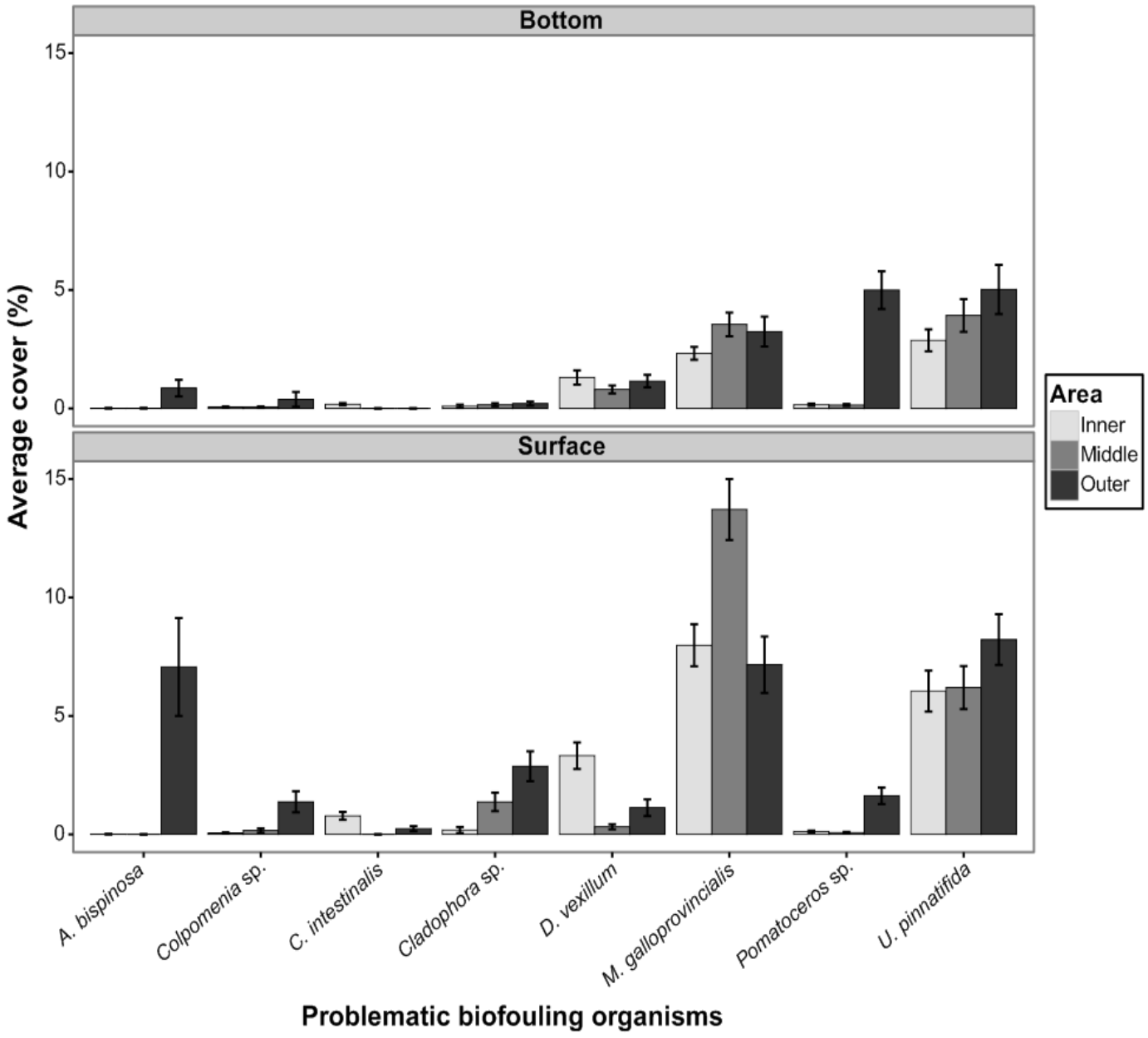

Figure 2.7. Average percent cover (± SE) of eight biofouling organisms known to be, or that have been, problematic for shellfish aquaculture in Pelorus Sound. The cover of *Amphisbetia bispinosa, Colpomenia* sp., *Ciona intestinalis, Cladophora* sp., *Didemnum vexillum, Mytilus galloprovincialis, Pomatoceros* sp.**,** and *Undaria pinnatifida* across areas in Pelorus Sound, within different depths are shown.

2.3.5 Univariate analyses

There was no significant difference in taxonomic richness across the fixed factors, areas and depth, nor was there a significant interaction between these factors (Appendix III, Table A3.1). However, there was a trend for lower average richness in the middle area across both depths (Figure 2.8a). A significant difference in richness was detected across farms nested within areas (Appendix III, Table A3.1: Farm (Area) effect). Richness also significantly differed on long-lines across farms, in different areas (Appendix III, Table A3.1: Long-line (Farm(Area)effect)), and this varied with depth (Appendix III, Table A3.1: Depth x Long-line(Farm(Area)effect)).

Evenness in biofouling communities significantly differed across areas with depth (Appendix III, Table A3.2: Depth x Area interaction effect), which pairwise comparisons revealed was due to significantly lower evenness within the surface 3 m of mussel long-lines in middle and outer areas (Appendix III, Table A3.2 and Figure 2.8b). The *k*-dominance species abundance curves revealed high biological diversity (indicated by the lines increasing reach along the x-axis) and greater evenness (indicated by their initial placement lower down the y-axis) for all areas and depths (Figure 2.9). However, there was a slight trend for higher taxa dominance in the middle area, at the surface, which is in accordance with the result from the evenness analyses (Figure 2.9).

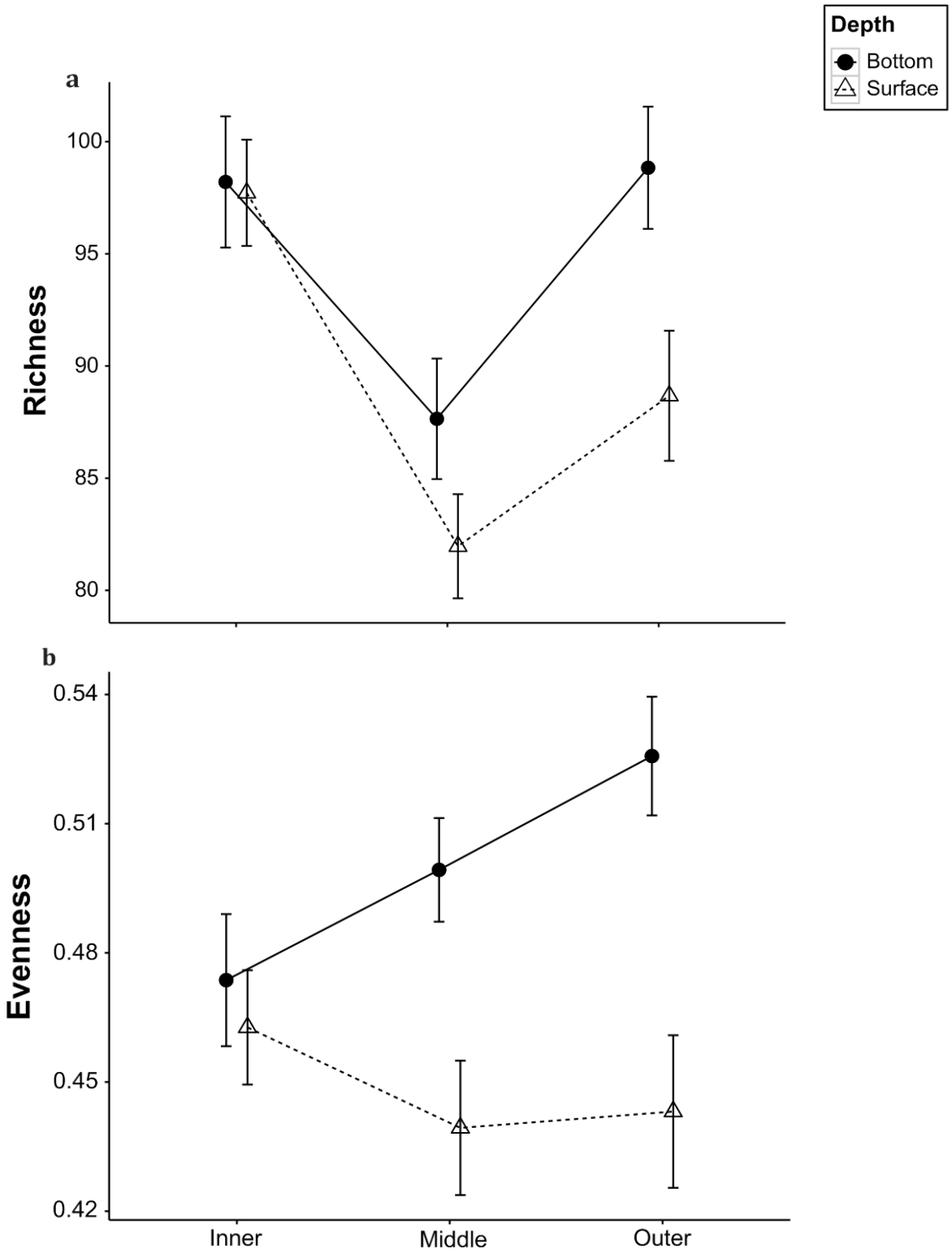

Figure 2.8. The average difference (±SE) in (a) taxonomic richness, based upon rarefaction (*N*=6), and (b) taxonomic evenness, based on a power transformation, across areas in Pelorus Sound, in accordance with depth.

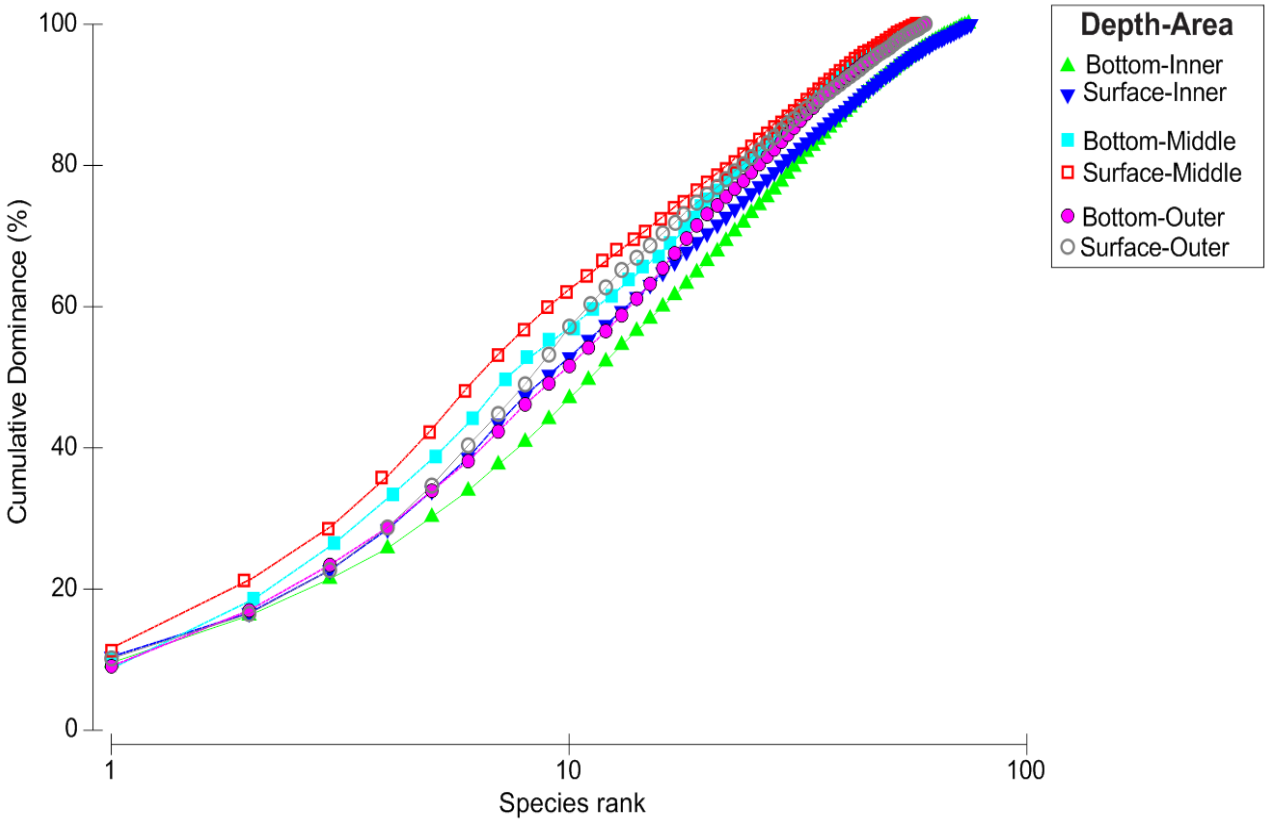

Figure 2.9. *k*-dominance species abundance curves for the Bray-Curtis transformed data of the average within-area/across-depth cover of biofouling taxa. The y-axis was subjected to a modified logistic transformation.

2.3.6 Multivariate analyses

Multivariate patterns of spatial variation in community structure were clearly depicted in the PCO plot, with clustering and separation of the inner area from middle and outer areas, which were partially overlapped (Figure 2.10). Patterns of spatial variation were confirmed by PERMANOVA analyses, which showed that depth had a significant effect on community structure, which was area dependent (Appendix III, Table A3.3: Depth x Area effect). Furthermore, community structure significantly differed with depth between long-lines nested in farms in different areas (Appendix III, Table A3.3: Depth x Long-line (Farm(Area)effect)). There was also a strong significant relationship between crop age and community structure (Appendix III, Table A3.3: Age effect).

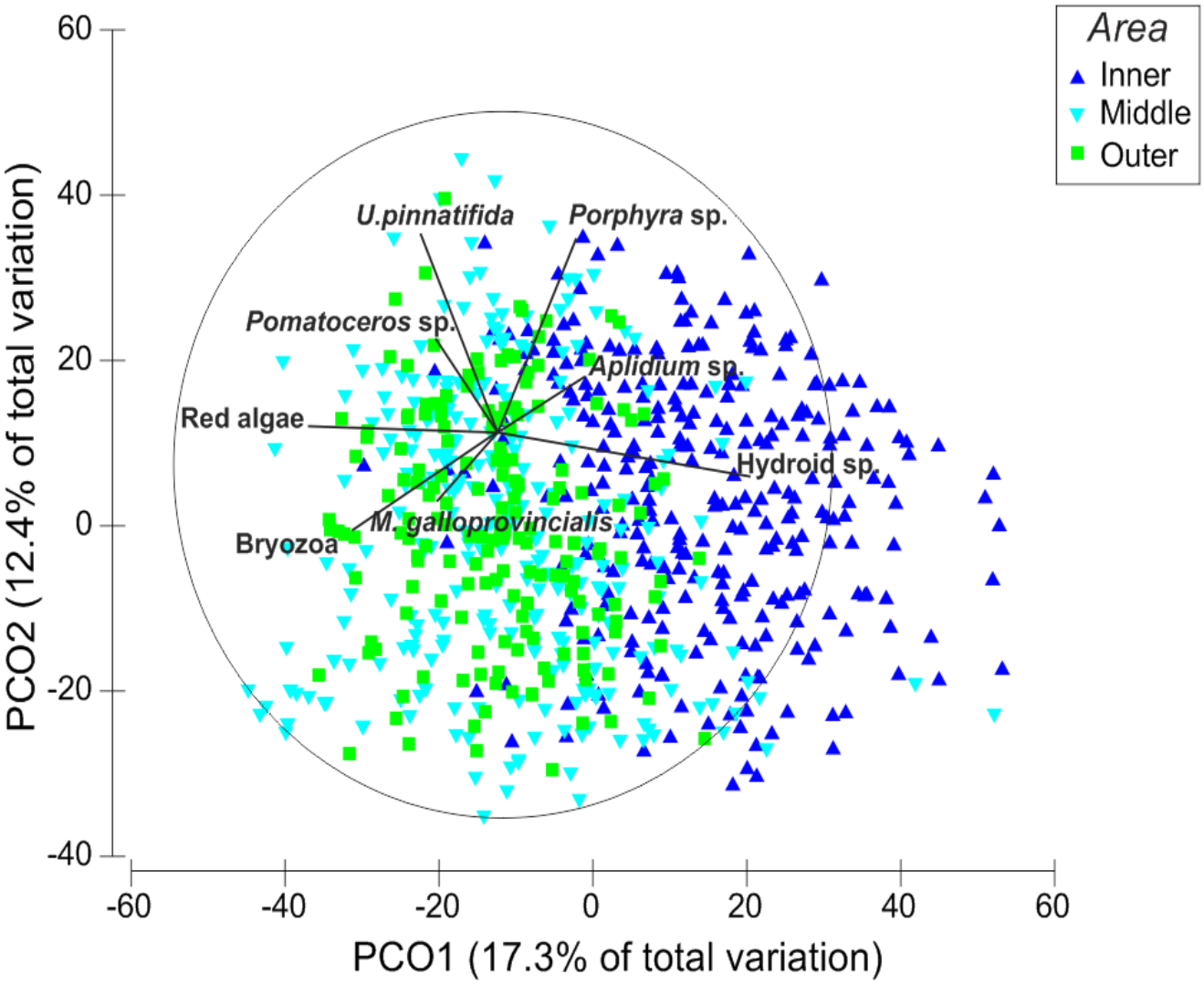

Figure 2.10. PCO plot on the basis of Bray-Curtis dissimilarities of the square root transformed abundance data of biofouling assemblages across the inner, middle and outer areas. All photoquadrat results across the three areas in Pelorus Sound are presented, along with the species contributing to differences in biofouling assemblages (correlation >0.3).

The greatest component of variation for biofouling community structure occurred at the lowest spatial scale (the residual, 28%, Appendix III, Table A3.3.), followed by between farm variation within different areas (i.e., the Farm (Area) effect, 21%, Appendix III, Table A3.3.). Moderate variability was observed among areas (16%, Appendix III, Table A3.3.), and the lowest variance component was estimated among crop age (5%, Appendix III, Table A3.3.). Pairwise comparisons revealed that community structure significantly differed across all areas, with depth (Appendix III, Table A3.3.). Although average percent similarity (AS) in community structure was lowest between inner and outer areas, across both depths (Appendix III, Table A3.3.), indicating that areas in closer proximity were more similar in community structure. Switching the order of the factors had no effect on result interpretations.

PERMDISP analyses showed significant differences in multivariate dispersion between areas, which varied with depth for community structure (Appendix III, Table A3.4: Area x Depth effects). Pairwise comparisons revealed that this was due to significant dispersion within the inner area, with greater dispersion at lower depths (Appendix III, Table A3.4 and Figure 2.11.). There was also a trend of lower dispersion within the middle area, although this did not consistently result in significant differences (Appendix III, Table A3.4 and Figure 2.11.).

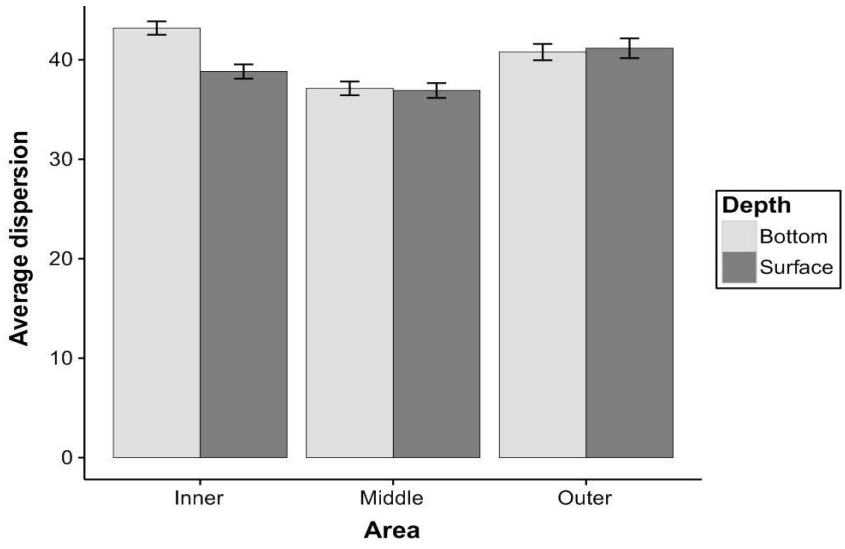

Figure 2.11. Average multivariate dispersion (±SE) from the PERMDISP pairwise comparisons for dispersion among and within areas, across different depths.

The SIMPER analysis revealed that variation in the cover of six taxa contributed consistently to the dissimilarities (>6%) in community structure between areas, and across depths (Figure 2.12). Taxa included red filamentous algae, hydroid species-1, encrusting bryozoans, *U. pinnatifida*, *Porphyra* sp. and *M. galloprovincialis*. However, the contribution made by these taxa was relatively low (a maximum of 11%). The largest average dissimilarity in community structure was between the inner area at the bottom depth (IB) and outer area at the surface depth (OS) (70%, Figure 2.12).

Patterns revealed by SIMPER analyses were confirmed by univariate permutational ANOVAs done on the cover of the most prominent taxa contributing to dissimilarities, and on taxa with a high correlation (>0.3) with the PERMANOVA PCO axis (Appendix III, Tables A3.5. and A3.6.). Depth had a significant effect on the cover of encrusting bryozoans, hydroid species-1 and *M. galloprovincialis*, which was area dependent (Appendix III, Table A3.5: Area x Depth effect). Encrusting bryozoa spp. tended to have high cover within the bottom 3 m of mussel long-lines, in the middle and outer areas (Figure 2.13), whereas hydroid species-1 and *M. galloprovincialis* displayed a trend of high cover at the surface of mussel long-lines in the inner and middle areas, respectively (Figure 2.13). Depth and area also had a significant effect on the cover of red filamentous algae and *U. pinnatifida*, but there was no interaction between these factors (Appendix III, Table A3.5: Area effect and Depth effect). Red filamentous algae and *U. pinnatifida* had a trend for high cover within the outer area (Figure 2.13). Area alone had a significant effect on the cover of *Porphyra* sp., with a tendency for high cover in the inner area (Appendix III, Table A3.5: Area effect). Univariate PERMANOVA tests also revealed that the cover of the two taxa, *Aplidium* sp. and *Pomatoceros* sp., which had a high correlation (>0.3) with the PERMANOVA PCO axis (Figure 2.10), significantly differed with depth and across areas (Appendix III, Table A3.6: Area x Depth effect). The cover of *Aplidium* sp. and *Pomatoceros* sp. tended to be highest at the bottom depth, but within the inner and outer areas, respectively (Figure 2.13).

	IB	IS	MS	MB	OS
OB	Average dissimilarity: 67.01 Red algae Hydroid Bryozoa Porphyra	Average dissimilarity: 62.63 Hydroid Bryozoa Red algae Porphyra	Average dissimilarity: 59.26 Mytilus Red algae Bryozoa Hydroid	Average dissimilarity : 57.47 Bryozoa Red algae Hydroid Porphyra	Average dissimilarity: 60.91 Red algae Bryozoa Hydroid
OS	Average dissimilarity: 70.17 Red algae Hydroid Porphyra Bryozoa Undaria	Average dissimilarity: 63.63 Hydroid Red algae Porphyra Bryozoa Undaria	Average dissimilarity: 56.98 Mytilus Red algae Hydroid Bryozoa	Average dissimilarity: 60.02 Red algae Hydroid Bryozoa	
MB	Average dissimilarity: 63.48 Hydroid Bryozoa Red algae Porphyra	Average dissimilarity: 59.21 Hydroid Bryozoa Porphyra	Average dissimilarity: 55.57 Mytilus Porphyra Hydroid Bryozoa		
MS	Average dissimilarity: 66.59 Red algae Hydroid Mytilus Porphyra Bryozoa	Average dissimilarity: 59.10 Hydroid Mytilus Porphyra Bryozoa			
IS	Average dissimilarity : 60.14 Porphyra Bryozoa Red algae				

Note: All taxa listed consistently contributed (%) to group dissimilarity

Figure 2.12. Summary of similarity percentage analysis (SIMPER) showing taxa with the highest percent contribution (reflected in the taxa order) towards the dissimilarity between areas, within different depths across Pelorus Sound (IB=Inner Bottom, IS=Inner Surface, MB=Middle Bottom, MS=Middle Surface, OB=Outer Bottom, OS=Outer Surface). Contributing taxa were; Red algae=red filamentous algae, Hydroid=hydroid species-1, Bryozoa=encrusting bryozoans, Porphyra=*Porphyra* sp., Mytilus=*Mytilus galloprovincialis*, Undaria=*Undaria pinnatifida*. Taxa % contribution was ≥6%.

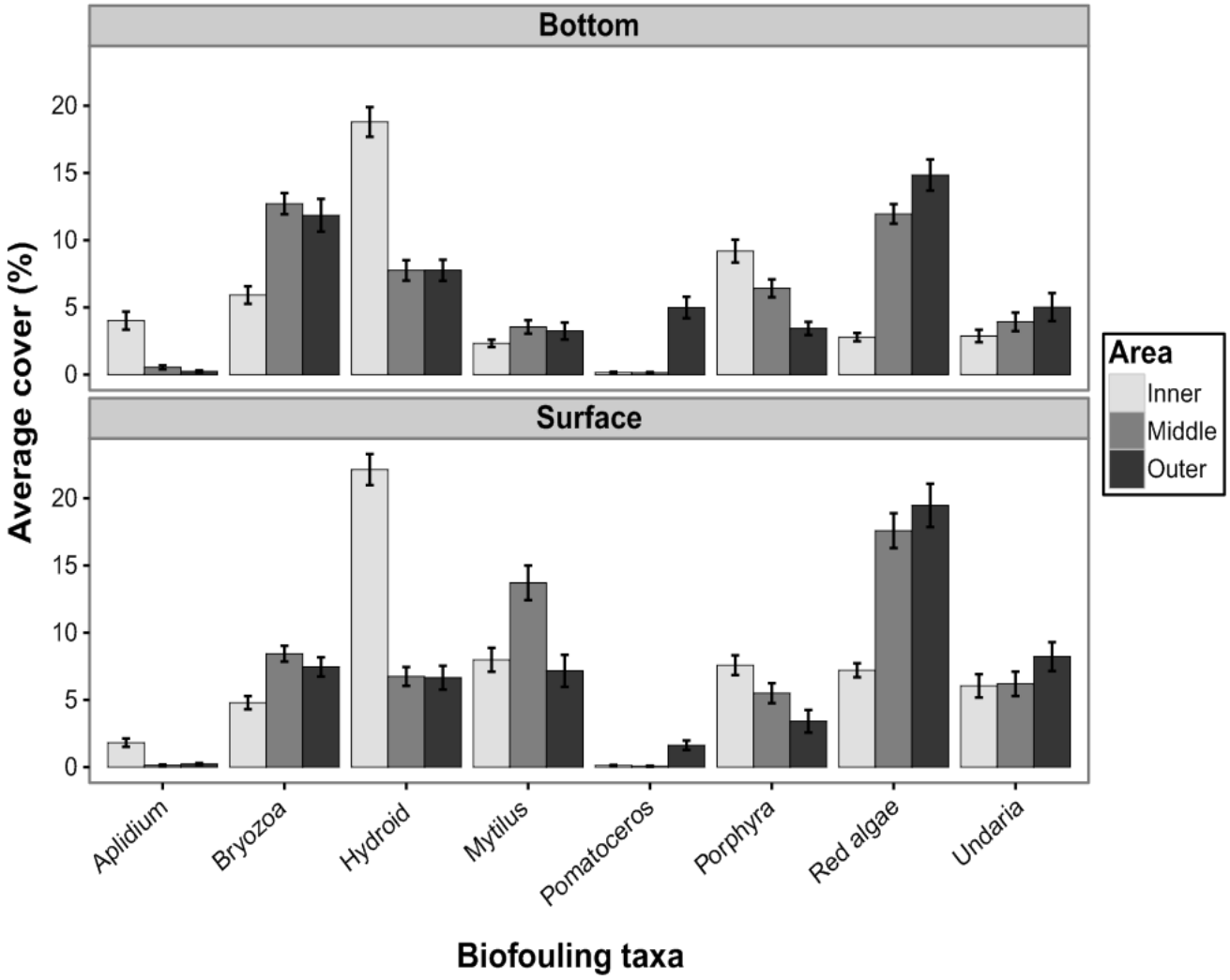

Figure 2.13. SIMPER analysis results for the average abundance of taxa contributing to dissimilarity between areas, within different depths across Pelorus Sound. The highest contributing taxa were; Red algae=red filamentous algae, Hydroid=hydroid species-1, Bryozoa=encrusting bryozoans, Porphyra=*Porphyra* sp., Mytilus=*Mytilus galloprovincialis*, Undaria=*Undaria pinnatifida*. The average abundance of taxa with a correlation >0.3 included Aplidium=*Aplidium* sp., and Pomatoceros=*Pomatoceros* sp.

2.3.7 Distance-decay assessment

There was no significant relationship between the geographical distance of sampled mussel farms across Pelorus Sound and the dissimilarity in taxonomic richness (rarefied, *N*=6) of biofouling communities at these sites (Appendix III, Table A3.7 and Figure 2.14a). However, a relatively weak, but highly significant positive relationship was detected between dissimilarity in the structure of biofouling assemblages and the geographical distance of study sites (Appendix III, Table A3.7and Figure 2.14b). Specifically, dissimilarity in the structure of biofouling assemblages increased, with a steep slope, with geographical distance (Figure 2.14b).

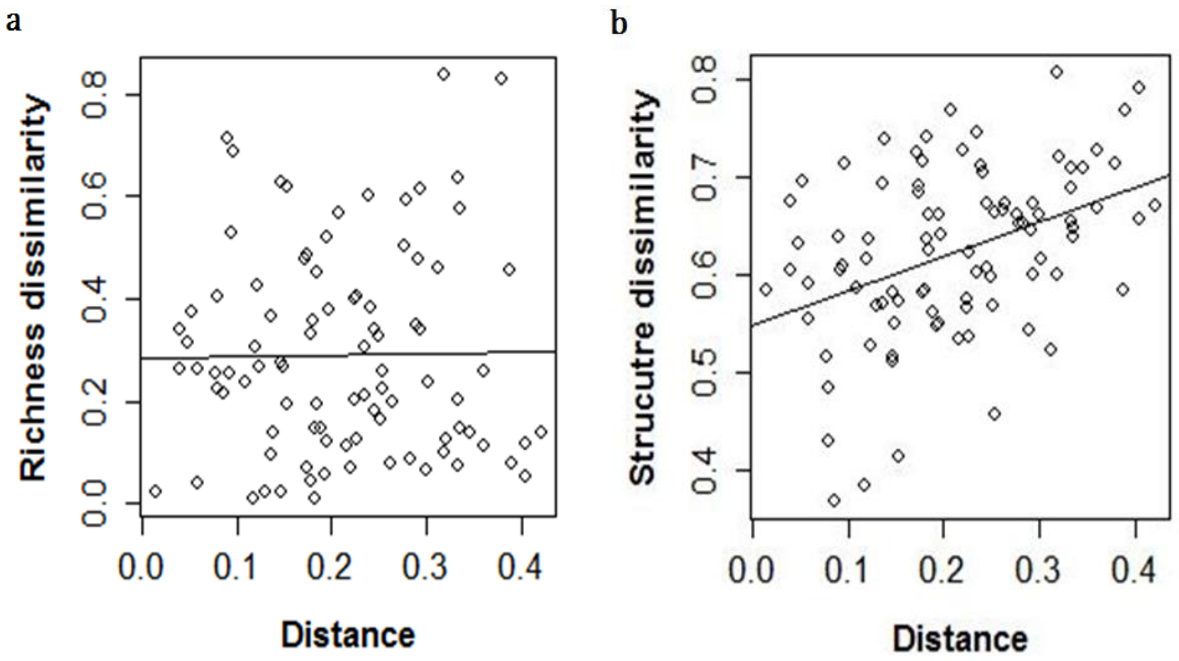

Figure 2.14. Distance-decay relationships for (a) the matrix of rarefied biofouling richness and (b) the Bray-Curtis matrix of biofouling dissimilarity against the Euclidean matrix of the geographic distances between sampled marine farms.

2.4. Discussion

2.4.1 Description of biofouling assemblages

The shell of green-lipped mussels provides an ideal substratum for colonisation by various biofouling organisms (McKindsey *et al.* 2007; Dürr & Watson 2010; Adams *et al.* 2011; Fitridge *et al.* 2012; Antoniadou *et al.* 2013; Sievers *et al.* 2013). This study has demonstrated that mussel farm long-lines in Pelorus Sound support diverse biofouling communities, which substantially contribute to excess long-line wet weight. A total of 81 distinct taxa were identified on mussel long-lines, with biofouling cover dominated by photosynthetic macroalgae and suspension-feeders (>60% of the total cover) such as other bivalves (specifically blue mussels), hydroids, bryozoans, and ascidians**.** Epibenthic communities are generally composed of suspension-feeders and macroalgae, although this is dependent upon water depth and location (Cook *et al.* 2006). Suspension-feeders are recognised for substantially contributing to the overall biomass of biofouling communities on artificial structures (Lesser *et al.* 1992; Cronin *et al.* 1999; Howes *et al.* 2007; Fitridge *et al.* 2012). Therefore, these findings are not unexpected, and a dominance of macroalgae and suspension-feeders within biofouling communities have been found on other marine farms, including oyster cultures and tuna farms (Cronin *et al.* 1999; Mazouni *et al.* 2001).

A number of important problematic pests were identified on mussel long-lines including the solitary and colonial ascidians, *Ciona intestinalis* and *Didemnum vexillum*, macroalgae, *Undaria pinnatifida*, *Cladophora* sp. and *Colpomenia* sp., the tube-building polychaete *Pomatoceros* sp., the blue mussel *Mytilus galloprovincialis*, and hydroid species *Amphisbetia bispinosa*. These species have had, or currently have, detrimental impacts on commercial industries in New Zealand. For example, *A. bispinosa* has been problematic for mussel cultures in the south Hauraki Gulf, adding weight which can enhance crop loss, and damaging shells, reducing their aesthetic value (Heasman & de Zwart 2004). In the Marlborough Sounds a conspicuous green filamentous alga, identified by molecular analyses as *Cladophora ruchingeri* (Xavier Pochon,

Cawthron Institute, pers. comm.), has recently increased in abundance, becoming problematic for mussel long-line processing operations.

2.4.2 Patterns of spatial variation in biofouling communities

In agreement with the three study hypotheses, it has been demonstrated that: (1) spatial variation exists in the structure of biofouling communities, and the relative abundance of pest species on mussel long-lines in Pelorus Sound, (2) biofouling cover is greater within the surface 3 m of mussel long-lines, and (3) there is a decrease in the similarity of community structure with increasing geographical distance between sampled mussel farms.

Findings from this study are consistent with recent research conducted by Woods *et al.* (2012), who detected spatial variability in biofouling accumulation on two mussel farms in Pelorus Sound, and observed greater levels of biofouling biomass associated with older crop, and less biofouling with increasing water depth. Similarly, PVC fouling plates suspended from mussel backbone lines in Australia (Sievers *et al.* 2014), and offshore oil platforms in Western Asia (Stachowitsch *et al.* 2002) displayed spatial variation in biofouling communities, with a dominance of suspension-feeding organisms. Evidence of reduced biofouling cover with increasing depth in this study is also in agreement with reports that biofouling communities generally decrease in biomass and become less diverse with increased water depth (Cronin *et al.* 1999; Braithwaite *et al.* 2007; Guenther *et al.* 2010; Fitridge *et al.* 2012).

This study is the first account of distance-decay in biofouling communities associated with artificial structures and supports the paradigm of distance-decay, which was first underlined by geographers (Tobler 1970), and is inherent in the pioneering ecological research (Whittaker 1960; Preston 1962). Distance-decay has also been reported for a variety of microorganisms and macroorganisms (Brouat & Duplantier 2007; Soininen *et al.* 2007; Jobe 2008), including marine fish parasite communities (Oliva & Teresa 2005; Poulin *et al.* 2011), demersal fish assemblages (Anderson *et al.* 2013), plant communities (Nekola & White 1999), communities of ectomycorrhizal fungi (Bahram *et al.* 2013) and macroinvertebrate communities (Thompson &

Townsend 2006). The slope of the relationship between distance and community similarity in this study was greater than those recorded for marine fish communities (Oliva & Teresa 2005). This contrast makes sense given that the rate of decline of similarity would be greater for organisms with a lower dispersal rate (e.g., ascidian species with a short-lived larval stage) in open and continuous marine systems, compared to mobile organisms (Soininen *et al.* 2007). Furthermore, smaller organisms, which respond more intensively to fine scale environmental variation due to their shorter generation times, may have lower similarity at small distances (Gillooly *et al.* 2002).

2.4.3 Potential explanations: niche versus neutral

Patterns of distance-decay and community structure can have many explanations, but often are attributed to either: (1) niche-related processes, such as environmental filtering and biotic interactions (Chesson & Case 1986; Chesson 2000; Condit *et al.* 2002), (2) neutral processes including chance colonisation, dispersal limitations, random extinction and ecological drift (Bell 2001; Hubbell 2001; Chave 2004; Leibold & McPeek 2006), or a combination of both (Thompson & Townsend 2006; Caruso *et al.* 2011; Chase & Myers 2011; Stegen *et al.* 2012; Kitching *et al.* 2013).

Niche-related processes may govern detected variability in the structure of biofouling communities growing on mussel long-lines in Pelorus Sound, with differential environmental stress selecting for the growth of site-specific species. For example, mussel farms near the entrance of Pelorus Sound are located within deep, exposed areas, with strong winds from the north and north-west generating high energy storm waves and promoting extensive coastal erosion (Mcintosh 1958). In contrast, mussel farms near the head of the Pelorus Sound, are in shallower waters and sheltered areas, experiencing low wave action and periods of reduced or stagnant water flow, which may induce warmer summer temperatures (Gibbs *et al.* 1991). Furthermore, episodic storm events can generate high freshwater inputs from the Pelorus and Kaituna rivers into areas near the head of Pelorus Sound (Gibbs *et al.* 1991).

Differential cover of certain biofouling taxa in this study could therefore be a reflection of tolerance to these local environmental conditions. For example, *U. pinnatifida* is well adapted for life on exposed coastlines, maintaining firm attachment from its holdfast (Curiel *et al.* 1998; Russell *et al.* 2008; Nelson 2013). Nanba *et al.* (2011) found that *U. pinnatifida* sporophytes grown in exposed sites attained larger sizes and had faster growth rates compared to those grown in sheltered sites in Japan. Similarly, the firm attachment of calcareous tubeworms to hard substrate by cementational adhesion makes them well adapted for high wave energy environments (Moate 1985; Callow & Callow 2002; Bromley & Heinberg 2006). In contrast, some ascidians, such as *C. intestinalis*, are more commonly found fouling sheltered habitats, as hydrodynamic processes in exposed areas preclude successful larval settlement (Howes *et al.* 2007). Furthermore, while it has been documented that ascidians perform best at salinities above 25 PSU and are rarely tolerant of brackish conditions (Lambert 2005), some may be well adapted to fluctuations in ion concentrations. For example, in laboratory experiments *D. vexillum* showed higher growth rates and survival under low salinities, ranging from 10-20 PSU (Gröner *et al.* 2011). However, a higher cover of *D. vexillum* near the head of Pelorus Sounds was unexpected in this study, given that *D. vexillum* is more abundant in deep-water open coast sites overseas, which was attributed to growth limitations associated with warmer water temperatures during the summer in protected, shallow-water embayments (Osman & Whitlatch 2007).

Biotic interactions, including competition and displacement, have important consequences for species distribution. For example, *U. pinnatifida* out-competes native species on artificial structures in Torquay Marina (Devon, UK), reducing the distribution of two kelp species, *Laminaria digitata* and *Laminaria saccharina*, and smothering the solitary ascidian *Styela clava* (Farrell & Fletcher 2006). Similarly, competitive interactions with rapidly growing kelps, including *L. saccharina*, caused displacement in the red alga *Iridaea cordata* to areas below its normal distribution in the Pacific Northwest waters (Hruby 1976). Competition between some species, such as *U. pinnatifida* and *D. vexillum*, could contribute to their opposing patterns of

cover across areas in Pelorus Sound. However, biotic interactions can also promote coexistence or enable the establishment of new species. Large colonies of the solitary ascidian *Pyura praeputialis* harbour over 116 species of macroinvertebrates and algae in Northern Chile and 55% of these are exclusively found within the ascidian matrices (Castilla *et al.* 2004). Enhanced habitat complexity generated by biofouling species, such as solitary ascidians or hydroids, may therefore contribute to the distribution of biofouling species in Pelorus Sound as well.

Light intensity and associated primary production appear to be major factors in the spatial distribution of biofouling organisms in this study, with photosynthetic macroalgae and sessile filter-feeders dominating biofouling communities within the surface 3 m of the long-line droppers, where light, nutrients and oxygen are not a limiting resource. Similarly, Cronin *et al.* (1999) found that macroalgae dominated fouling communities at shallow depths on tuna cage nets. Most problematic biofouling species in Pelorus Sound also displayed greater cover within shallow water depths, with the exception of the suspension-feeding calcareous tubeworm *Pomatoceros* sp., a taxon which is known to inhabit a range of depths, including some of the deepest parts of the Ocean (Kupriyanova *et al.* 2011). For example, *Pomatoceros triqueter* has been reported in depths of over two hundred metres (Moate 1985). However, on natural rocky substrates in some southern fiords in New Zealand, *Pomatoceros* species have been documented to occur in dense aggregations in depths of 15 m (Grange *et al.* 1981).

In accordance with the stochastic niche theory, periodic disturbance, and other processes allowing species to overcome resource-dependent recruitment limitation are expected to increase local species diversity and richness (Tilman 2004). Mussel production occurs over 12 to 24 month cycles from initial spat settlement to the harvesting of adult mussels (Woods *et al.* 2012). During intermediate and final seed crop stages, culture ropes are stripped and reseeded to reduce the density of the mussels for grow-out to market size and reduce mussel biofouling (Woods *et al.* 2012). This process can facilitate species coexistence by decreasing the abundance of competitively dominant species and creating niche opportunities for inferior competitors

(Huston 1979; Sousa 1979; Chesson & Huntly 1997). It is therefore likely to be an important component contributing to differences in biofouling communities and to the reduced levels of species dominance, and undetected differences in richness across sampled areas in Pelorus Sound. Consequently, community structure in this system may be more of a function of disturbance than a function of competition.

Neutral-based processes align with the pattern of distance-decay detected in this study, with similarity in the occurrence and abundance of biofouling communities being negatively correlated with the spatial arrangement of mussel farms (Bell 2001; Gilbert & Lechowicz 2004; Soininen *et al.* 2007; Diniz-Filho *et al.* 2012). As benthic invertebrates with long-lived planktonic larval phases are generally expected to disperse over greater distances than species with short-duration pelagic larval stages, differential dispersal capabilities may substantially contribute to evident patterns of distance-decay. For instance, the dispersal of ascidian larvae is typically limited to scales of only 10 to 100s of metres (Berrill; Ayre *et al.* 1997; Lambert 2002). In contrast, annual algae, such as *U. pinnatifida*, have the potential for both short and long-range dispersal via microscopic propagules and drifting reproductive thalli (Dayton 1973; Forrest *et al.* 2000; Valentine *et al.* 2007a; Schiel & Thompson 2012). Bivalves, on the other hand, develop in the plankton for weeks or months and thereby have the potential to be dispersed over large geographic ranges (Hilbish *et al.* 2002).

Artificial structures can restrict dispersal by modifying wave propagation and tidal currents, thereby affecting the recruitment of species in surrounding habitats (McNeill *et al.* 1992; Floerl & Inglis 2003; Madin *et al.* 2010). For example, Waite (1989) found that water movement through mussel farms is reduced to approximately 30% of the velocity of the water approaching it, with the remainder forced below and around the farm. Farms situated in areas with reduced water flow (e.g., areas within the Kenepuru Sound), may therefore have an enhanced potential for propagule retention, increasing the growth of specific biofoulers. For example, reduced water flow around and within marine finfish cages has been found to increase biofouling growth

of sessile organisms (Madin *et al.* 2010). Although water current velocity was not measured in this study, variability in flow regimes due to differences in the bottom hydrography at certain farm sites, and the specific influence of farm infrastructure, would potentially alter physical living conditions and influence the number of larvae that settle on substrate, resulting in variability in the distribution of biofouling communities (Leichter & Witman 1997).

2.4.4 Limitations in discerning explanations

Distinguishing the relative importance of niche and neutral-based processes from spatial configuration data alone is a difficult and potentially unattainable challenge. When using distance-decay relationships as an indicator of neutral processes, the distance between sites is often positively correlated with differences in local environmental factors and, therefore, ecological adaptations (Gilbert & Lechowicz 2004; Diniz-Filho *et al.* 2012). The limitations of this study, which include one-off sampling (restricted to the summer season) and the use of coarse-level environmental surveys, prevent confident differentiation between the relative importance of these processes. However, given the findings of this study it can be speculated that both niche and neutral processes contribute to spatial variability in biofouling structure, with site-specific variation in environmental filtering, biotic interactions, dispersal limitations and the influence of artificial structures on propagule retention across Pelorus Sound.

2.4.5 Concluding remarks

This study is the first attempt to quantify biofouling patterns on a regional scale in association with aquaculture infrastructure in one of New Zealand's most important growing regions. Results provided evidence of spatial variation in biofouling community structure, with an increasing dissimilarity between communities along the Pelorus Sound, and a prominence of problematic species within surface depths, near the entrance of Pelorus Sound.

Distributional patterns observed in this study could be used by aquaculture industries to improve marine farm management in the presence of biofouling. For example, mussel farm locations near the entrance of Pelorus Sound may be less suitable for spat holding. This is due to

the higher cover of several problematic species, such as *Colpomenia* sp. (bubble weed), which can extensively cover long-line droppers during warm-summer periods and prevent the successful settlement of young mussel spat, with an increased loss of spat due to their direct settlement onto *Colpomenia* sp. (SpatNZ pers. comm.) In addition, higher overall cover of biofouling organisms, such as *M. galloprovincialis* (blue mussels), within the upper region of long-line droppers may indicate the need to position mussel long-lines in deeper waters to avoid the over-settlement of problematic biofoulers. Knowing where groups of taxa (e.g., colonial and solitary ascidians or barnacles) succeed, from distribution and density patterns, could also be used to predict where incoming problematic taxa, such as ascidian species (e.g., *Styela clava*) or barnacle species (e.g., *Balanus trigonus*), may become predominantly problematic. These species have been documented to negatively influence aquaculture industries elsewhere. For example, the barnacle *B. trigonus* has recently emerged as a pest for cultured mussel farms in the southern Hauraki Gulf, causing problems with automatic mussel opening equipment, which relies on smooth shell surfaces (Jeffs & Stanley 2010).

Further work is required to optimise management strategies and predict the distribution of future problematic species: including: (1) seasonal, long-term data on biofouling patterns, with concurrent recordings of local environmental variables, (2) improved hydrodynamic models to enable predictions of farm connectivity and potential for marine pest spread, and (3) to make this information more accessible to marine farmers and assist with management, online databases could be constructed to share information attained from ongoing biofouling monitoring. This has previously been suggested by Sievers *et al.* (2014) and is already used in many shellfish aquaculture industries for the detection of harmful phytoplankton (Trainer *et al.* 2003).

Chapter III
Connectivity between Biofouling Populations on Artificial Substrata

3.1. Introduction

The invasion of high-profile pest species to regions worldwide has increased the need for understanding physical and biological qualities facilitating the establishment and secondary spread of non-indigenous species (NIS) (Carlton 1996; Floerl & Inglis 2005; Lockwood *et al.* 2005; Bock *et al.* 2011; Reinhardt *et al.* 2012). These qualities include propagule pressure, vector availability, dispersal potential, local biotic resistance and environmental conditions (Rilov & Crooks 2009). Following incursion, establishment success is dictated by post-border processes, including a species ability to survive, reproduce and spread (Forrest *et al.* 2009). Post-border spread is dependent upon natural dispersal, as well as anthropogenic transport mechanisms at local scales (Wasson *et al.* 2001; Branch & Nina Steffani 2004). Identifying the extent of introductory pathways, the role of vectors and the rate of post-border spread, are of importance to conservationists and managers, as they determine the control measures that can be employed (Shanks *et al.* 2003; Hampton *et al.* 2004; Forrest *et al.* 2009).

As many benthic invertebrates have sedentary adult life stages, dispersal of pelagic early life-stages is of critical importance (Cowen & Sponaugle 2009). Human activities and man-made structures provide an added dimension to dispersal and enhance opportunities for spread (Grosberg 1987). Artificial structures are recognised as critical vectors for secondary spread,

acting as novel corridors or 'stepping-stones' for NIS dispersal, and generating connectivity among isolated populations (Johannesson & Warmoes 1990; Airoldi *et al.* 2005; Bulleri & Airoldi 2005; Glasby *et al.* 2007; Vaselli *et al.* 2008; Fauvelot *et al.* 2009; Bulleri & Chapman 2010). Increased connectivity among populations on artificial structures may result in biogenic homogenization (Olden *et al.* 2004) and can enhance gene flow, reducing genetic diversity at a regional scale (Airoldi *et al.* 2005; Fauvelot *et al.* 2009; Fauvelot *et al.* 2012). Compared to natural substrates, populations associated with artificial structures have been shown to have reduced genetic diversity. For instance, the genetic diversity of populations of two native species, the tube-building keel worm *Pomatoceros triqueter* and the rayed Mediterranean limpet *Patella caerulea* were found to be lower on offshore gas platforms compared to nearby natural habitats in the Adriatic Sea (Fauvelot *et al.* 2009; Fauvelot *et al.* 2012). However, this pattern is not universal. There was no evidence of a difference in the genetic diversity of populations of the invasive solitary ascidian *Microcosmus squamiger* growing on artificial breakwaters compared to those within natural rocky outcrops in the Mediterranean Sea (Ordóñez *et al.* 2013), although low genetic diversity within these introduced populations may have made genetic differences harder to detect.

Among marine invertebrates, ascidians are well recognised as a biofouling nuisance, with a number of cosmopolitan NIS (Lambert 2002; Dijkstra *et al.* 2007; Lambert 2009; Locke & Carman 2009). Many non-indigenous ascidian species have been used to investigate pre-border processes with the aid of genetic tools, focused on the relatedness of established populations and on inferring the source population of NIS (de Barros *et al.* 2009; Zhan *et al.* 2010; Goldstien *et al.* 2011; Lejeusne *et al.* 2011; Rius Viladomiu *et al.* 2012; Stefaniak *et al.* 2012). However, fewer studies relating to post-border dispersal have been conducted (Goldstien *et al.* 2010; Pérez-Portela *et al.* 2012; Zhan *et al.* 2012).

D. vexillum is an aggressive colonial ascidian native to the northwest Pacific (Lambert 2009; Stefaniak *et al.* 2009; Stefaniak *et al.* 2012), with expanding populations in Europe (Minchin &

Sides 2006; Gittenberger 2007), New Zealand (Kott 2002), both coasts of North America (Bullard *et al.* 2007), and more recently, the Mediterranean (Tagliapietra *et al.* 2012). In New Zealand, since its initial discovery in Whangamata Harbour in 2001 (Coffey 2001), *D. vexillum* has established in Tauranga, Whangarei, Tarakohe, Wellington, the Marlborough Sounds, Nelson and Lyttelton Harbour (McDonald & Acosta 2012; Fletcher *et al.* 2013a). Its arrival into Shakespeare Bay (Picton) and subsequent spread within the Marlborough Sounds was of particular concern, being New Zealand's largest area for green-lipped mussel (*Perna canaliculus*) aquaculture and given *D. vexillum's* demonstrated fouling capabilities (Coutts & Forrest 2007). Two events were instrumental in the spread of *D. vexillum* around the Marlborough Sounds: (1) the movement of an infected aquaculture structure to the outer regions of Queen Charlotte Sound, and (2) the inter-regional transfer of infected mussel seed-stock in Pelorus Sound (Forrest & Hopkins 2013) (Figure 3.1). Subsequent local spread was attributed to the movement of aquaculture equipment and vessels, as well as natural 'stepping-stone' dispersal between man-made structures (Fletcher *et al.* 2013c). In 2002, a management programme was initiated in Shakespeare Bay to minimize the spread of *D. vexillum* (Coutts & Forrest 2007), and while efforts to reduce its abundance were successful, consequential spread was inevitable.

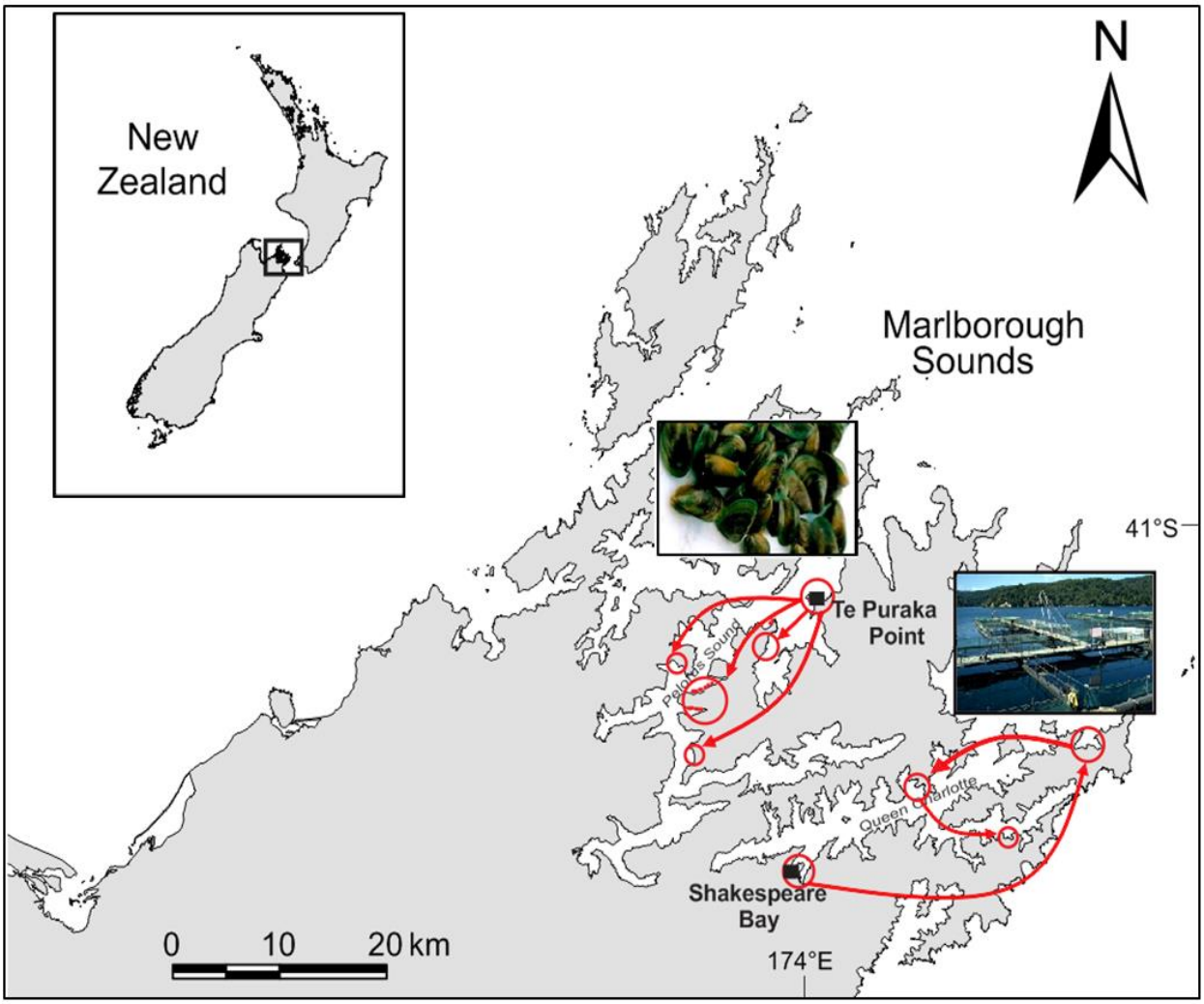

Figure 3.1. Map of the Marlborough Sounds, indicating the subsequent spread of *Didemnum vexillum* (red arrows) within Queen Charlotte, following its initial incursion into Shakespeare Bay (black square), and within Pelorus Sound, following its incursion into Te Puraka Bay (black square). Spread was facilitated by the movement of aquaculture infrastructure and infected seed stock; images are located on the map in accordance to the areas influenced by these movements (photo credit: Cawthron Institute). Unfilled red circles indicate the location of marine farms that were instrumental in the spread of *D. vexillum*. Figure modified from L. Fletcher, Cawthron Institute.

D. vexillum is a strong spatial competitor, capable of overgrowing, outcompeting or displacing indigenous and previously established organisms (Sinner & Coutts 2003; Bullard *et al.* 2007; Valentine *et al.* 2007b; Fletcher *et al.* 2013b) For example, in the north-east United States, cultured blue mussels *Mytilus edulis* displayed reduced shell lengths and lower condition values with the overgrowth of *D. vexillum* in experimental systems (Auker 2010). It can rapidly colonise hard substrate including docks, pilings, rock outcrops and gravel habitats (Bullard *et al.* 2007). In New Zealand, however, colonies do not inhabit natural environments and display poor survival following transplantation to the seabed, but they do inhabit biogenic structures (e.g., horse mussels and seaweeds) (Hopkins *et al.* 2011). The life history of *D. vexillum* includes sexual and asexual reproductive strategies (Sakai *et al.* 2001). The reproductive season of *D. vexillum* ranges from five to nine months (in the northern and southern hemispheres, respectively). Reproduction is strongly correlated with water temperature and colonies undergo periods of degeneration during colder winter months, when temperatures fall below 14°C (Valentine *et al.* 2007b; Fletcher *et al.* 2013a). The pelagic larval duration of most ascidians is limited to a few hours, making natural dispersal less consequential for regional spread (Osman & Whitlatch 2007; Herborg *et al.* 2009; Morris & Carman 2012). However, *D. vexillum* is potentially capable of natural dispersal from hundreds of metres to kilometres, with 10% of experimental larvae remaining viable in the water column for up to 36 hours under laboratory conditions (Fletcher *et al.* 2013c).

In addition to larval settlement, *D. vexillum* is able to form and expand colonies asexually by means of budding and fragmentation (Morris & Carman 2012; Reinhardt *et al.* 2012). Fragmentation, the breaking-off of smaller colony fragments, is common in colonial ascidians and results from natural or anthropogenic-mediated disturbance (Ben-Shlomo *et al.* 2001; Ben-Shlomo *et al.* 2008; Acosta & Forrest 2009; Westerman *et al.* 2009). The dispersal of fragmented colonies of *D. vexillum* may accelerate this species' spread, as fragments may be less susceptible to competition and predation than settled larvae, and brooded larvae in fragments could be released prior to, or following reattachment (Bullard *et al.* 2007). Furthermore, Morris and

Carman (2012) found that under laboratory conditions *D. vexillum* fragments remain viable after suspension in the water column for three weeks, with only one zooid required for the successful attachment and regrowth of colonies. However, while cited as an important mechanism of secondary spread (Carver *et al.* 2006; Bock *et al.* 2011; Morris & Carman 2012), the natural dispersal of colonial fragments (i.e., the current-based dispersal of fragments) can be limited. Under laboratory conditions it was found that sinking rates of detached *D. vexillum* fragments ranged from 3.11 $cm.s^{-1}$ to 8.68 $cm.s^{-1}$, varying with the size of the fragment (Fletcher *et al.* 2013c). Therefore, given hypothetical water current speeds of 10 $cm.s^{-1}$ and a water depth of 25 m, fragments with the lowest sinking potential rates would only travel within 80 m of the release point, making its contribution predominantly local. Nevertheless, fragmentation may still be important for the regional spread of *D. vexillum* if disturbed fragments are able to 'hitch-hike' to nearby habitats aboard vessels.

Aside from fragmentation, *D. vexillum* possesses another trait that can enhance its invasive capacities; it has the ability to form chimeric entities through fusion (Smith *et al.* 2012). While traditionally thought of as a rare event due to potential genetic conflicts (Strassmann & Queller 2004), literature now shows that following fragmentation or preferential larval settlement near related colonies, colonial ascidians can fuse with other genetically compatible colonies, resulting in stable chimeras (Grosberg 1987; Sommerfeldt & Bishop 1999; Puill-Stephan *et al.* 2009; Westerman *et al.* 2009; Smith *et al.* 2012; Rinkevich & Fidler 2014). For example, a single highly polymorphic locus strongly influences allorecognition, dictating chimeric fusion in *Botryllus schlosseri* (Saito *et al.* 1994; De Tomaso *et al.* 2005; Ben-Shlomo *et al.* 2008). Chimerism is also known to occur naturally in some algae, sponges, hydroids, corals and bryozoans (Grosberg 1981; Buss 1982; Grosberg 1988; Puill-Stephan *et al.* 2009).

These chimeric entities are thought to be important biological attributes, increasing the invasive potential of NIS by generating larger colonies with a greater store of genetic variability, potentially increasing the colonies adaptive and competitive capabilities (Grosberg 1988;

Rinkevich & Yankelevich 2004; Puill-Stephan *et al.* 2009; Smith *et al.* 2012; Rinkevich & Fidler 2014). Despite the range of literature discussing chimerism, studies using genetic markers, such as microsatellites, often fail to utilise results arising from chimeric colonies which can have more than two alleles per locus. Instead, analyses are restricted to the two alleles with the highest peaks (diploid analyses), potentially underestimating genetic diversity and population structure within study systems (Ben-Shlomo *et al.* 2008; Ben-Shlomo *et al.* 2010; Smith 2012; Meirmans & Van Tienderen 2013; Reem *et al.* 2013a). The limited availability of analytical programs for datasets from chimeric organisms has undoubtedly hindered progress in this area, but packages designed for polyploid data sets are now available (Obbard *et al.* 2006; Clark & Jasieniuk 2011; Guichoux *et al.* 2011; Dufresne *et al.* 2014).

As a dominant biofouler of artificial structures and due to its enhanced potential for spread, *D. vexillum* represents a valuable model to study the connectivity of NIS between artificial structures. In this study, eight polymorphic loci were used to characterise the genetic structure of 15 *D. vexillum* populations from artificial structures in the top of the South Island. This study had three aims. The first was to investigate the effect of including chimeric loci data on estimates of genetic diversity, compared to using diploid data alone. It was expected that the exclusion of chimeric data would underestimate allelic diversity within the study system. Using the diploid data, the second aim was to examine connectivity and gene flow across sampling sites (Pelorus Sound, Queen Charlotte and Port Nelson). Due to the abundance of vessels in the region, the potential for vessel movements between sites, and the wealth of artificial substrates, it was expected that populations would be well connected, reflected by genetic homogeneity. Thirdly, patterns of genetic diversity within populations in Pelorus Sound and Port Nelson were compared to suspected source populations in Queen Charlotte Sound. According to population genetic theory, smaller founder populations typical for NIS contain a fraction of the total genetic variance present in any source population (Nei *et al.* 1975; Barrett *et al.* 1990; Sakai *et al.* 2001; Dlugosch & Parker 2008). Therefore, suspected source populations were predicted to have greater genetic diversity. However, within this system (with asexual capacity) genetic diversity

could also be low, masking differences between source and founder populations, especially given that *D. vexillum* went through a major bottleneck when originally introduced into New Zealand (Smith 2012). The fourth aim was to assess gene flow among *D. vexillum* populations growing on mussel farms within Pelorus Sound, in relation to expected larval connectivity based upon a mathematical matrix. This matrix modelled variable pelagic larval duration and water currents within Pelorus Sound.

3.2. Materials and Methods

3.2.1 Sampling Didemnum vexillum populations

Tissue samples from colonies morphologically identified as *D. vexillum* were collected from artificial structures (mussel ropes, floats, wharfs, buoys) in April 2013. A total of thirty specimens were collected per site (N=15), with sites located in Port Nelson (N=1), Pelorus Sound (N=9) and Queen Charlotte Sound (N=5), New Zealand (Figure 3.2). This sample size was in accordance to the recommended number of individuals per population required to accurately estimate allele frequencies (Hale *et al.* 2012) and detect rare alleles (Sjogren & Wyoni 1994).). A larger sample size was also collected to increase the power of tests used for detecting genetic differentiation of subpopulations, such as analysis of molecular variance (AMOVA), and for detecting genetic clusters within species, such as STRUCTURE, which increase with increasing sample size (Hudson *et al.* 1992; Leberg 2002; Fitzpatrick 2009). Samples were collected from colonies or colony fragments situated ≥ 2 m apart to minimise the chance of pseudoreplication due to sampling from clonally related colonies (Smith et al., 2012). Tissue samples (ca. 100-500 mg) were preserved in approx. 1.0 ml of 100% (v/v) ethanol and stored at -20 °C.

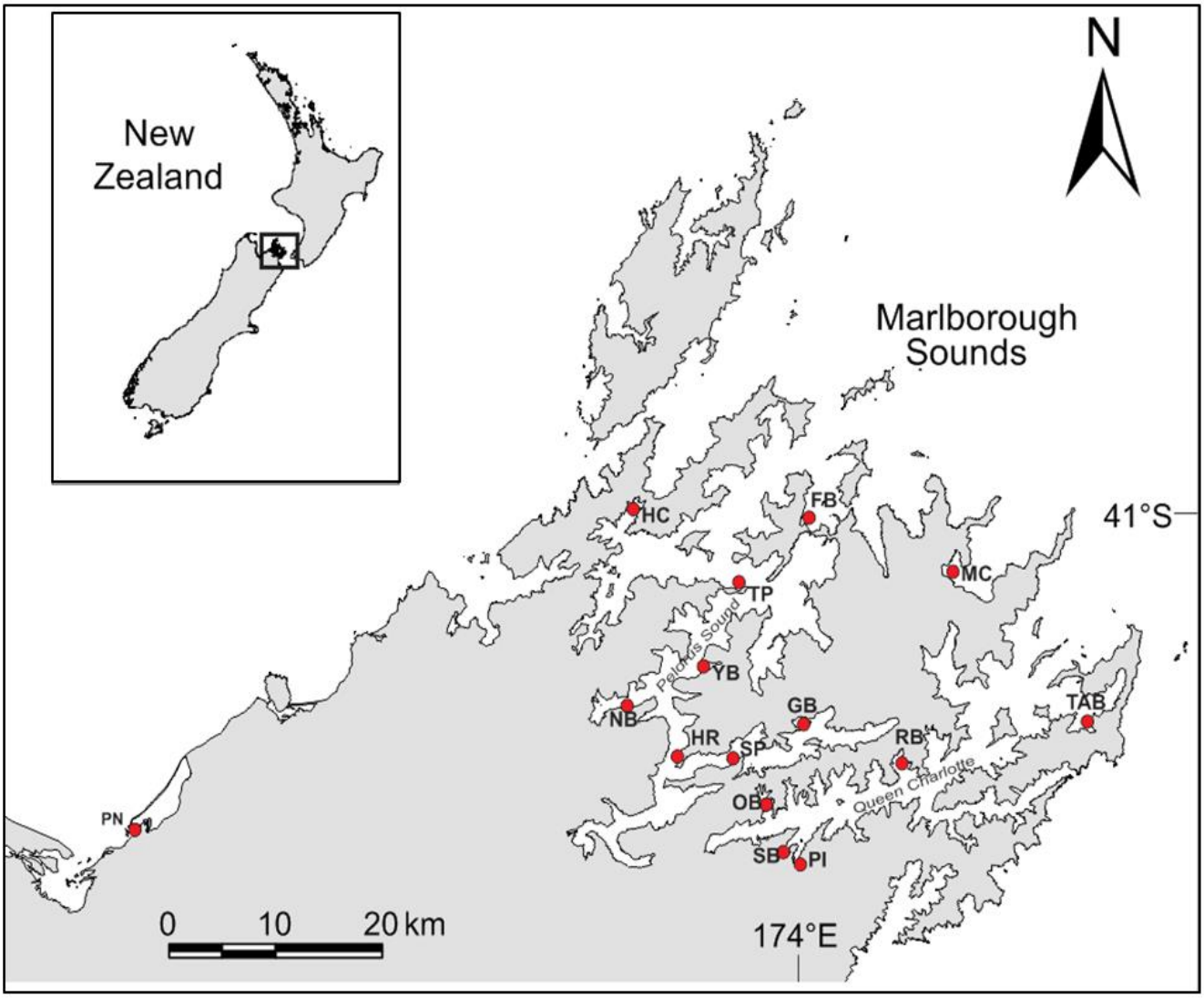

Figure 3.2. Map of the Tasman and Marlborough Sounds region where *Didemnum vexillum* samples were collected for microsatellite genotyping. Each sampling site is indicated by a red circle and initials. Queen Charlotte sampling sites: SB=Shakespeare Bay, PI=Picton Marina, OB=Opuaha Bay, RB=Ruakaka Bay, TAB= Te Aroha Bay. Pelorus Sound sampling sites: SP=Schnapper Point, GB=Goulter Bay, HR=Hikapu Reach, NB= Nydia Bay, YB= Yncyca Bay, TP=Tawero Bay, HC=Hallam Cove, FB=Forsyth Bay, MC=Melville Cove. Nelson sampling site: PN=Port Nelson.

3.2.2 DNA extraction and amplification

For each specimen a 2 mm^2 section of tissue was cut for DNA extractions. The tissue was macerated using flame-sterilised forceps and a scalpel. Total genomic DNA for polymerase chain reactions (PCR) amplification was extracted using a lithium chloride/chloroform protocol (Gemmell & Akiyama 1996)(Appendix IV, A4.1.). Extracted DNA samples, along with a 1:10 dilution of the DNA, were stored at -20° C.

3.2.3 Primer development

The initial set of primer candidates for microsatellite analyses contained thirty nine markers, developed by Ecogenics Ltd (http://www.ecogenics.ch: Switzerland) using a 454 sequencing protocol. Preliminary selection of all primers was conducted at the University of Canterbury by honours student, Sarah Redlich (Appendix IV, A4.2). Redlich's work identified a subset of twenty four primers which amplified consistently across the *D. vexillum* DNA samples she examined. Preliminary work I conducted in this study, however, identified three of the twenty four primers as monomorphic, six did not amplify consistently, and one had a very small allele range, causing diminished ability to distinguish between allele peaks and primer dimer, and leaving a total of fourteen primers.

All loci were amplified in polymerase chain reactions (PCRs) using the Qiagen Type-it Microsatellite PCR kit and M13 tags to label each forward primer, with the addition of M13 fluorescent-labelled primers (FAM,PET,VIC,NED) (Schuelke 2000). Initially loci were amplified singularly in PCRs using annealing temperature gradients (52-60 °C) for the optimisation of protocols (Appendix IV, A4.3). During preliminary optimisation, primer concentrations were optimised, DNA dilutions were examined and the suitability and repeatability of microsatellite loci was established using individual amplifications (repeating 10% of the single PCRs). Following this, microsatellite loci were multiplexed (Schuelke 2000) and organised to ensure allele size ranges did not overlap (Appendix IV, Table A4.1.). Loci were assigned using the MULTIPLEX MANAGER 1.0 software (Holleley & Geerts 2009). The multiplexing protocol was

performed with a final volume of 4 µl and contained 2x Type-it Multiplex PCR Master Mix, 0.0216 µm of each M13-labelled, locus-specific forward primer, 0.0864 µm of each locus-specific reverse primer, 0.135 µm of M13 5'-end labelled with an Applied Biosystems (ABI) dye (FAM, NED, PET or VIC), 0.82 µl of RNase-free water and 2 µl of 5.5 - 8.5ng/µl diluted DNA (Appendix IV, A4.4).

The thermocycling parameters for all PCRs included an initial denaturation at 94°C for 15 minutes, 94°C for 30 seconds, 58°C for 90 seconds and 72°C for 60 seconds for 8 cycles, followed by 89°C for 30 seconds, 56°C for 90 seconds and 72°C for 60 seconds for 25 cycles, with a final 30 minute extension at 60°C (Schuelke 2000). Post-PCR products were diluted with 5 µl of MilliQ water, producing a total volume of 9 µl. 2 µl of the diluted PCR products were then taken from each multiplexed loci and pooled with other multiplexed loci to form genotyping groups (Appendix IV, Table A4.1.), to a volume of 6-8 µl. 10 µl formamide and 0.4 µl of GeneScan 500LIZ internal size standard ABI (per individual) were then added for genotyping. PCR products were resolved by the University of Canterbury Sequencing Service (Christchurch, New Zealand) on an ABI 3100 DNA analyser. Alleles for each locus were scored using GENE MARKER v.1.6 (SoftGenetics LLC). Replicates (minimum 10% of the sample size) were assessed for amplification errors and the repeatability of scoring. Scores from individual amplifications were also compared to those acquired via multiplexing to detect possible multiplexing errors. To ensure genotyping accuracy for each PCR reaction, a control sample was included with each PCR run. However, there were inconsistencies in the control results for three of the ten PCR runs. Preliminary investigations of *D. vexillum* zooids (Appendix IV, A4.5 and Figure A4.1) within the suspected chimeric control sample revealed that the variation in this sample was likely to be the result of the chimeric characteristics of this ascidian (Appendix IV, A4.6, Table A4.2). Genotyping results for all three control genotypes were included in analyses.

Following this optimisation process, eight out of the fourteen primers were found to be consistent and successful across all samples using the multiplexing method, and these were selected to use within further analyses (Appendix IV, Table A4.1).

3.2.4 Statistical methods

3.2.4.1 The influence of including chimeric loci

Chimeric colonies can have more than two alleles per locus present due to the fusion of tissues from conspecific organisms. Therefore, the locus scores were separated into two datasets. One dataset incorporated the two alleles with the highest peaks (diploid dataset), as per Smith (2012), and the other incorporated all detected peaks within 50% of the height of the two main peaks (chimeric dataset). The analyses that follow were conducted using only the former (diploid) dataset, which is the standard procedure used for genetic analyses of chimeric organisms, due to the limited availability of programs able to analyse microsatellite data with more than two alleles per locus (e.g., polyploidy data) (Obbard *et al.* 2006; Clark & Jasieniuk 2011; Guichoux *et al.* 2011). However, neglecting to account for multiple alleles in population genetics not only results in a loss of information, but may bias estimates of genetic diversity and population structure (Meirmans & Van Tienderen 2013). The R package 'POLYSAT' is a recently developed tool for analysing autopolyploid and allopolyploid microsatellite data (Clark & Jasieniuk 2011). It handles genotype data of any ploidy level, including mixed-ploidy populations, and assumes allele copy is always ambiguous in partial heterozygotes. While this package has been used in recent publications for vertebrate work (Münzbergová *et al.* 2013), and plant analyses (Lo *et al.* 2009; Sampson & Byrne 2012), it has rarely been used in studies involving chimeric organisms. In accordance with the nature of polysomy, it is expected that greater heterozygosity and associated diversity should be maintained in a polyploid, and specifically autopolyploid population compared to a diploid population (Moody *et al.* 1993). In this study, POLYSAT in R v.3.0.2 (R Core Team 2013) was used to assess differences in allelic diversity of *D. vexillum* populations when incorporating allele peaks resulting from chimeric

colonies, to results obtained from their exclusion. As it was not feasible to isolate the different individual alleles, or genotypes in chimeric results, chimeric colonies were treated as polyploids for the analysis.

3.2.4.2 A characterisation of the diploid dataset

All microsatellite markers and populations were assessed for deviations from Hardy-Weinberg equilibrium (HWE) and for linkage disequilibrium (LD) using ARLEQUIN v.3.5.1.3 (Excoffier & Lischer 2010). ARLEQUIN v.3.5.1.3 uses a test analogous to Fisher's exact test to detect departures from HWE at each locus, and a likelihood ratio test to assess LD, the presence of non-random associations between pairs of loci (Slatkin & Excoffier 1996; Excoffier & Slatkin 1998; Excoffier & Lischer 2010). HWE and LD comprise the basic assumptions of most statistical tests, and although deviations can designate underlying biological processes, such as inbreeding or non-random mating, they often increase potential bias in results. All calculations were conducted per locus and population. For analyses conducted in ARLEQUIN v.3.5.1.3, 10,000 permutations were used and 95% confidence intervals for F-statistics were obtained by bootstrapping over loci 20,000 times. In addition, MICRO-CHECKER v.2.2.3 (Van Oosterhout *et al.* 2004) was used to assess all loci for null alleles, as well as genotyping errors, such as large allele drop outs and stutter (1000 randomizations) (Van Oosterhout *et al.* 2004; Arif *et al.* 2010). These technical errors can inflate measures of genetic differentiation and decrease the overall power of statistical analyses (Bonin *et al.* 2004; Hoffman & Amos 2005; Carlsson 2008).

To account for multiple comparisons and control alpha inflation when two or more statistical tests on the same data were performed, a False Discovery Rate (FDR) correction was applied (Benjamini & Yekutieli 2001). Traditional correction methods, such as Bonferroni corrections, aim to control family-wise error rates, i.e., the probability of incorrectly rejecting one or more null hypotheses. However, FDR methods control the expected fraction of incorrectly rejected hypotheses out of the total number of hypotheses rejected (Benjamini & Yekutieli 2001). FDR corrections are less conservative, more consistent in terms of whether a particular hypothesis is

rejected, and have greater power than traditional correction methods (García 2004; Narum 2006). Using the following equation: $CPV = \alpha / \sum_{i=1}^{k}(1/i)$ the corrected *p*-value (*CPV*) was obtained. Values below this were considered significant for multiple comparisons. Using this equation, α=0.05, k=the number of tests performed, and i=the ith observation (Narum 2006).

Population-specific numbers of alleles and allelic diversity, incorporating allelic richness (A_{RN}) and private allelic richness (A_{RP}), were estimated for all markers and populations in HP-RARE v.1.1 (Leberg 2002; Kalinowski 2005). Rarefaction, based upon the lowest sample size (N=19), was used to account for variable sample sizes and allow comparisons between datasets (Leberg 2002). To evaluate differences in allelic diversity (A_{RN}, A_{RP} and H_E) based upon site (Pelorus Sound, Queen Charlotte, Nelson), and based upon populations within sites, separate single-factor analysis of molecular variance tests (ANOVAs) were computed in R v.3.0.2 (R Core Team 2013). Observed (H_O) and expected (H_E) heterozygosity were estimated using GENALEX v.6.5 (Peakall & Smouse 2006). The population-specific inbreeding coefficient (F_{IS}) was obtained using ARLEQUIN v.3.5.1.3 (Excoffier & Lischer 2010).

3.2.4.3 Genetic structure and colonisation routes

A hierarchical analysis of molecular variance (AMOVA) was performed using ARLEQUIN v.3.5.1.3 (Excoffier & Lischer 2010) to assess differences in allele frequencies between sites (Pelorus Sound, Queen Charlotte Sound and Nelson). Molecular variance was partitioned into three levels: between sites, among populations within sites and within populations. No more than two loci were missing per individual and 10% missing data per loci was selected as an acceptable missing data rate (Pritchard *et al.* 2000). Molecular variance was also assessed using AMOVA following the removal of the Port Nelson population.

Using the AMOVA framework to test statistical hypothesis requires randomisation of the data (unique permutations), which is dictated by sample size (Fitzpatrick 2009). Calculations were conducted in R v.3.0.2 (R Core Team 2013) to ensure sample sizes used in this study were

adequate for main and pairwise AMOVAs, with a rejection of the null hypothesis set at $\alpha = 0.05$ and no hierarchical population structure (Fitzpatrick 2009).

Isolation-by-distance (IBD) was assessed by the correlation between the matrix of pairwise genetic distances and matrix of pairwise geographic distances between populations within Queen Charlotte and Pelorus Sound. The Port Nelson population was excluded from the IBD assessment, as spatial disharmony with sites would generate structural information, not pattern, in the results. MANTEL tests in GENALEX v.6.5 (9999 permutations) were performed with a geographic Euclidean distance matrix (kilometres). IBD results were then compared to those attained from STRUCTURE v.2.3.4 (Pritchard *et al.* 2000), which was used to infer population structure between Queen Charlotte, Pelorus Sound and Port Nelson. STRUCTURE is a Bayesian genetic clustering program that aims to delineate clusters of individuals into populations based on their inferred allelic frequencies, using multilocus genotypes (Evanno *et al.* 2005; François & Durand 2010). For this program, admixture with correlated frequencies were used, with no *a priori* definitions of population identification. *A priori* definitions were also assigned in accordance to populations, for comparison (Falush *et al.* 2003; François & Durand 2010). For all STRUCTURE analyses, a burn-in period of 10,000 iterations and 100,000 Markov Chain Monte Carlo (MCMC) replicates was selected for and simulations were run five times for each simulated population cluster (K), ranging from 1-10 (incorporating the actual number of populations). The optimal number of clusters (K) was estimated by comparing the log-likelihood of the data [$\ln P(X \mid K)$] (Pritchard *et al.* 2000), as implemented in STRUCTURE HARVESTER (Earl 2012).

An assignment test was conducted in GENALEX v.6.5 to determine whether the patchy structure among Pelorus Sound and Queen Charlotte Sound populations, and for the Port Nelson population, as generated by STRUCTURE, reflected the structure of suspected source populations identified within Queen Charlotte sound (Shakespeare Bay, Picton Marina and Te Aroha Bay).

3.2.4.4 Connectivity model development

To assess gene flow among *D. vexillum* populations growing on mussel farms in relation to expected larval connectivity, connectivity scores for mussel farms throughout Pelorus Sound were obtained using a GIS-based connectivity matrix. This matrix was constructed by incorporating estimated current velocities (Knight and Beamsly, 2012), and pelagic larval duration times (PLD clusters) (Cawthron Institute, unpublished data). For this study, two, twelve, twenty four and thirty six hour PLD cluster groups (Figure 3.3) were selected in order to incorporate the entire range of *D. vexillum's* natural dispersal capability (up to 36 hours, with 10% survival) (Fletcher *et al.* 2013c).

To develop the connectivity matrix, a hydrodynamic model (Knight *et al.* 2010) was used to estimate time-averaged current data throughout Pelorus Sound at a 100 m grid resolution, over two semi-diurnal tides (~24 hours). Current data derived from the model was combined in ArcMap 10.1 (ESRI 2012) with geographical information (i.e., the coastline) and present farm location data, provided by the New Zealand Marine Farming Association. A table of pairwise distances between farms, measured in hours, was then generated using the ArcMap 'cost distance' tool, which calculates the shortest path between nominated points (ESRI 2012). Path length was determined by the time taken to travel each step along that path, where each step comprised one grid cell in a cost matrix. For this thesis, the 'steps' were representative of operational mussel farms, and the cost matrix was the inverse of the average current speed. A path was nominated as quicker if it traversed high current areas. The table of pairwise distances was imported into R v.3.0.2 (R Core Team 2013) to create a network of connectivity between all farms. The connectivity network represented the travel time between each pair of farms, and all operational farms were included.

Farm connectivity was analysed using the network of connectivity for a given PLD time. Lines of 'connectivity' in association with currents, PLD and farm locations were then imbedded onto a coastal map (Appendix IV, Figure A4.2a, b). The complete 'network' then separated into two or more 'subnetworks' or, as they are defined here as 'clusters', depending upon the PLD time. These 'clusters' were represented by different colours on the map (Appendix IV, Figure A4.2c) and farms within each cluster were effectively connected. The final map provided a visualisation of potential 'stepping-stone' movements between farms, with connectivity represented by lines and colours representing isolated 'clusters' (Appendix IV, Figure A4.2d).

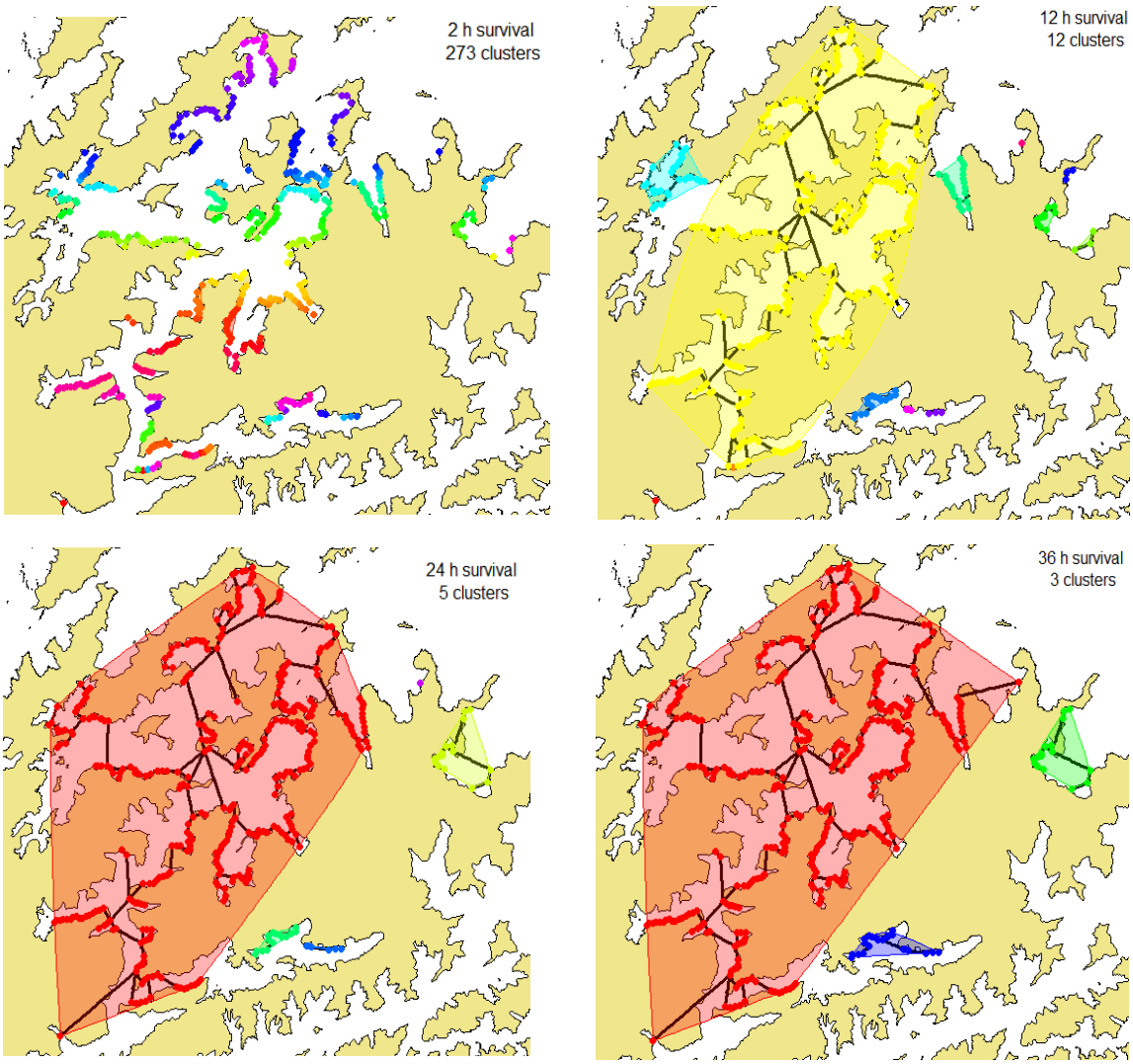

Figure 3.3. Maps displaying variable amounts of connectivity between mussel farms in Pelorus Sound in accordance with the connectivity matrix (Cawthron, unpublished data). Four different pelagic larval duration times, and their associated cluster groups, are presented (a=2 hours, b=12 hours, c=24 hours, d=36 hours). Each cluster is represented by a different colour; connected clusters are denoted by lines and an overlaid, similar-coloured image.

3.2.4.5 Genetic connectivity in relation to model predictions

Two separate AMOVAs were performed using ARLEQUIN v.3.5.1.3 to assess genetic differentiation among sampled populations within PLD cluster groups of 12 or 24 hours. As the number and identity of sampled farms in this study in the 24 hour cluster groups, were identical to the sampled farms within the 36 hour cluster group (generated by the matrix), the 24 hour cluster group was representative of 24-36 hours PLD potential. To investigate differentiation between populations within cluster groups, pairwise F_{ST} (Wright 1965), using ARLEQUIN v.3.5.1.3 (Excoffier & Lischer 2010), and Jost's *D* (Jost 2008), using the R package DEMEtics (Gerlach *et al.* 2010), were calculated. Pairwise tests also provided a way to determine differentiation between populations within the two hour PLD cluster group. DEMEtics generates Jost's *D* by bootstrapping over loci 1,000 times, the package also applies a modified Benjamini-Hochberg False Discovery Rate correction for multiple statistical tests to *P*-values for a family-wise error of $\alpha=0.05$ (Benjamini & Yekutieli 2001; Narum 2006; Jueterbock *et al.* 2011; Jueterbock *et al.* 2013). Jost's *D* was used as a measure of differentiation, as it performs better with polymorphic markers and uneven sample sizes (Jost 2008). The fixation index, F_{ST}, was also employed to enable better comparisons with previous research, because despite its dependency upon within-population diversity and controversy surrounding its use, which is still in debate, (Jost 2009; Ryman & Leimar 2009; Gerlach *et al.* 2010; Meirmans & Hedrick 2011; Whitlock 2011; Wang 2012), F_{ST} has remained in practice for several decades (Meirmans & Hedrick 2011). Furthermore, its combined use with recent estimates (such Jost's *D*) is suggested to generate robust analyses of population structure (Meirmans & Hedrick 2011). A Pearson correlation between F_{ST} and Jost's *D* values was used to ensure both estimators provided comparable information.

3.3. Results

3.3.1 The influence of including chimeric loci

A high proportion of chimeric colonies were identified across all populations (19-47%), with the highest proportions evident in Nelson (35%) and Queen Charlotte Sound (35%) (Table 3.1). While not statistically significant (df=14, t=2.15, P=0.803), there was a trend for increased allelic diversity when allele peaks representing chimeric colonies were considered (Table 3.1). Furthermore, when incorporating data from chimeric colonies, allele frequencies can be modified. In this study, the frequency of more common alleles was reduced, less common allele frequencies increased, and rare alleles were introduced when data was incorporated (Appendix V, Tables A5.1 and A5.2). It should be noted that for many native species the introduction of rare alleles (frequencies < 0.01) is a common feature of microsatellite loci, and very rare alleles are often uninformative for population-based analyses, as their presence may be due to reoccurring mutations rather than historical association or contemporary gene flow (Hale *et al.* 2012). However, based on the mutation rate of microsatellite loci for invertebrates (between 10^{-2} and 10^{-6} mutations per locus per generation, and on average $5x10^{-4}$) (Selkoe & Toonen 2006) it would be against evolutionary theory to expect to find mutations within the timescale of invasions.

Table 3.1. Allelic diversity, measured as the average number of alleles (A_N), and the proportion of chimeric colonies (*Cc*), found at each locus per population (*N*=sample size) using all loci for polyploid and diploid data over 15 *Didemnum vexillum* populations. Measurements were taken across all populations. All means ± SE.

Site	*N*	*Polyploid* A_N	*Diploid* A_N	*Cc*
Pelorus Sound	240	5.32 (± 0.14)	5.17 (± 0.16)	82 (24%)
Goulter	26	5.54 (± 0.60)	5.38 (± 0.60)	11 (42%)
Schnapper	24	5.50 (± 0.63)	5.50 (± 0.63)	6 (25%)
Hikapu	29	4.75 (± 0.59)	4.75 (± 0.59)	7 (24%)
Nydia	30	5.37 (± 0.80)	5.00 (± 0.65)	11 (37%)
Yncyca	26	5.00 (± 0.65)	5.00 (± 0.65)	5 (19%)
Tawero	27	5.25 (± 0.67)	5.12 (± 0.64)	11 (40%)
Hallam	21	4.88 (± 0.67)	4.50 (± 0.55)	10 (47%)
Forsyth	28	5.62 (± 0.73)	5.12 (± 0.55)	9 (32%)
Melville	29	6.14 (± 1.00)	6.13 (± 1.00)	12 (41%)
Queen Charlotte	124	5.00 (± 0.14)	4.85 (± 0.15)	44 (35%)
Picton	24	5.14 (± 0.54)	4.88 (± 0.61)	5 (20%)
Shakespeare	26	5.25 (± 0.62)	5.25 (± 0.62)	12 (46%)
Ruakaka	28	4.88 (± 0.58)	4.75 (± 0.49)	11 (39%)
Te Aroha	27	5.25 (± 0.67)	5.00 (± 0.59)	7 (26%)
Onahau	19	4.50 (± 0.53)	4.38 (± 0.49)	9 (47%)
Port Nelson	24			
Nelson	24	5.13 (± 0.58)	5.25 (± 0.37)	9 (36%)
Overall	388	5.20 (± 0.03)	5.07 (± 0.12)	

3.3.2 A characterisation of the diploid dataset

Across all 15 populations, most samples amplified successfully for all eight loci (Table 3.2). All populations showed significant deviations from Hardy-Weinberg equilibrium (HWE) for some of the eight loci, mainly due to homozygote deficits, which may increase the probability of type I error for AMOVA statistics (Appendix V, Table A5.3.). Hikapu Reach and Port Nelson showed the greatest deviations (with five of the eight loci out of HWE), attributed to a deficit of homozygotes for four loci and deficit of heterozygotes for one locus (Appendix V, Table A5.3.). MICRO-CHECKER suggested no evidence of stutter or genotyping errors, but detected the potential presence of null alleles for four markers, although this was not consistent across all populations within each locus (Appendix V, Table A5.3). To limit the influence of the detected deviations from HWE and suggested null alleles, subsequent analyses were repeated with and

without loci Dvex10, and Dvex19. These two loci (Dvex10 and Dvex19) were selected from the four loci with detected null alleles because these loci had null alleles present in ≥ half of all the populations. Out of the total of 420 tests for linkage disequilibrium (LD), 11 showed evidence for loci pairs with LD after FDR correction (*CPV* = 0.008). Similarly, two out of the 90 tests showed evidence for loci pairs with LD when loci Dvex10 and Dvex19 were excluded (*CPV*=0.01). The test for LD in ARLEQUIN assumes Hardy-Weinberg proportions of genotypes and therefore the significance of these tests may be a result of departures from HWE (Excoffier & Slatkin 1998). However, loci pairs were not consistently linked across populations, and therefore physical chromosomal linkage appears unlikely, and all markers were considered as independent replicates of the *D. vexillum* genome.

All eight loci were polymorphic, with a total range of 4 (Dvex01) to 13 (Dvex30 and Dvex33) alleles (Appendix V, Table A5.4). Overall, genetic diversity was consistently high, with average observed and expected heterozygosity ranging from 0.63 ± 0.10 to 0.74 ± 0.06 and 0.56 ± 0.05 to 0.66 ± 0.05, respectively (Table 3.2). Queen Charlotte populations exhibited the lowest levels of observed and expected heterozygosity on average (H_O=0.065 ± 0.07, H_E=0.59 ± 0.04, Table 3.2), though expected heterozygosity slightly increased (H_E=0.63 ± 0.01) with the removal of Dvex10 and Dvex19 (Appendix V, Table A5.5). While t-tests showed no significant differences between observed and expected heterozygosity, all populations exhibited homozygote deficiency, with no evidence of significant inbreeding (Table 3.2). Allelic and private allelic richness ranged from 3.74 ± 0.34 (Ruakaka Bay) to 4.57 ± 0.60 (Melville Cove) and from 0.00 ± 0.00 (Hallam cove and Hikapu Reach) to 0.34 ± 0.13 (Melville Cove), respectively (Table 3.2). Private allelic richness was greatest for Melville Cove 0.34 ± 0.13 and Forsyth Bay 0.22 ± 0.09 (Table 3.2). Private alleles were evident when inspecting population-specific allele frequencies across loci for the diploid dataset. For example, only Forsyth Bay had allele 174 within Dvex10 and allele 115 within Dvex01 (Appendix V, Table A5.1). On average, allelic and private richness were similar across all sites (Pelorus Sound, Queen Charlotte and Nelson), although the Port Nelson site had a slightly higher proportion of private alleles (A_{RP}=0.17±0.08,

Table 3.2). There were no significant differences in allelic diversity (A_{RN}, A_{RP} and H_E), when compared across sites (Pelorus Sound, Queen Charlotte and Port Nelson), or compared across populations within Sounds (Pelorus Sound and Queen Charlotte Sound). These patterns were consistent when loci Dvex10 and Dvex19 were removed (Appendix V, Table A5.5).

Table 3.2. Estimates of genetic diversity for 15 *Didemnum vexillum* populations across three sites in New Zealand (Pelorus Sound, Queen Charlotte and Port Nelson) using all loci. Statistics are shown for: N=sample size, A_N=mean number of alleles, A_{RN}=rarefied mean allelic richness, A_{RP}=rarefied mean private allelic richness (based upon *N*=19), H_O=observed heterozygosity, H_E=expected heterozygosity under Hardy-Weinberg equilibrium, and F_{IS}=the inbreeding coefficient. All means ± SE.

Site	N	A_N	A_{RN}	A_{RP}	Ho	H_E	F_{IS}	
Pelorus Sound	240	5.15 (± 0.65)	4.14 (± 0.09)	0.14 (± 0.06)	0.68 (± 0.07)	0.60 (± 0.05)	-0.162328	ns
Goulter	26	5.38 (± 0.60)	4.40 (± 0.46)	0.09 (± 0.06)	0.66 (± 0.08)	0.63 (± 0.05)	-0.064510	ns
Schnapper	24	5.50 (± 0.63)	4.56 (± 0.50)	0.18 (± 0.11)	0.73 (± 0.07)	0.64 (± 0.05)	-0.181090	ns
Hikapu	29	4.75 (± 0.59)	3.95 (± 0.49)	0.00 (± 0.00)	0.69 (± 0.08)	0.58 (± 0.06)	-0.218780	ns
Nydia	30	5.00 (± 0.65)	4.11 (± 0.45)	0.14 (± 0.06)	0.66 (± 0.06)	0.60 (± 0.05)	-0.151910	ns
Yncyca	26	5.00 (± 0.65)	3.96 (± 0.50)	0.15 (± 0.08)	0.64 (± 0.10)	0.57 (± 0.07)	-0.138230	ns
Tawero	27	5.13 (± 0.64)	4.03 (± 0.47)	0.11 (± 0.05)	0.74 (± 0.06)	0.61 (± 0.05)	-0.201120	ns
Hallam	21	4.50 (± 0.50)	3.82 (± 0.37)	0.00 (± 0.00)	0.68 (± 0.06)	0.58 (± 0.04)	-0.244860	ns
Forsyth	28	5.13 (± 0.55)	3.87 (± 0.43)	0.22 (± 0.09)	0.67 (± 0.09)	0.57 (± 0.04)	-0.197950	ns
Melville	29	6.00 (± 1.02)	4.57 (± 0.60)	0.34 (± 0.13)	0.68 (± 0.06)	0.66 (± 0.05)	-0.062500	ns
Queen Charlotte	124	4.85 (± 0.57)	4.01 (± 0.08)	0.08 (± 0.06)	0.65 (± 0.07)	0.59 (± 0.04)	-0.153766	ns
Picton	24	4.88 (± 0.61)	4.09 (± 0.53)	0.06 (± 0.05)	0.66 (± 0.05)	0.61 (± 0.04)	-0.191170	ns
Shakespeare	26	5.25 (± 0.62)	4.19 (± 0.46)	0.14 (± 0.08)	0.68 (± 0.07)	0.62 (± 0.03)	-0.096280	ns
Ruakaka	28	4.75 (± 0.53)	3.74 (± 0.34)	0.06 (± 0.06)	0.66 (± 0.10)	0.56 (± 0.05)	-0.192110	ns
Te Aroha	27	5.00 (± 0.60)	4.04 (± 0.40)	0.07 (± 0.04)	0.63 (± 0.05)	0.59 (± 0.04)	-0.141150	ns
Onahau	19	4.38 (± 0.50)	3.97 (± 0.44)	0.08 (± 0.07)	0.63 (± 0.10)	0.57 (± 0.04)	-0.148120	ns
Port Nelson	24							
Nelson	24	5.00 (± 0.53)	4 (± 0.40)	0.17 (± 0.08)	0.73 (± 0.05)	0.62 (± 0.03)	-0.311290	ns
Overall	388	5.04 (± 0.10)	4.09 (±0.064)	0.06 (± 0.01)	0.68 (± 0.03)	0.60 (± 0.01)		

Note: ns = F_{IS} was not significant at $P>0.05$

3.3.3 Genetic structure and colonisation routes

AMOVA results revealed significant genetic differentiation among sites (Pelorus Sound, Queen Charlotte and Nelson) and among populations within sites, although this explained only a small proportion of the genetic variation (1.48% and 3.49%, respectively, Table 3.3). Highly significant variation within populations was detected (F_{ST}=0.050; P<0.001; Table 3.3), and this explained a large proportion of the variation in the data (95%, Table 3.3). However, for all levels of the AMOVA, F_{ST} values were low (F_{ST}=0.05), indicating little to moderate genetic differentiation, as defined by Wright (1978). When the Port Nelson population was removed from AMOVA analyses, no significant genetic variation was detected among sites (Pelorus Sound and Queen Charlotte) (Table 3.3). These results were consistent when excluding loci Dvex10 and Dvex19 (Appendix V, Table A5.6).

Bayesian clustering in STRUCTURE incorporating all populations suggested two population clusters (ΔK=2, Figure 3.4), (indicated by the smallest K value before the plateau of [ln P(X | K)], which is considered the best model (Pritchard *et al.* 2007). Evident sub-structure was patchy, with clusters spread among all three sites (Pelorus Sound, Queen Charlotte and Port Nelson). The first cluster comprised populations from Schnapper Point, Nydia Bay, Yncyca Bay, Picton and Port Nelson (dominant colour: red, Figure 3.4); while the other comprised Tawero Point, Forsyth Bay, Shakespeare Bay, Ruakaka Bay and Te Aroha Bay populations (dominant colour: green, Figure 3.4). The remaining populations appeared to have high levels of admixture, with dominant assignment to each of the two clusters (Figures 3.4 and 3.5). Results were consistent following the removal of the loci Dvex10 and Dvex19, and following the addition of information regarding population locations (ΔK=2, Appendix V, Figures A5.1 and Figure A5.2). There was no evidence for isolation by distance among populations in Pelorus Sound and Queen Charlotte Sound (rxy=0.132, P=0.260, Appendix V, Table A5.7) and this was consistent with the removal of Dvex10 and Dvex19 (Appendix V, Table A5.7). Similarly, there was no evidence or trend for isolation by distance among the Pelorus Sound populations and

among Queen Charlotte Sound populations with all loci, and without loci Dvex10 and Dvex19, when areas were analysed separately (Appendix V, Table A5.7).

Likelihood assignments indicated a moderate proportion of 'self' assignments for source populations within Queen Charlotte Sound, 66% for Picton, 50% for Shakespeare Bay and 48% for Te Aroha Bay. Within Queen Charlotte Sound and Pelorus Sound populations, and for the Port Nelson population, the greatest proportion of assignment was to the Picton and Te Aroha Bay populations. Yncyca Bay, Nydia Bay, Schnapper Point and Port Nelson populations had the greatest proportion of assignment to the Picton population (70%, 67%, 58% and 66% respectively, Figure 3.5). While Ruakaka Bay (78%), Forsyth Bay (51%) and Tawero Point (48%) were assigned to the Te Aroha Bay population (Figure 3.5). Of the remaining populations, Goulter Bay, Melville Cove and Orchard Bay all had close assignment to Picton and Shakespeare Bay (with differences ranging from 3-10%, respectively), Hikapu Reach was assigned to both Picton and Te Aroha Bay populations (a difference of 9%), and the Hallam Cove population was assigned to Shakespeare Bay and Te Aroha Bay populations (difference of 12%) (Figure 3.5). These findings support the patchy substructure and mixed grouping of the populations as indicated by STRUCTURE, which revealed high levels of admixture in populations from Goulter Bay, Melville Cove, Orchard Bay, Hikapu Reach and Hallam Cove (Figure 3.4). However, assignment results may be an artefact of the selection of specific-suspected source populations and the missed sampling from 'true' source populations, such as Te Puraka Bay in Pelorus Sound.

Table 3.3. Analysis of molecular variance (AMOVA) results for *Didemnum vexillum* microsatellite data for all loci, including *F*-statistics (FCT, FSC, and FST), associated 95% confidence intervals and percentages of explained variation. Four separate AMOVA results are presented, looking at differences across sites (Pelorus Sound, Queen Charlotte and Port Nelson) and across two different cluster groupings (24 and 12 hour pelagic larval duration times).

Spatial Scale		F_{CT}	F_{SC}	F_{ST}
Queen Charlotte, Pelorus Sound and Port Nelson	F-statistic	0.0148 *	0.03547 ***	0.04975 ***
	(95% CI)	(0.00439, 0.02943)	(0.02585, 0.05544)	(0.03252, 0.07588)
	Variation (%)	1.48	3.49	95.03
Queen Charlotte and Pelorus Sound	F-statistic	0.0108	0.03515***	0.0456***
	(95% CI)	(0.00321, 0.01881)	(0.02110, 0.05578)	(0.02871, 0.06949)
	Variation (%)	1.09	3.48	95.44
Pelorus Sound Only - Cluster 24 hours	F-statistic	-0.00791 ns	0.04009 ***	0.03250 ***
	(95% CI)	(-0.02419, 0.00475)	(0.02205, 0.06593)	(0.01921, 0.05224)
	Variation (%)	-0.79	4.04	96.75
Pelorus Sound Only - Cluster 12 hours	F-statistic	-0.01669 na	0.04575 ***	0.02982 ***
	(95% CI)	(-0.02676, 0.0098)	(0.02856, 0.07010)	(0.01901, 0.05108)
	Variation (%)	-1.67	4.65	97.02

Note: ns= not significant *P<0.05, **P<0.01, ***P<0.001

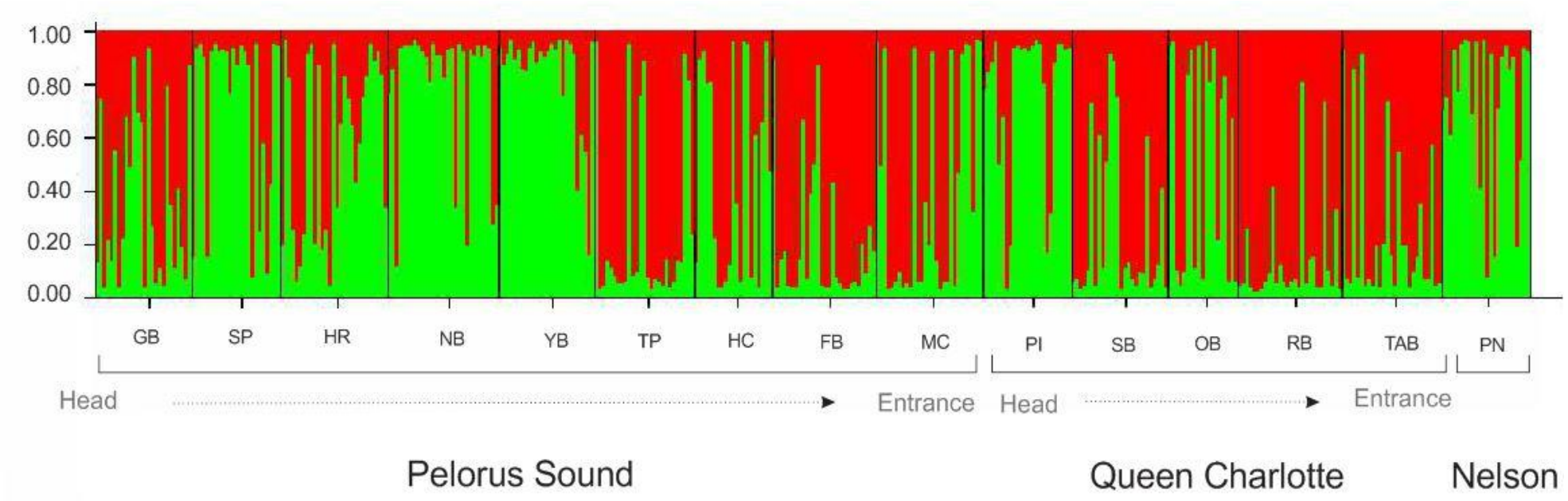

Figure 3.4. Population structure according to Bayesian clustering of *Didemnum vexillum* genotypes performed in STRUCTURE for all populations and all loci, within each of the three sampling sites (Pelorus Sound, Queen Charlotte and Port Nelson). Populations within each sampling site, from the head to the entrance of each Sound, are indicated. Pelorus Sound sampling sites: SP=Schnapper Point, GB=Goulter Bay, HR=Hikapu Reach, NB=Nydia Bay, YB=Yncyca Bay, TP=Tawero Point, HC=Hallam Cove, FB=Forsyth Bay, MC=Melville Cove. Queen Charlotte sampling sites: SB=Shakespeare Bay, PI=Picton Marina, OB=Opuaha Bay, RB=Ruakaka Bay, TAB=Te Aroha Bay, Nelson sampling site: PN=Port Nelson.

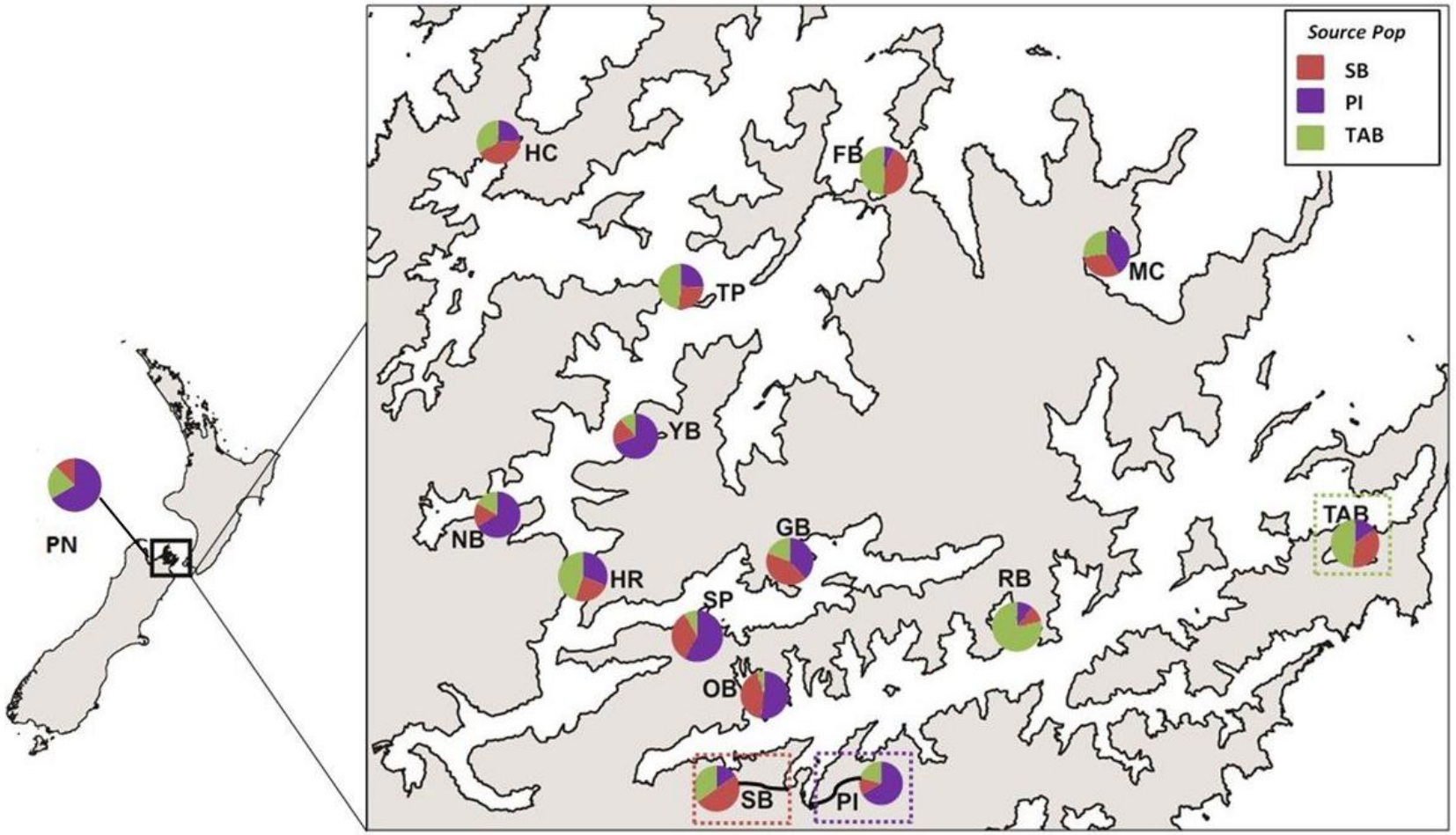

Figure 3.5. Results from likelihood assignment tests for 15 *Didemnum vexillum* populations indicating the proportion of populations within Pelorus Sound, Queen Charlotte Sound, and Port Nelson assigned to potential source populations located within Queen Charlotte Sound. Three source populations were sampled and tested, SB=Shakespeare Bay, represented by red proportions of the pie charts, PI=Picton Marina, represented by purple proportions of the pie charts and TAB=Te Aroha Bay, represented by the green proportion of the pie charts.

3.3.4 Genetic connectivity in relation to model predictions

AMOVA results revealed no significant genetic differentiation among cluster groups for the 24 and 12 hour PLD clustering times (24hr F_{CT}=-0.00791, 12hr F_{CT}=-0.01669, P>0.05, Table 3.3). However, there was significant genetic differentiation among populations within cluster groups for the 24 and 12 hour PLD clustering times (24 hr F_{SC}=0.04009, 12 hr F_{SC}=0.04575 respectively, P>0.05, Table 3.3). Most of the variation (%) within the data was found within population clusters (97%, Table 3.3). The lack of genetic differentiation among cluster groupings, including a cluster grouping of 2 hours PLD, was mirrored in the pairwise fixation index (F_{ST}) and distance measures (Jost's *D*) (Appendix V, Table A5.8a, b and c), which showed that each cluster group (e.g., Clust 1) had at least one population that was present in another cluster group (e.g., Clust 2), from which it was not significantly differentiated (Appendix V, Table A5.8a, b and c).

When looking at each population within the three PLD cluster groups independently, it is evident that there was no significant genetic differentiation between Goulter Bay, Schnapper Point and Tawero Point, between Schnapper Point and Yncyca Bay, and also between Hikapu Reach and Nydia Bay (Appendix V, Table A5.8a, b and c). Hallam Cove was also not significantly different from Goulter Bay, Forsyth Bay or Melville Cove (Appendix V, Table A5.8a, b and c). However, significant genetic differentiation was observed between Forsyth Bay, Hikapu Reach, Nydia Bay and Yncyca Bay for both F_{ST} (P≤0.01) and Jost's *D* (P≤0.01) (Appendix V, Table A5.8a, b and c). These findings were consistent across all PLD cluster groups (24, 12 hours and 2 hours, Appendix V, Table A5.8a, b and c). Genetic differentiation was therefore not reflective of PLD and current movements alone. Pearson correlation between F_{ST} and Jost's *D* was highly significant (r=0.0970, P<0.01), ensuring both estimators provided comparable results. The removal of loci Dvex10 and Dvex19 did not alter the overall results (Appendix V, Table A5.9a, b and c).

3.4. Discussion

3.4.1 A characterisation of the diploid dataset

Microsatellite analyses revealed that overall allelic richness in *Didemnum vexillum* populations was low and expected heterozygosity was relatively high. Values were similar to those found for other New Zealand populations of *D. vexillum* (mean allelic richness 3.28, expected heterozygosity 0.68; Smith (2012)), as well as other non-indigenous colonial ascidians, such as *Botrylloides violaceus* (mean allelic richness 3.55, expected heterozygosity 0.61; Bock *et al.* (2011)), and solitary ascidians, such as *Styela clava* (mean allelic richness 3.06, expected heterozygosity 0.54; Goldstien *et al.* (2010). However, compared with this study, higher allelic richness was detected for some European populations of *Botryllus schlosseri*, such as Scandinavian populations (mean allelic richness 5.29, expected heterozygosity 0.62; Reem *et al.* (2013b)), while for other European populations, including Scotland, Germany and parts of England, richness and expected heterozygosity was relatively low (mean allelic richness 1.75-4.0, expected heterozygosity 0.17-0.42) (Ben-Shlomo *et al.* 2006)). Lower genetic variation in these areas was attributed to ecological factors, traced directly to the last ice age, as well as site-specific selection pressures (Ben-Shlomo *et al.* 2006).

An excess of heterozygosity in this study, resulting in Hardy Weinberg disequilibrium, is similar to findings by Cohen (1990), who detected higher levels of heterozygosity then expected in two self-fertile solitary ascidians, *Corella inflata* and *Chelyosoma productum,* in Washington. This was attributed to latency in gamete activation, and variable timing in the release of sperm and eggs observed under laboratory conditions, generating a predominance of outcrossing (Cohen 1990). However, results were dissimilar to those found by Smith (2012), with no deviations from HWE detected within *D. vexillum* populations from samples taken across New Zealand. As results taken from samples across New Zealand were pooled to present one area it is plausible that the scale of sampling reduced the ability of this study to detect heterozygote excess or deficiency present within localised populations, such as those taken from the Marlborough Sounds.

It is more common within ascidian research to find heterozygote deficiency. For example, regional studies of NIS solitary ascidians, both in New Zealand and overseas, exhibited ancestral admixture and heterozygote deficiency (Dupont *et al.* 2009; Goldstien *et al.* 2010; Ordóñez *et al.* 2013). Similarly, heterozygote deficiency was detected in populations of the colonial ascidian *B. schlosseri* from the coasts of the North and South Islands of New Zealand, (Ben-Shlomo *et al.* 2001), the east and west coast of the USA (Stoner *et al.* 1997; Stoner *et al.* 2002), the Mediterranean Sea (Paz *et al.* 2003) and the Adriatic Sea (Rinkevich *et al.* 2001). Heterozygote deficiency in these studies was attributed to non-random mating and inbreeding, resulting not from self-fertilisation, but from the aggregated settlement of sibling *B. schlosseri* larvae (Ben-Shlomo *et al.* 2001; Rinkevich *et al.* 2001; Stoner *et al.* 2002; Paz *et al.* 2003), a characteristic which has not been studied in *D. vexillum*. These studies were conducted both in areas with high levels of potential connectivity generated by shipping activity between busy marinas and harbours (Stoner *et al.* 1997; Ben-Shlomo *et al.* 2001; Goldstien *et al.* 2010; Ordóñez *et al.* 2013), and areas where shipping activity was restricted and populations were more isolated (Rinkevich *et al.* 2001).

3.4.2 Three key findings and potential explanations

Three overall conclusions can be drawn from the findings of this study. Firstly, chimerism is an important feature in the life history of *D. vexillum* and the exclusion of chimeric data may alter the results obtained in analyses of genetic diversity and population structure. Secondly, populations sampled from artificial structures in Port Nelson, Pelorus Sound and Queen Charlotte form a larger metapopulation, with outcrossing (mating between non-relatives) and some substructure. This is evident in the data due to: low, but significant, genetic variation among sampled sites and within populations; the detection of two genetic clusters; no significant inbreeding; and heterozygote excess. Thirdly, parameters of the connectivity matrix (dispersal potential and water current movements) were not good predictors of genetic structure in Pelorus Sound. An alternative explanation for genetic structure is that in the absence of gene flow, region-wide selection is maintaining similar frequencies of common

alleles across populations during periods of environmental stress, generating genetic similarity despite environmentally-driven bottleneck events. This explanation was considered by Cohen (1990) when genetic differences were not detected between short-dispersing populations of *C. inflata* on a scale of 100 km. However, in this study the random survival and the regeneration of colonies following stressful periods (i.e., colder winter months) may contribute to genetic dissimilarity between some sites by generating site-specific private alleles.

These findings are consistent with molecular studies of the Mediterranean sponge *Scopalina lophyropoda*, shown to form chimeric entities and reported to have high levels of genetic diversity, outcrossing and heterozygote excess, despite a restricted PLD and patchy population structure (Uriz *et al.* 1998; Blanquer & Uriz 2010; Blanquer & Uriz 2011). However, they contradict findings from genetic studies of colonial ascidians, such as *B. schlosseri*, that regularly report heterozygote deficiency associated with inbreeding (Stoner *et al.* 2002; Paz *et al.* 2003; Ben-Shlomo *et al.* 2010). This contradiction is interesting, given that *D. vexillum* and *B. schlosseri* have similar reproductive strategies, and experience periods of die-back during colder seasons (Skerman 1958). However, unlike New Zealand populations of *D. vexillum*, *B. schlosseri* is able to settle on natural substrates, generating a constant source of propagules to surrounding substrates (Rinkevich *et al.* 1998; Ben-Shlomo *et al.* 2001; Ben-Shlomo *et al.* 2008). Additionally, the aggregated settlement of sibling *B. schlosseri* planktonic larvae generates small scale subpopulations, promoting mating between sibling colonies (Sabbadin 1978; Grosberg & Quinn 1986; Rinkevich *et al.* 1998).

Potential explanations for the genetic patterns found in this study include: (i) the formation of chimeric entities, which may alter genotypic frequencies, (ii) multiple incursions, generated by commercial and recreational vessel movements between artificial structures, (iii) different ancestral sources and admixture, and (iv) selection pressures for advantageous alleles during winter die-back. Each potential explanation will be discussed in turn. Null alleles did not

influence the overall conclusions of this study and were therefore excluded as a potential explanation for detected genetic patterns.

3.4.3 Reduced genetic diversity when excluding chimeric loci

It is evident from the results of this study that chimerism is an important biological feature in the life history of *D. vexillum*, with a high proportion of chimeric colonies detected across all sampled populations within the Marlborough Sounds region (19-47%). Chimerism in other ascidians, such as *Diplosoma listerianum* and *B. violaceus*, has also been found to be extremely common (Sommerfeldt & Bishop 1999; Sommerfeldt *et al.* 2003; Ben-Shlomo *et al.* 2008; Westerman *et al.* 2009). However, this calculation may be underestimated because, (i) colonies were sampled ≥ 2 m apart to avoid sampling the same colony/genotype more than once, and (ii) colonies defined as heterozygotes may actually be undetected chimeras. Such underestimations have been encountered in previous research (Ben-Shlomo *et al.* 2001) and have been attributed to allelic diversity, with greater diversity increasing the probability of detecting all present chimeras.

Given that *D. vexillum* larvae are brooded within the colony tunic (Lambert 2009; Fletcher *et al.* 2013a), additional microsatellite peaks may have resulted from DNA contamination of stored larvae. However, larval DNA would only comprise a small percentage of the total DNA. Further analyses into this relationship is beyond the scope of this study, but will be conducted following the completion of this thesis. A substantially greater proportion of chimeric colonies were detected in this study compared with earlier studies conducted across New Zealand on *D. vexillum* populations (12%) (Smith 2012) and populations of *B. schlosseri* (8-14%) (Ben-Shlomo *et al.* 2001). However, Smith (2012) speculated that the actual proportion of chimeric colonies in New Zealand would be higher than those detected because reduced genetic diversity resulting from founder effects increases the rate of inter-colony fusion. In addition, chimeric estimates for *B. schlosseri* were underestimated due to the specific avoidance of sampling these colonies (Ben-Shlomo *et al.* 2001). Despite the range of literature describing

chimerism within colonial ascidians, studies often exclude results with multiple alleles (Ben-Shlomo *et al.* 2008; Ben-Shlomo *et al.* 2010; Smith *et al.* 2012; Reem *et al.* 2013a). In accordance with my first hypothesis, there was a trend for decreased allelic diversity, with a reduced frequency of otherwise more common alleles when chimeric data was excluded from analyses. This exclusion could have implications for the interpretation of genetic results, given that most population analyses are allele frequency based (Hale *et al.* 2012). Moreover, restricting analyses to the two alleles with the highest peaks may generate null alleles. Therefore, sampled allele frequencies need to be representative of the true population allele frequencies (Hale *et al.* 2012), which will only result from including all allelic diversity. This is now viable with the development of programs capable of handling data with multiple alleles per locus (e.g., polyploidy), such as the 'POLYSAT' package (Clark & Jasieniuk 2011) and these avenues of analyses should be explored.

3.4.4 The influence of multiple introductions

In contrast to my second hypothesis, weak, but significant, genetic differentiation was detected across study sites, indicating reduced gene flow. However, following the removal of the Port Nelson population, differentiation between sites was no longer evident. On average, Port Nelson had the highest private allelic richness, as well as expected and observed heterozygosity compared to the other sites. One explanation for this pattern may be the reduced potential for gene flow, due to the large geographical distance between Port Nelson and areas within the Marlborough Sounds. Alternatively, the extensive number of vectors and pathways available for movement in the Marlborough Sounds region would provide ample opportunity for multiple NIS introductions. If these incursions originate from different sources, this could generate novel allelic combinations and enhance local heterozygosity, thereby contributing to genetic differentiation (Kolbe *et al.* 2004; Voisin *et al.* 2005; Kelly *et al.* 2006; Geller *et al.* 2010).

Artificial structures and the movement of vessels between these structures can contribute to the primary introduction and secondary spread of NIS (Lambert & Lambert 1998; Wasson *et al.*

2001; Floerl & Inglis 2005). The Marlborough Sounds is an important area for recreational and commercial activities. It is the capital of New Zealand's aquaculture, holds one of the busiest ports in this region (Port Picton), facilitates an active tourism industry, and fosters numerous residential and holiday dwellings. Port Nelson is also a major hub for shipping, with 500 foreign ship visits per year, 100 of which represent the first port of call for that vessel in New Zealand (Inglis 2006). This may explain the trend for higher private allelic richness in Port Nelson compared to Pelorus Sound and Queen Charlotte Sound, and why including Port Nelson in the AMOVA analysis may have led to significant genetic differentiation among cluster groups. Ports within the Marlborough Sounds are, however, well connected by domestic shipping traffic. For example, there are more than two domestic vessel visits per week between Port Nelson and Picton Marina (Inglis 2006).

3.4.5 Different ancestral sources and admixture

In contrast to my third hypothesis, reduced genetic diversity resulting from founder effects was not detected in Pelorus Sound or Port Nelson populations when compared to the diversity of potential source populations in Queen Charlotte Sound. This is not entirely surprising, as reduced genetic diversity in invasive populations is not as common as initially expected, with only 37% of studies on aquatic NIS reporting evidence of a significant loss in the genetic diversity of introduced populations (Roman & Darling 2007). Given heterozygote excess detected in this system, and the potential for multiple introductions, genetic exchange between populations, either by natural dispersal, fragmentation, or as a result of repeated migration events, potentially involves a large enough number of individuals to avoid reduced genetic diversity in regional dispersal. The random regeneration of *D. vexillum* individuals following degeneration during colder winter months (Valentine *et al.* 2007b; Fletcher *et al.* 2013a) may also contribute to the maintenance of genetic diversity.

When all populations were pooled, STRUCTURE analyses showed two genetic clusters. These clusters are likely to be a reflection of differences in allele frequencies between ancestral source

populations due to drift, as indicated by likelihood assignments. However, potential source populations in Pelorus Sound that were not sampled in this study (i.e., Te Puraka Bay) may generate incorrect or bias assignment results. Likelihood assignments revealed that Picton Marina and Te Aroha Bay (Queen Charlotte) were the most likely ancestral source populations in this study. Shakespeare Bay was the original site of incursion in the Marlborough Sounds by *D. vexillum,* and it was postulated that secondary spread took place from this site following the movement of aquaculture infrastructure (Forrest & Hopkins 2013). However, eradication attempts in Shakespeare Bay would have enforced the effects of seasonal die-back, reducing not only species abundance, but also allelic diversity, making it more likely that new incursions in Picton Marina and Te Aroha Bay played a central role in secondary spread. More comprehensive sampling is needed to confirm this theory.

3.4.6 Connectivity parameters, selection and boat movements

In contrast to my fourth hypothesis, partitioning *D. vexillum* populations in accordance to the connectivity matrix did not reflect the observed genetic structure. This is evident by the lack of detectable genetic differentiation among all PLD cluster groups. However, for certain populations within cluster groups, such as Schnapper Bay and Yncyca; Nydia Bay and Hikapu Reach, parameters of the connectivity matrix may explain a lack of among-population genetic differentiation. These sites are in close proximity, incorporating the estimated PLD dispersal potential of *D. vexillum,* and are surrounded by high flowing water currents, providing the opportunity for natural dispersal. However, a lack of genetic variation or detected genetic variation between other populations may be: (1) an artefact of the model's simplicity, resulting in incorrectly assigned clusters and over or underestimated site-to-site connectivity, (2) different environmental variables driving selection for regional, or site-specific, advantageous alleles, or (3) recreational and/or commercial vessel movements (including spat transfers and movements of farm infrastructure, such as ropes, floats, nets and cages).

The connectivity matrix was simplistic, since post-border spread was only dictated by larval dispersal duration and water current movements. Limited time and data availability prevented the use of a full hydrodynamic model in this study. Therefore, temporal variability in current movements and interactions between water currents and wind were excluded, also cross-channel connectivity was exaggerated. Other factors not considered here, but that may impact the successful recruitment of larvae to predicted mussel farm locations, include dispersal behaviour, local biotic resistance and the health and abundance of the source population (Ronce 2007; Clobert *et al.* 2009; Cote *et al.* 2010). The limitations of this matrix may have caused an overestimation in potential natural dispersal, underestimating site-site connections generated through other vectors, such as vessel and equipment movements. Caution is therefore warranted in the interpretation of the matrix results and population clusters, as they may be incorrectly biased. However, the matrix does provide a good spatially explicit null hypothesis against which genetic patterns can be tested.

Different environmental variables across Pelorus Sound may be generating novel selection pressures for site-specific advantageous alleles. For example, Forsyth Bay and Melville Cove were not genetically differentiated from Hallam Cove and all are located in deep, exposed embayments with similar topography, situated near the entrance of Pelorus Sound. Similarly, Schnapper Point and Goulter Bay were not differentiated and are found in shallow, sheltered locations within the Kenepuru Sound. Overall, Hallam Cove had no private alleles, but shared all common alleles and most of the rare alleles across all loci with Forsyth Bay and Melville Cove (Appendix V, Table A5.1). For example, alleles comprising 89% of allele frequencies for Dvex10 (alleles:180,183,186,192) were shared between Hallam Cove, Melville Cove and Forsyth Bay, but within Dvex10, only Forsyth had allele 174, making it a private allele potentially differentiating this site. In contrast, alleles comprising 99% of all alleles for Dvex01, (alleles: 106,112) were shared across Hallam Cove, Melville Cove and Forsyth Bay, but only Forsyth Bay had allele 115 and only Melville Cove had allele 118. This pattern is evident across all loci. It is also interesting that, despite their proximity (30 km), their similar environments and the

sharing of common alleles, Melville Cove and Forsyth Bay were genetically differentiated. This pattern may be a reflection of the high number of private alleles detected for each population, driven by multiple incursions from different sources or differential ancestry. However, incomplete sampling and high levels of admixture between potential source populations (Picton and Te Aroha Bay), as evident in the Melville Cove population, preclude the determination of differential ancestry.

An alternative explanation could be the frequent movement of small commercial and recreational vessels between Melville Cove and Forsyth Bay, vectors which have received increasing attention in the past decade for spreading NIS (Minchin *et al.* 2006; Acosta & Forrest 2009; Floerl *et al.* 2009; Davidson *et al.* 2010). For instance, the movements of recreational vessels have been implicated in pre-border incursions by *S. clava* in New Zealand (Goldstien *et al.* 2010). In the Pelorus Sound, the regular movements of aquaculture vessels may contribute to the post-border spread of NIS and resultant genetic patterns. For example, the removal of infected structures from the water, such as aquaculture lines, and disturbance of *D. vexillum* fragments, which break off and land on vessel decks, may be released into the water column during cleaning or bilge water discharge, increasing the potential for NIS spread (Acosta & Forrest 2009). Therefore, the movement of commercial vessels carrying *D. vexillum* fragments could contribute to observed genetic patterns in this study that were unexplained by natural dispersal alone, such as genetic similarities between Tawero Point and Goulter Bay. However, commercial vessel movements are restricted by the distance between marine farms and Havelock Marina, where vessels are moored. For instance, during the two week period of biofouling sampling on-board the Sanford mussel-sourcing vessel (refer to Chapter 2: Materials and Methods), it took a long time to travel from Havelock Marina to mussel farms in outer areas, such Melville Cove, which restricted activities to that one area during that day (Figure 3.6). Therefore, it is unlikely that the movements of this vessel are driving the signature of connectivity between specific sites, such as Melville Cove and Hallam Cove. However, as the activities of local harvesting, reseeding and spat transfer vessels were not recorded in this

study, they cannot be excluded as potential vectors contributing to the signature of connectivity. Neither can recreational vessels, which may move between these outer marine farm sites from local residential dwellings.

Reproduction in *D. vexillum* is strongly correlated with water temperature, with colonies undergoing periods of degeneration during colder winter months, when temperatures fall below 14°C (Valentine *et al.* 2007b; Fletcher *et al.* 2013a). During these periods of stress there may be selection for specific alleles enabling over-winter survival. This would result in an increased frequency of these alleles in each population, with the subsequent loss of rare alleles by chance. These common alleles may then be driving the signature of connectivity and low genetic differentiation between some populations in Pelorus Sound. However, if the survival and regeneration of individuals following seasonal die-back was random, this could contribute to genotypic differentiation within and among some sampling sites, as found by Johnson and Woollacott (2012) for inter-annual genotypic differentiation in colonies of the colonial bryozoan *Bugula stolonifera.*

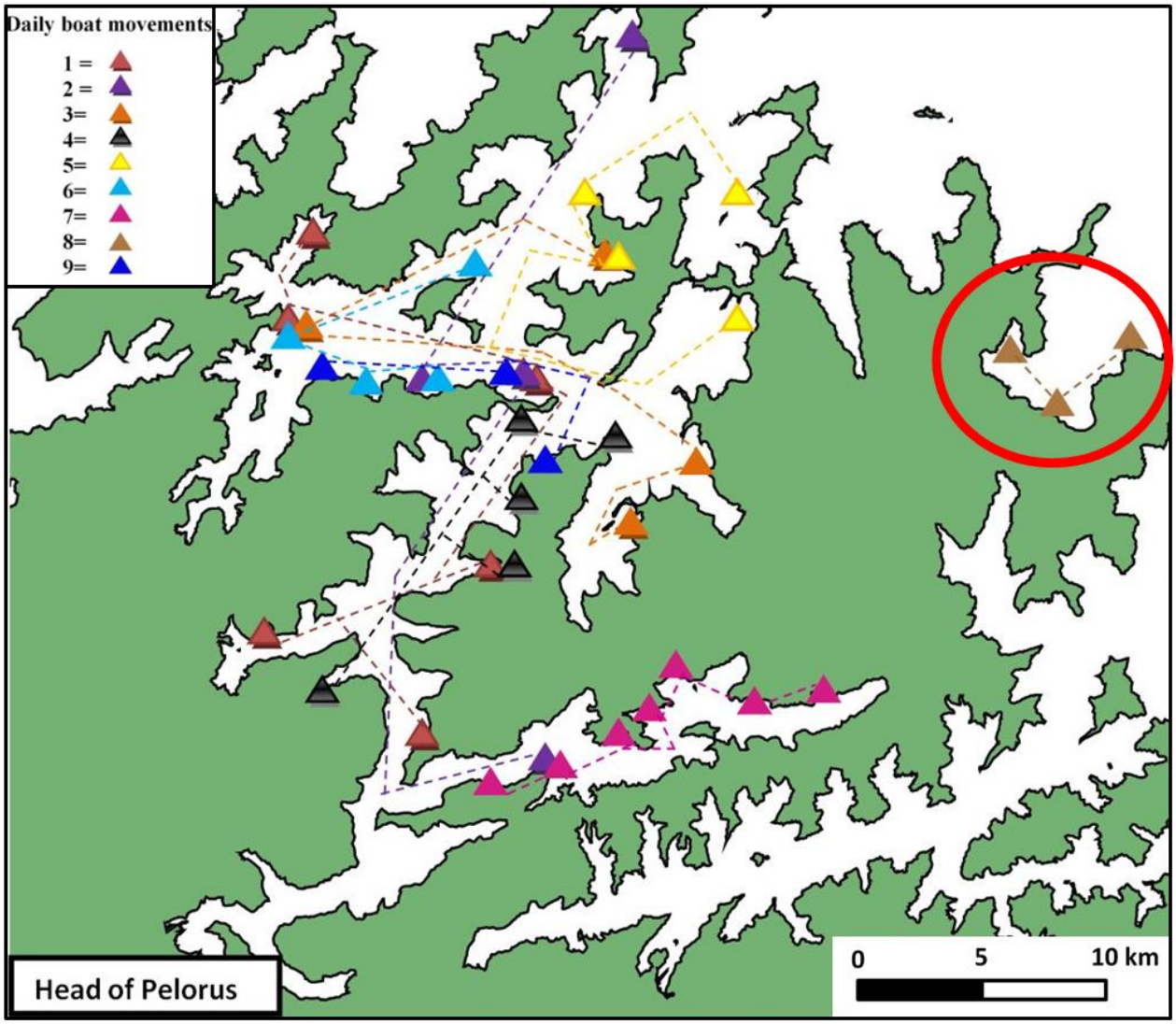

Figure 3.6. Daily movements conducted by the Sanford sourcing vessel over a two week period in January 2013. Triangles denote a new day and dashed lines indicate the movements of that day. Each day began and ended at the head of Pelorus Sound. The red circle represents restricted boat movements.

3.4.7 Concluding remarks

Genetic patterns found in this study show that *D. vexillum* populations in Pelorus Sound were not as clonal as expected, but exhibited extreme outcrossing, genetic substructure and low levels of connectivity. Findings here illustrate the problem of conducting population genetic surveys using a single sampling period. For species that are subject to inter-annual changes in growth and reproduction, such as winter die-back, seasonal changes in population genetic structure may be a major driver of observed genetic patterns. Hence, multiple collections over time are required to fully understand the population dynamics of these types of species and to informatively manage post-border spread. Investigations into fusion rates and the influence of DNA contamination from stored larvae would also increase our understanding of chimeras in nature. This study also demonstrates the need for monitoring vessel movements in high-value areas, such as aquaculture regions, as they can generate multiple incursions, which may enhance NIS genetic diversity or maintain gene flow between populations within these systems.

Differences in population connectivity substantially contribute to spatiotemporal patterns in the distribution of marine pest organisms, and needs to be considered for efficient and effective management strategies (Palumbi 2003; Treml *et al.* 2008). Findings from this study may therefore have important implications for the control of NIS biofouling organisms associated with artificial structures. Using genetic tools, populations were identified which may be self-sustaining and less reliant upon connectivity with nearby populations to persist, indicated by higher average private allelic richness. These populations, such as Melville Cove and Forsyth Bay, are of specific interest because they provide an opportunity for site-specific management. Given that the reproduction and growth of *D. vexillum* populations is strongly correlated with water temperature and degeneration during colder winter months, eradication attempts may be optimal and achievable during these periods when the population's abundance is greatly reduced. These findings may be applicable to other pest species influenced by seasonal regression, warranting further investigation into the use of population genetics within the post-border management of biofouling organisms.

Chapter IV
General Discussion and Synthesis

4.1. A synthesis of biofouling community structure

The occurrence of biofouling is recognised as a significant threat to commercial operations worldwide. For the aquaculture industry, biofouling causes management issues resulting in increased operational expenses and, in some instances, inflicts deleterious impacts on cultured species directly (Denny 2008; Terlizzi & Faimali 2010; Fitridge *et al.* 2012; Fitridge & Keough 2013; Fletcher *et al.* 2013b; Sievers *et al.* 2013). Considering the high impact of biofouling in a growing global industry, sparse information exists around the distribution and dynamics of biofouling communities directly growing on aquaculture infrastructure, and the processes facilitating their regional and local proliferation in New Zealand (Dodgshun *et al.* 2007; Goldstien *et al.* 2010; Woods *et al.* 2012). The research described in this thesis addresses these gaps in knowledge by providing information on the spatial variability of biofouling communities associated with marine farms, and investigating connectivity between populations of *Didemnum vexillum*, a cosmopolitan biofouling organism, growing on artificial structures in the Marlborough Sounds region, New Zealand.

Sampling biofouling assemblages in Pelorus Sound demonstrated distributional patterns in community structure and in the cover of problematic taxa associated with aquaculture structures. Community similarity decreased as the geographical distance between marine farms

increased; the cover of biofouling taxa decreased with increasing depth; and for most of the problematic taxa percentage cover was highest near the entrance of Pelorus Sound. In addition, despite distributional variability, macroalgae and suspension-feeders comprised the dominant component of the biofouling communities that were sampled.

Biofouling accumulation can increase the loading stress and drag forces experienced by floating structures. In this study, biofouling taxa added substantial weight to mussel long-lines in Pelorus Sound, ranging from 4877 $\pm$ 1223 kg (at low levels of estimated biofouling) to 10226 $\pm$ 1713 kg (at high biofouling levels). This additional weight can be detrimental for aquaculture industries, increasing the loss of mussel crops, causing interference with the mechanical handling of equipment and reducing the service lifetime of buoyancy and anchoring systems (Claereboudt *et al.* 1994; Guenther *et al.* 2009; Guenther *et al.* 2010). For example, the added weight of the hydroid *Amphisbetia bispinosa* (known as mussel's beard) increases crop loss during the harvesting of mussel cultures in the south Hauraki Gulf (Heasman & de Zwart 2004). Similarly, in the Marlborough Sounds, excessive weight associated with the over-settlement of blue mussels *Mytilus galloprovincialis* leads to crops sloughing off mussel long-line droppers, especially when lifting lines from the water (observed in this study). Other problematic taxa included the solitary and colonial ascidians (*Ciona intestinalis* and *D. vexillum*), macroalgae, (*Undaria pinnatifida*, *Cladophora* sp., and *Colpomenia* sp.), and tubeworms (*Pomatoceros* sp.).

High spatial variation observed in this study and a dominance (in terms of cover) of suspension-feeders aligns with previous studies of biofouling accumulation on mussel farms in New Zealand (Woods *et al.* 2012), on PVC fouling plates suspended from mussel crop back-bone lines in Australia (Sievers *et al.* 2014), and offshore oil platforms in Western Asia (Stachowitsch *et al.* 2002). Patterns of observed biofouling also support general trends reported for the distribution of biofouling organisms worldwide, including a reduction in biofouling biomass with increasing depth (Cronin *et al.* 1999; Braithwaite *et al.* 2007; Guenther *et al.* 2010; Fitridge

et al. 2012), and evidence of distance-decay relationships, which have been reported for a variety of other freshwater, terrestrial and some marine organisms (Oliva & Teresa 2005; Astorga *et al.* 2012; Bahram *et al.* 2013).

4.2. A synthesis of genetic structure for a biofouling pest

Microsatellite analysis on *D. vexillum* populations revealed metapopulation dynamics, with significant population structure, heterozygote excess and outcrossing (mating between non-relatives) in the Marlborough Sounds region. These findings were in contrast to those reported from a previous analysis of *D. vexillum* populations in New Zealand (Smith *et al.* 2012), and other studies on colonial (Ben-Shlomo *et al.* 2001) and solitary (Goldstien *et al.* 2010) ascidians in New Zealand and overseas (Rinkevich *et al.* 2001; Stoner *et al.* 2002; Paz *et al.* 2003), which found heterozygote deficiency and evidence of inbreeding among colonial ascidian populations.

Due to small size and weak swimming capabilities of most marine larvae, it has been argued that given oceanographic advection, dispersal potential is largely determined by the length of a species pelagic stage (Scheltema 1971; Eckert 2003; Shanks *et al.* 2003). Accordingly, recent reviews and general ecological theory suggest that pelagic larval duration (PLD) can be a good predictor of the magnitude of gene flow and geographical scale of population structure in marine systems (Jablonski 1986; Doherty *et al.* 1995; Bohonak 1999), especially when combined with oceanographic data (Largier 2003; Diehl *et al.* 2007). In this study, PLD and water current movements (combined for basic GIS-modelling) did not explain genetic variability. Therefore, the movement of artificial structures, such as commercial vessels or stock transfers, may play a more prominent role in genetic and population structure. This supports the widely held view that human-mediated movements contribute more to species spread than natural dispersal, which is often restricted by oceanographic barriers, increasing the need for vector management (Dodgshun *et al.* 2007; Forrest *et al.* 2009).

Although the movement of aquaculture stock and vessels (commercial and recreational) may be important for examining biofouling patterns and genetic structure in this system, a range of

other interacting variables are likely to be at play. For example, in classical successional theory there is a well-known, though highly debated notion that communities develop toward a stable 'climax' community over time (Clements 1916; Allee *et al.* 1949). However, in aquaculture environments, operations generate high levels of disturbance (i.e., through the stripping and reseeding of crop ropes), which renew limiting resources and promote local co-existence, preventing the potential attainment of a climax community. Community structure across the Pelorus Sound may therefore be largely governed by the chance timing of larval availability within the water column and the timing of local disturbances, as well as the random regeneration of species following disturbance events (Petraitis *et al.* 1989). Additionally, variation in the environmental and physical conditions across Pelorus Sound (i.e., site-specific variations in temperature, turbidity, water flow and wave action), as described by Gibbs *et al.* (1991) and Heath (1982), are likely to interact with the impacts of localised disturbance, selecting for more tolerable species to certain conditions and for the survival of site-specific alleles (private alleles).

4.3. Potential study limitations

More confidence in the findings of this study could be generated from conducting long-term temporal sampling, which would account for inter-annual variation in this system. Seasonal variation in species growth and reproduction would then be accounted for, which is important for species such as the colonial ascidian *D. vexillum* (Valentine *et al.* 2007a; Fletcher *et al.* 2013a), and the macroalga *U. pinnatifida* (Hay & Villouta 1993). Monitoring vessel and industry movements within this area, as well as implementing the use of a full hydrodynamic model may also enable a clearer understanding of the different roles that vectors (artificial and natural) play in connecting biofouling communities. In addition, the physical collection of biofouling taxa would enable higher taxonomic resolution and more certainty in species identification than can be attained from images alone. In this study, the goal was to capture information along the length of Pelorus Sound at multiple lines within farms, at two depths. Therefore, the physical

collection of samples was not practically feasible, nor was the development of a full hydrodynamic model given the time requirements of this thesis.

Using the tools available (i.e., photographic processing, genetic analyses and simple GIS-based modelling), this study has provided baseline information for informing management practice by marine farmers and for future research. It also supports the notion that research tools can be integrated to provide new insight into the spatial ecology of marine populations (Galindo *et al.* 2006; Galindo *et al.* 2010; Selkoe *et al.* 2010; Coscia *et al.* 2013).

4.4. Integrating ecological, genetic and oceanographic tools

This study represents the first attempt to use a combination of research approaches, including simple GIS-based modelling and genetic analyses, to provide insight into the spatial distribution and connectivity of biofouling organisms within an aquaculture system in New Zealand. The application of these 'multidisciplinary approaches' is especially important for marine species, as their large population sizes can resist genetic divergence, reducing the statistical power to detect population structure (Selkoe *et al.* 2008; Selkoe *et al.* 2010). The integration of genetic, oceanographic, behavioural and modelling approaches have already provided important insights into the patterns, causes and consequences of population structure and connectivity for many marine populations, guiding new approaches within fisheries management and for the design of marine protected areas (Callow & Callow 2002; Galindo *et al.* 2006; Stenseth *et al.* 2006; Selkoe *et al.* 2008). For example, Selkoe *et al.* (2010) used a combination of genetic analysis, environmental data and oceanographic modelling to show that a single spatial management strategy could be used to effectively protect genetic diversity of multiple species in the Southern California Bight, including the kelp bass *Paralabrax clathratus*, Kellet's whelk *Kelletia kelletii* and California spiny lobster *Panulirus interruptus* .

The value of integrating approaches in an aquaculture system is that information can advise the placement of susceptible stock (e.g., spat) and aquaculture infrastructure, so that distances between marine farms align not only with the occurrence of pest species, but with their

dispersal capabilities. In addition, this approach would be particularly suitable for aquaculture industries and other nodes of high shipping activity, such as ports and marinas, given that biofouling communities are often dominated by non-indigenous species (NIS) (Lambert & Lambert 1998; Tyrrell & Byers 2007). The careful placement of artificial structures, such as marine farms or buoys, may further reduce levels of localised natural spread. However, methods for monitoring biofouling or assessing connectivity would need to be modified to make them appropriate (i.e., more practical) for application within an industry setting.

4.5. Industry implications

4.5.1 Managing high risk species

In aquaculture, collecting information on biofouling patterns could better inform the timing and placement of husbandry practices, such as the stripping of mussel crop lines or placement of sensitive (younger-aged) spat, by outlining specific areas that may be more susceptible to the occurrence and proliferation of pest organisms (Fitridge & Keough 2013; Sievers *et al.* 2014). For example, if spat holding areas were placed away from the entrance of Pelorus Sound in deeper waters, this may avoid heavy settlement by notoriously detrimental species such as the brown alga *Colpomenia* sp., which creates problems for spat retention in the Marlborough Sounds (Dan McCall, SpatNZ pers. comm.), or the hydroid species *A. bispinosa*, which renders mussels unsuitable for half-shell trade (Heasman & de Zwart 2004). However, temporal variation in the cover of problematic species in Pelorus Sound would first need to be assessed before such advice could be confidently given.

Biofouling patterns could also be used to inform the management of other high risk organisms, such as diseases and parasites. Biofouling communities can serve as a reservoir for pathogenic microorganisms harboured by macrofouling species or microbial assemblages growing on finfish cages and other artificial structures (Braithwaite & McEvoy 2004; Dürr & Watson 2010). For example, *Prorocentrum lima* is a toxic dinoflagellate responsible for diarrhetic shellfish poisoning (DSP) in humans which has been shown to be associated with the filamentous brown

alga *Ectocarpus* sp. (Dürr & Watson 2010). Amoebic gill disease (AGD), which is a major health issue for salmon-farming caused by *Neoparamoeba pemaquidensis,* has also been associated with biofouling, specifically by the bryozoan *Scrupocellaria bertholetti* and solitary ascidian *C. intestinalis,* which act as vectors and reservoirs for this amoeboid protozoa (Tan *et al.* 2002).

4.5.2 Development of a biofouling scale

Using categorised levels to assess biofouling biomass in this study proved to be a reliable approach, generating increased wet weights (kg) with an associated increase in the categories of biofouling cover (low ≤ 30%, medium 31 – 70%, or high ≥ 71% cover). If modified, this method could be applied within an industry setting by marine farmers and contractors (e.g., mussel-sourcing boat staff). To achieve this, expert opinion-based approaches could be used to elicit industry's perception of what makes biofouling communities more or less 'pesty'. For example, a workshop could be undertaken by knowledgeable industry members (i.e., those working within the field, experiencing biofouling first-hand) and biosecurity ecologists. During this workshop, images displaying different levels of biofouling could be presented to industry members for pairwise comparisons. Images capturing the greatest levels of problematic fouling could then be selected by industry members and used to inform the construction of a biofouling scale, ranging from one to five, in accordance to increasing levels of 'pestiness'. Pairwise comparisons would be repeated numerous times to cover the range of biofouling assemblages that occur in an industry setting, and would cover the various stages of industry production (e.g., seeding, re-seeding, grow-out to market size, harvesting and marketing).

Following the workshop, images could be processed to attain the percent cover of different organisms (e.g., using CPCe methods, as discussed in Chapter 2) and determine the level of biofouling cover that equates to problematic biofouling for industry operations. This biofouling scale could be rapidly applied during farm maintenance and harvesting, providing industry members and scientists with a long-term record of yearly trends in the cover of biofouling taxa. These data could be continually uploaded to an online database, as suggested by Sievers *et al.*

(2014), and used to better inform overall industry practices, as is already undertaken for the detection of harmful phytoplankton (Trainer *et al.* 2003).

4.5.3 Applying GIS-based modelling to identify 'isolated' areas

GIS-based modelling used in this study, which incorporated larval competency periods and water currents, identified areas in Pelorus Sound which appeared to be reasonably 'isolated' from other areas across different PLD times (e.g., areas near the entrance of Pelorus Sound such as Melville Cove). Using this information, or information from manipulating the model to represent the potential connectivity of a wider range of short-dispersing species, lower connectivity between specific areas could be achieved by strategically removing potential "stepping-stone" farms (as indicated by the model). However, given that natural population spread is often restricted by physical or oceanographic barriers, the management of commercial vessel movements and aquaculture stock transfers has been recommended as a more effective management tool (Forrest *et al.* 2009). Vector and operational management is also particularly important for aquaculture industries as existing practices (i.e., declumping and washing biofouling from mussel long-lines) can enhance the dispersal of biofouling species, evident from the fragmentation and resultant spread of *D. vexillum* colonies and *U. pinnatifida* sporophytes in New Zealand (Forrest & Hopkins 2013).

Nevertheless, efforts to manage local biofouling communities in high-transport areas rely upon management processes at a national level. For example, managing aquaculture vessels and the movement of equipment could be seen as futile if heavily-fouled barges and other vessels were still frequently moving between areas with high levels of problematic biofouling (in accordance to the biofouling scale) or between areas which would otherwise be naturally isolated (in accordance with findings from GIS-based modelling). Biosecurity efforts in such regions would therefore need to be legislated by national government with coordination at the council and national government level. The need for regional pathway management is already part of the current changes being made to the Biosecurity Act, with local councils now responsible for

biosecurity management in their own backyard, and for the development of regional pathway management plans (Sinner *et al.* 2013). Synchronised management between local and governmental agencies, in order to reduce the spread of harmful pests, is not only important within the marine environment, but also applies to freshwater and terrestrial habitats.

4.6. Concluding remarks

While it may not be possible to eradicate biofouling species entirely, reducing their spread and impacts is imperative for aquaculture growers, and for the management of other artificial structures (Getchis 2006). It is well known that biofouling communities vary temporally and spatially (Fitridge & Keough 2013; Sievers *et al.* 2014), therefore efficiency in the management of marine pests requires a thorough understanding of their distribution and potential for spread. The integration of ecological monitoring, genetic and mathematical modelling tools provide the opportunity to gain new insights into the spatial ecology of biofouling communities, as demonstrated in this thesis. Findings have contributed to a growing range of literature investigating the accumulation and spread of marine pests associated with artificial structures by: (1) characterising biofouling assemblages associated with marine farms in Pelorus Sound, demonstrating clear distributional patterns in the cover of biofouling communities and marine pests, and (2) identifying patterns of connectivity and gene flow trajectories among populations of the biofouling colonial ascidian *D. vexillum*, demonstrating genetic structure and low levels of gene flow. Communication of these data and the exploration or application of the suggested tools (e.g., a biofouling scale or fine scale use of GIS-based modelling) may enable managers to prioritise which biofouling pests and what areas should receive management efforts, as well as when management applications may be most effective (i.e., aligned with low levels of cover of pest species). Provided local biosecurity efforts can be magnified at a national level, the results of such endeavours could provide more information for the development of guidelines for pest species management, and better inform efforts to control vectors and anthropogenic pathways that assist in the spread of pests in marine, freshwater and terrestrial systems.

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Appendices

Appendix I: CTD cast results

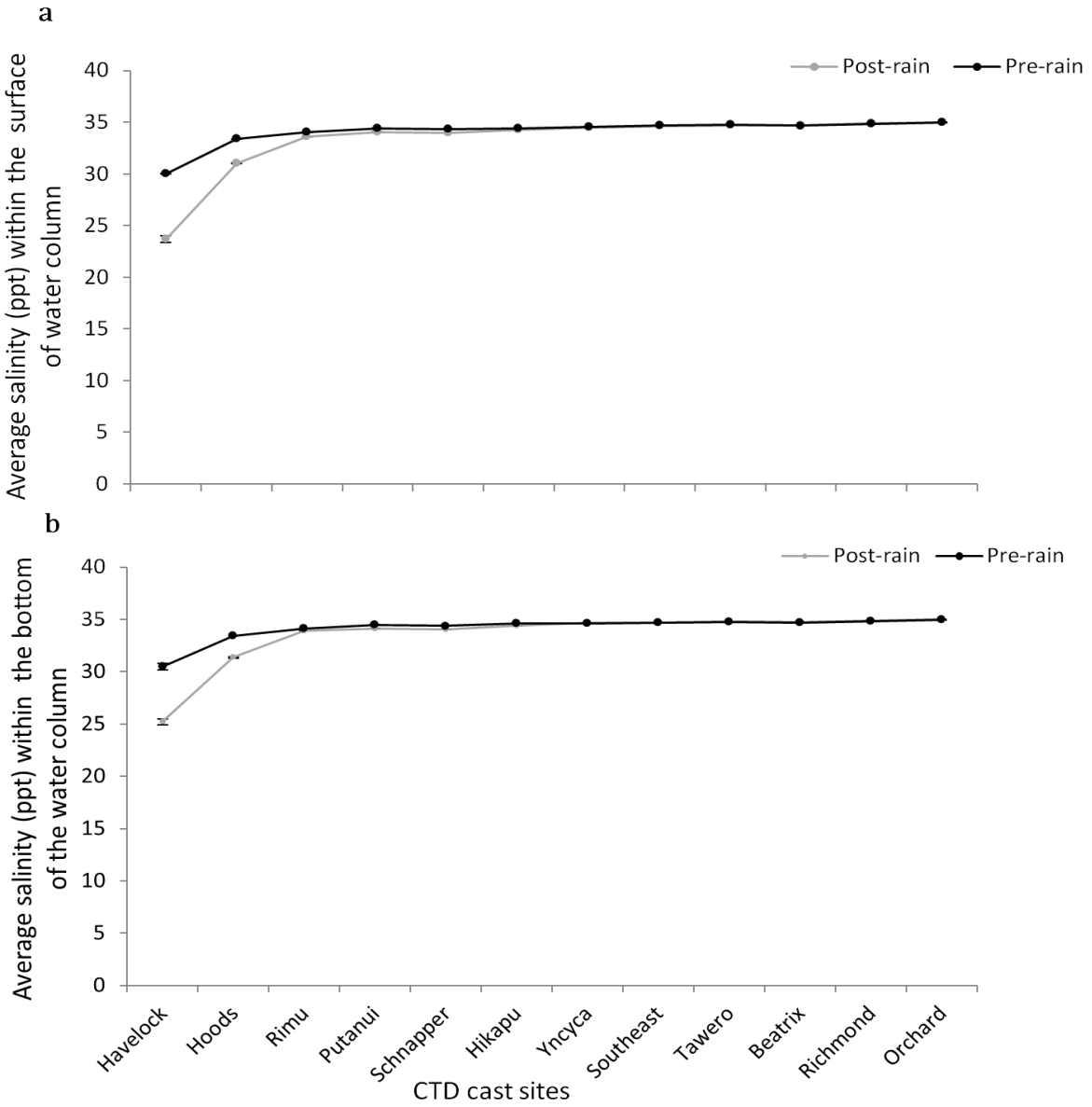

Figure A1.1. Average salinity (±SE) across 12 CTD cast sites within the surface 3 m of the water column (a) and within the bottom 3 m of the water column (b). Cast sites included areas near the head of Pelorus Sound (Havelock Marina, Hoods and Rimu Bay) to the entrance of Pelorus Sound (Richmond and Orchard Bay).

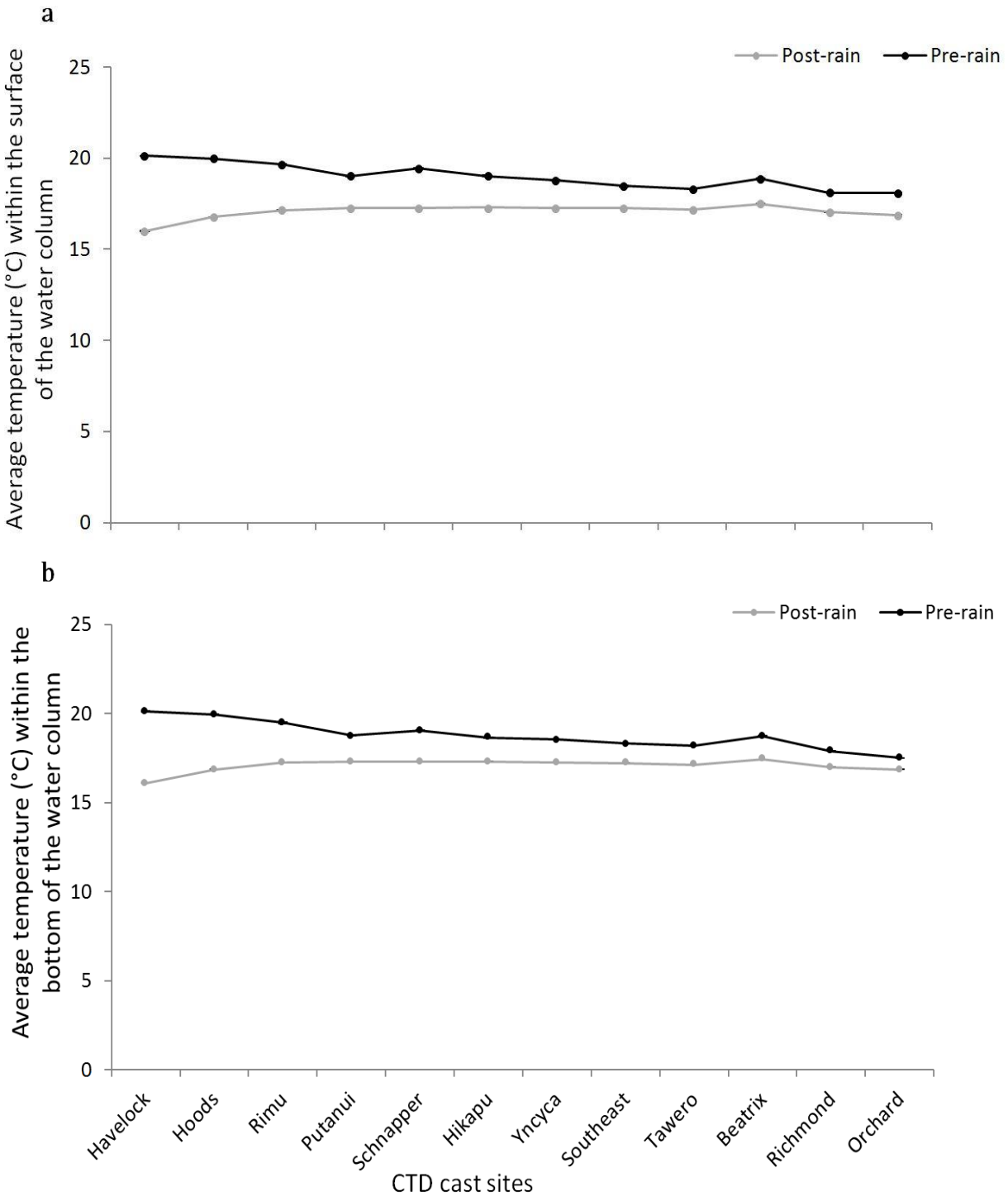

Figure A1.2. Average temperature (±SE) across 12 CTD cast sites within the surface 3 m of the water column (a) and within the bottom 3 m of the water column (b). Cast sites included areas near the head of Pelorus Sound (Havelock Marina, Hoods and Rimu Bay) to the entrance of Pelorus Sound (Richmond and Orchard Bay).

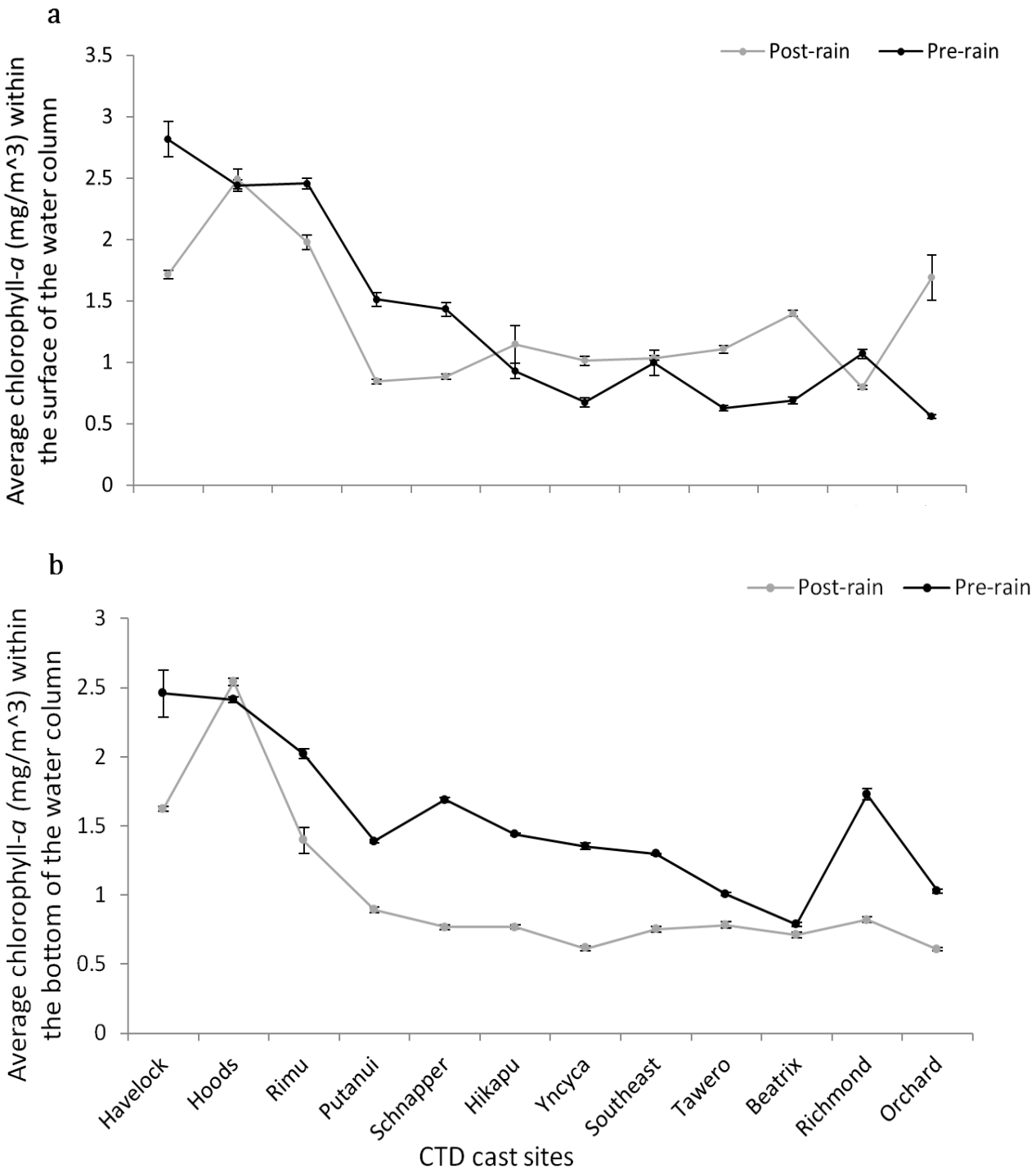

Figure A1.3. Average chlorophyll-*a* (±SE) across 12 CTD cast sites within the surface 3 m of the water column (a) and within the bottom 3 m of the water column (b). Cast sites included areas near the head of Pelorus Sound (Havelock Marina, Hoods and Rimu Bay) to the entrance of Pelorus Sound (Richmond and Orchard Bay).

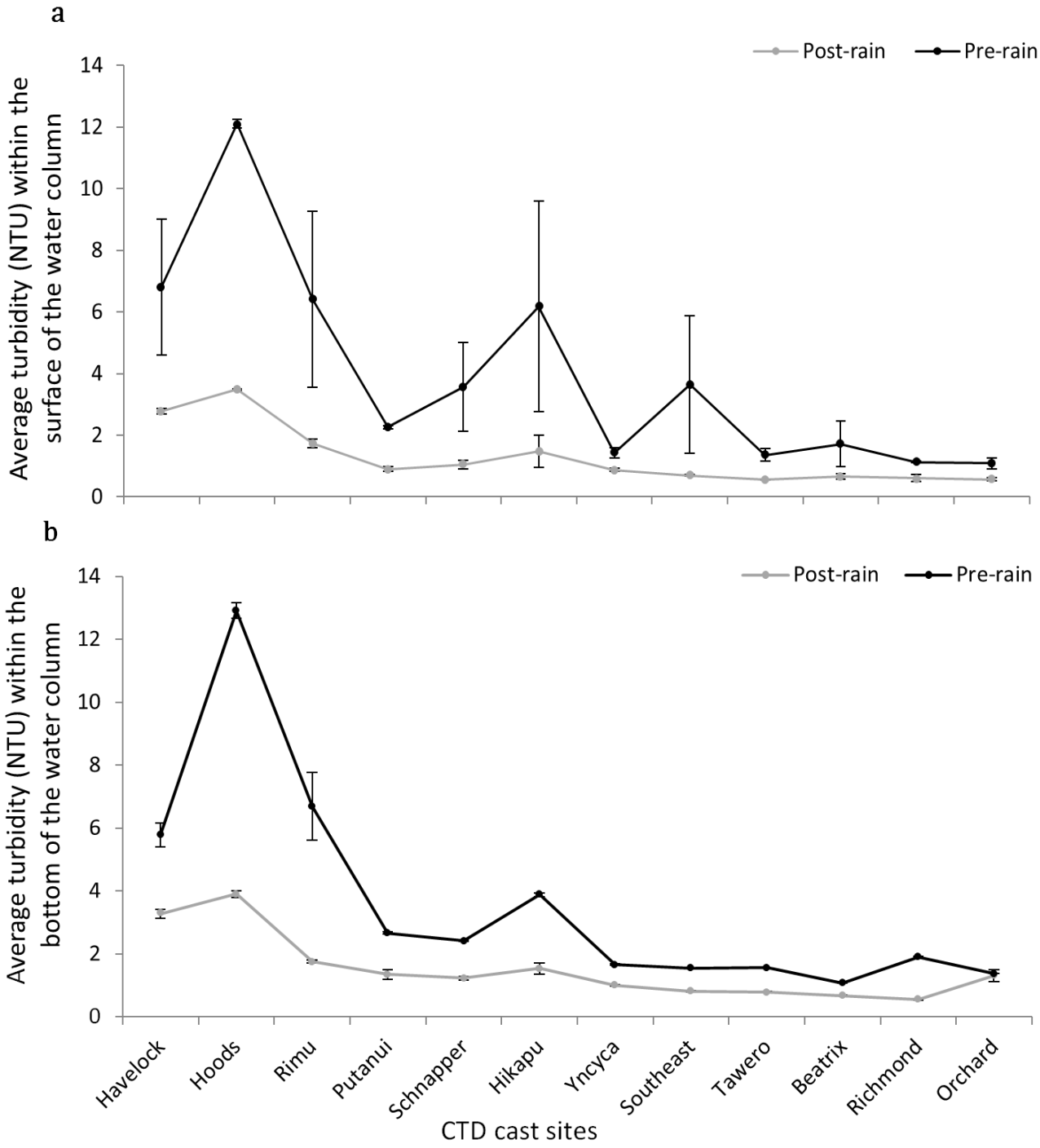

Figure A1.4. Average turbidity (±SE) across 12 CTD cast sites within the surface 3 m of the water column (a) and within the bottom 3 m of the water column (b). Cast sites included areas near the head of Pelorus Sound (Havelock Marina, Hoods and Rimu Bay) to the entrance of Pelorus Sound (Richmond and Orchard Bay).

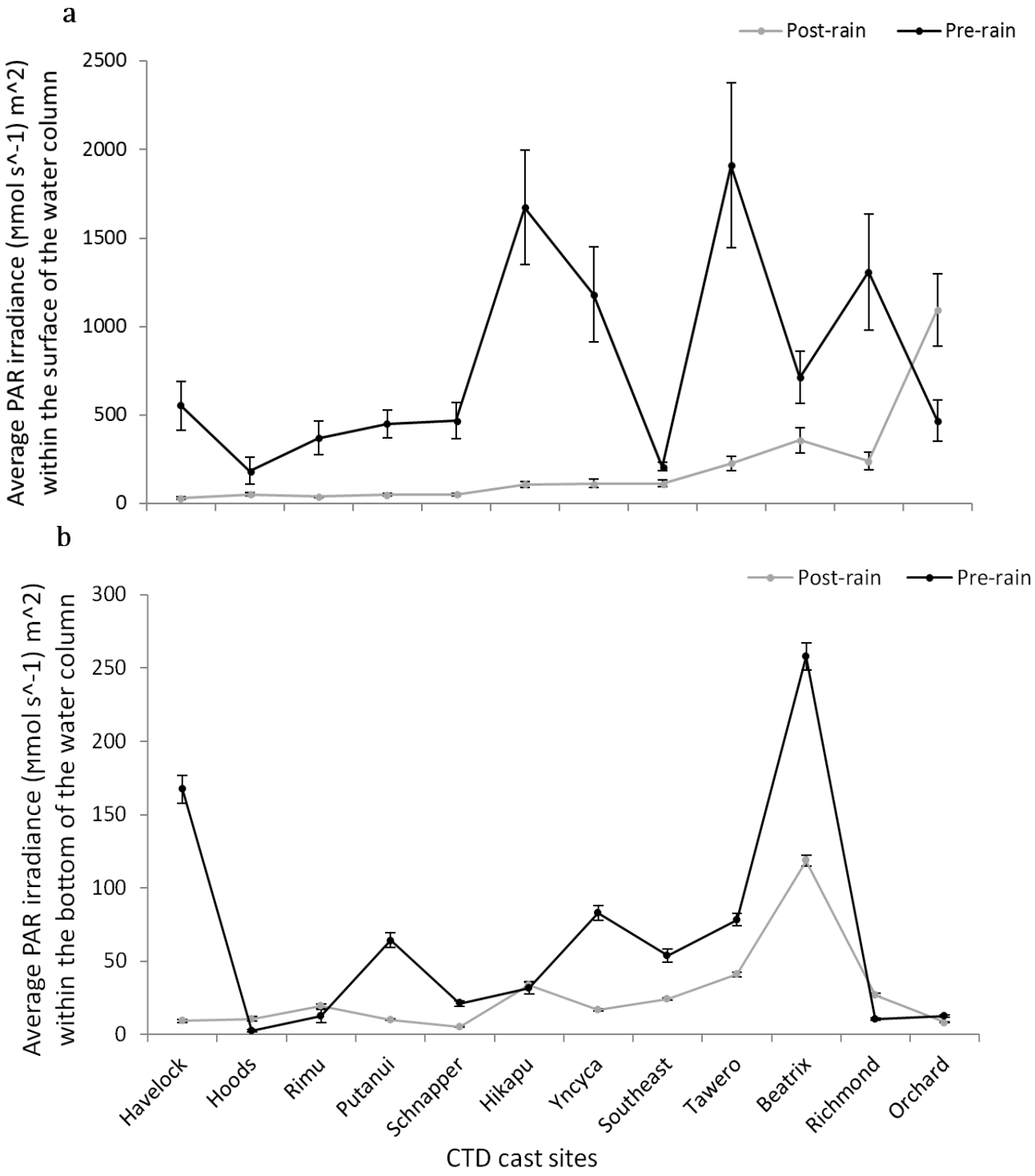

Figure A1.5. Average PAR irradiance (±SE) across 12 CTD cast sites within the surface 3 m of the water column (a) and within the bottom 3 m of the water column (b). Cast sites included areas near the head of Pelorus Sound (Havelock Marina, Hoods and Rimu Bay) to the entrance of Pelorus Sound (Richmond and Orchard Bay).

Appendix II: CPCe optimisation

A2.1 Methods

Percent cover and taxa richness of biofouling on mussel long-lines in Pelorus Sound were determined from photoquadrats using Coral Point Count with excel extension (CPCe) (Kohler & Gill 2006). Prior to processing the images from the main study, four random images were selected and preliminary analyses were undertaken in CPCe to determine: (1) whether point count analysis reflected the 'true' percent cover of selected taxa, which was represented by area-length analyses, and (2) whether increasing the number of points per image significantly altered the precision, accuracy and efficacy of the information gained from point count analyses. Ascidians and bryozoans were selected as they were the two dominant biofouling taxa in the images and represent different forms of biofouling coverage (i.e., clumped and homogeneous).

To compare the percent cover for ascidians and bryozoans attained by point count analysis with the 'true' percent cover, paired two sample t-tests were conducted in R v.3.0.2 (R Core Team 2013). To determine differences in the average cover of dominant biofouling taxa when using a different density of points per image (10-100 points) and differences in precision, accuracy and time efficiency, a series of univariate permutation analyses of variance (ANOVAs) were conducted in PRIMER 6 with PERMANOVA add-on (Clarke & Warwick 2001; Anderson *et al.* 2007). Precision was measured by the ratio of standard error divided by the mean, ($SE/\bar{x}$) (Andrew & Mapstone 1987). Prior to analyses, the distribution of percent cover data was examined for departures from normality and homogeneity. Data were transformed to achieve approximately unimodel symmetry and avoid right skewness. Using a distance-based approach, Euclidean distances were selected when running univarate permutation analyses of variance and 4999 non-restricted permutations of the appropriate units were used under a reduced model. A Type I SS was employed, although as the data was balanced the order in which factors entered the model did not affect the analyses (Anderson *et al.* 2007). No pairwise comparisons were required.

A2.2 Results

There was no significant difference between the percent cover estimates of the dominant biofouling organisms (i.e., ascidians and bryozoans) using point count analysis compared with area-length analyses in CPCe (Table A2.1). However, area-length analysis was more time consumptive. When using point count analysis, there was no significant difference between mean cover estimates for ascidians and bryozoans using different densities of points per image (Table A2.2), nor was there a significant difference in the accuracy and precision of these estimates. Precision is inversely related to the values of the ratios used to measure it and it is greatest when the standard error (SE) is small relative to the mean ($\bar{x}$) (Andrew & Mapstone 1987). Investigations into precision values indicated that photoquadrats sampled using 50 or more points gave the most precise estimates for species cover of ascidians and bryozoans (Figure A2.1). Furthermore, variation around percent cover estimates was larger when using less points per image, with the lowest variation occurring at 50 points (Figure A2.2). When comparing cover estimates produced from 50 or more points (50, 75 and 100 points), there was no significant difference in the estimated cover of ascidians and bryozoans (Table A2.3). However, using 50 points per image produced a consistent overestimate of species cover for ascidians and bryozoans (Figures A2.3a, b) and using 100 points per image in isolation was time consumptive. In contrast, using 75 points per image did not produce a systematic bias, both underestimation and overestimation occurred (Figures A2.3a, b), and points covered a large area across the image, reducing the potential for clumping and accounting for patchiness in the distribution of species across the image. Moreover, using 75 points per image (as adopted in this study) was more time efficient and just as precise as using a larger number of points.

Table A2.1. Results from paired two samples t-tests for differences in the percent cover of ascidians and bryozoans in four photographs, comparing true species cover produced from area-length analyses to estimated species cover produced using point count analyses in CPCe. The means (M), standard deviations (SD), *t*-statistics (t) and *P*-values (*P*) are shown. Significance was set at $P<0.05$.

Taxa	Photo ID	Points	M	SD	*t*	*P*
(a) Ascidians	1	50	19.00	12.00	3.18	0.09
		75	14.35	10.03	3.18	0.70
		100	16.50	3.67	3.18	0.42
	2	50	42.00	34.67	3.18	0.72
		75	45.00	38.52	3.18	0.29
		100	43.00	18.00	3.18	0.42
	3	50	27.00	22.67	3.18	0.19
		75	24.33	21.33	3.18	0.36
		100	21.50	3.67	3.18	0.36
	4	50	19.00	17.33	3.18	0.53
		75	20.33	7.62	3.18	0.15
		100	17.75	8.25	3.18	0.85
(b) Bryozoans	1	50	35.00	14.67	3.18	0.54
		75	38.65	5.90	3.18	0.06
		100	35.75	8.25	3.18	0.35
	2	50	20.50	11.67	3.18	0.34
		75	15.15	25.62	3.18	0.33
		100	19.75	4.92	3.18	0.39
	3	50	24.50	19.67	3.18	0.06
		75	7.62	14.22	3.18	0.18
		100	25.25	40.92	3.18	0.18
	4	50	30.50	9.00	3.18	0.49
		75	31.65	91.90	3.18	0.97
		100	30.25	14.92	3.18	0.44

Table A2.2. Results from two one-way analysis of variance tests (ANOVAs) testing for differences in the percent cover of ascidians and bryozoans in four photographs, using a different number of random points per image (10-100). Means sums of squares (MS), *F*-statistics (*F*) and *P*-values (*P*) are included. Significance was set at $P<0.05$.

Taxa	Source of variation	*df*	MS	*F*	*P*
Ascidians	Points per image	9	11.15	0.20	0.99
	Residuals	30	56.76		
Bryozoans	Points per image	9	15.70	0.19	0.99
	Residuals	30	83.95		

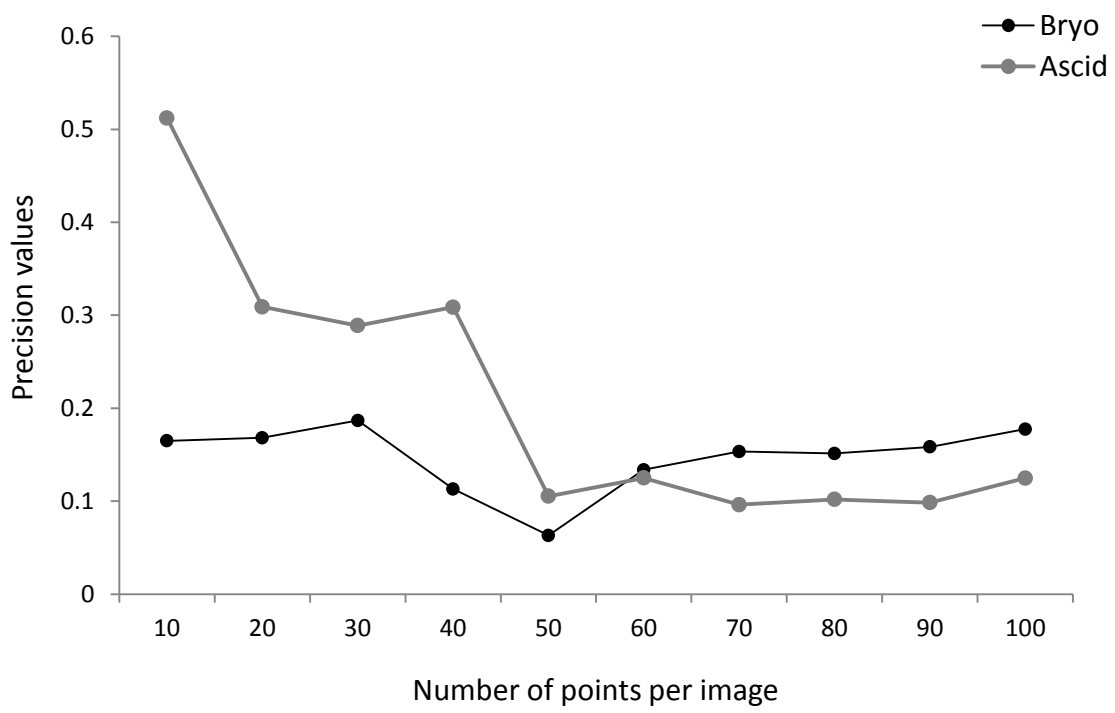

Figure A2.1. Precision estimates of the percent cover of ascidians (Ascid) and bryozoans (Bryo) using different numbers of random points per image (10-100) in point count analysis in CPCe. Precision values were calculated as $[SE/\bar{x}]$ of percent cover estimates using various numbers of random points.

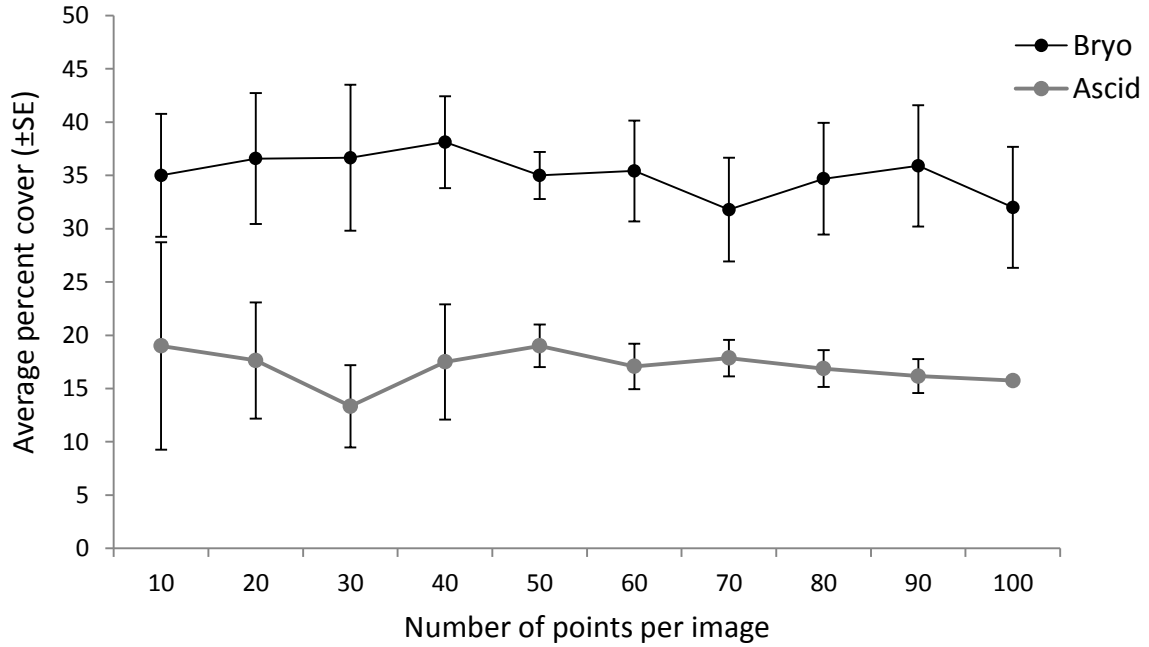

Figure A2.2. Average percent cover (± SE) of two biofouling taxa, bryozoans (Bryo) and ascidians (Ascid), as a function of the number of random points used per image in CPCe.

Table A2.3. Results from two separate one-way analysis of variance tests (ANOVAs) testing for differences in the percent cover of ascidians and bryozoans in four photographs, using different numbers of points per image (50, 75 and 100). Mean sums of squares (MS), *F*-statistics (*F*) and *P*-values (*P*) are included. Significance was set at $P<0.05$.

Taxa	Source of variation	*df*	MS	*F*	*P*
(A) Ascidian cover	Points per image	2	29.14	0.73	0.51
	Residuals	9	39.73		
(B) Bryozoans cover	Points per image	2	4.30	0.03	0.97
	Residuals	9	149.40		

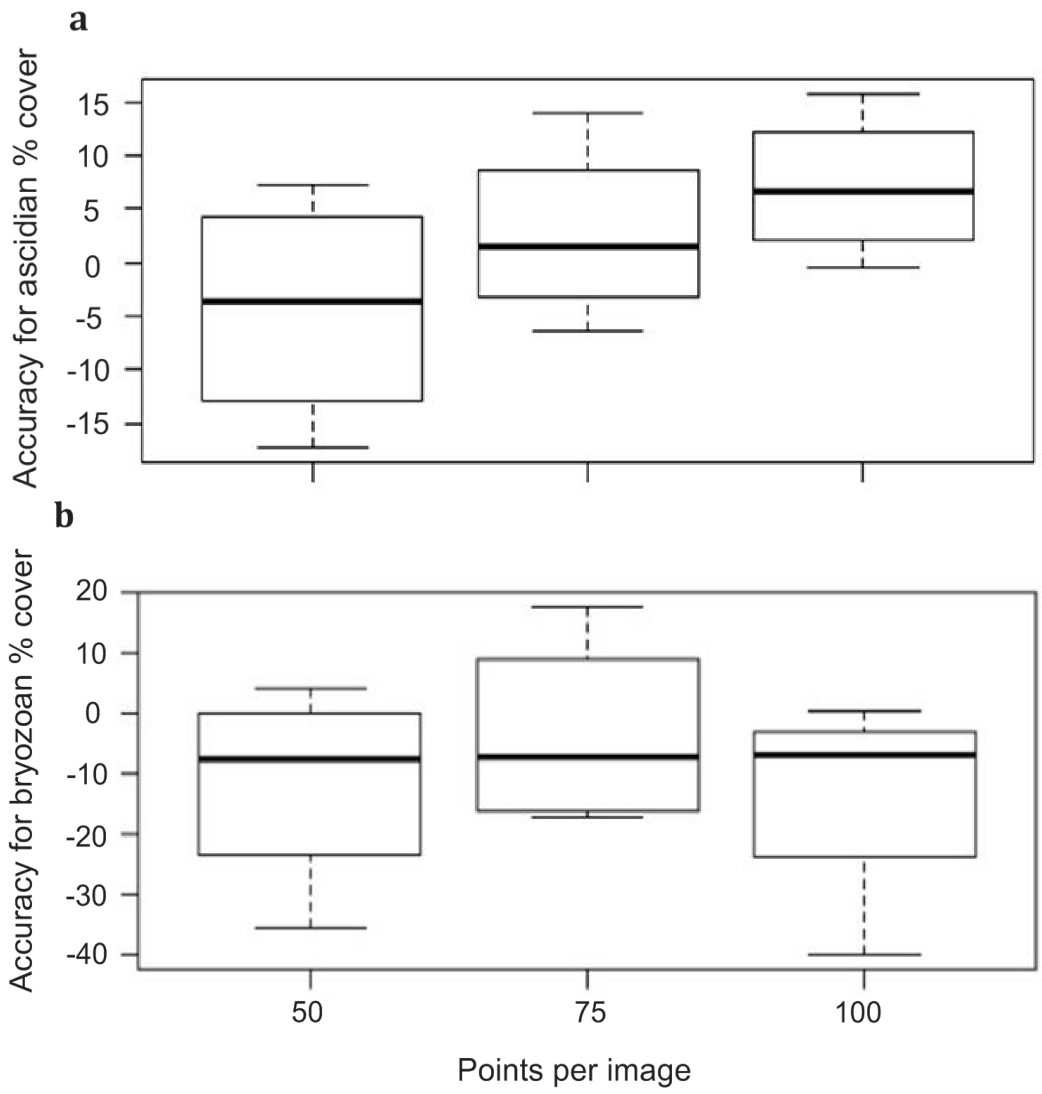

Figure A2.3. Accuracy estimates of the percent cover of ascidians (a) and bryozoans (b) using different numbers of random points per image (50, 75, and 100). Accuracy was calculated as [estimated % cover – true % cover]. Negative accuracy numbers indicate an overestimation of species cover (compared to the 'true' value obtained from area-length analyses), while positive accuracy values indicate an underestimation.

Appendix III: Data results

A3.1 Univariate Data

Table A3.1. Results of the two-way univariate permutational ANOVA testing for differences in taxonomic richness (rarefied, *N*=6), at scales of area and depth. Mean sums of squares (MS) and *F*-statistics (*F*) are included.

	Taxonomic Richness		
Source of variation	***df***	**MS**	***F***
Depth	1	409.16	2.67
Area	2	495	1.3
Farm(Area)	70	352.79	2.37***
Depth x Area	2	286.88	1.89
Long-line (Farm(Area))	52	149.14	2.74***
Depth x Farm(Area)	70	141.09	1.29
Depth x Long-line (Farm(Area))	52	109.57	2.01***
Residual	512	54.523	
Total			

***P<0.05;**P<0.01 and ***P<0.001**

Table A3.2. Results of the two-way univariate permutational ANOVA testing for differences in taxonomic evenness, at scales of area and depth, and the associated pairwise comparisons. Mean sums of squares (MS), *F*-statistics (*F*) and pairwise *t*-statistics (*t*) are included.

		Evenness	
Source of variation	***df***	**MS**	***F***
Depth	1	881.75	8.96***
Area	2	47.142	0.31
Farm(Area)	70	164.79	1.95***
Depth x Area	2	272.43	2.81*
Long-line (Farm(Area))	52	84.517	1.96***
Depth x Farm(Area)	70	88.803	1.51
Depth x Long-line (Farm(Area))	52	58.717	1.36*
Residual	512	43.185	
Total			

Pairwise comparisons

Groups	***t***
Inner	
Lower - Upper	0.45747
Middle	
Lower - Upper	2.5053*
Outer	
Lower - Upper	2.2631*

***P<0.05;**P<0.01 and ***P<0.001**

A3.2 Multivariate Data

Table A3.3. Results of the multivariate PERMANOVA based on Bray-Curtis dissimilarities for spatial differences in community structure (square root transformed), at the scales of area and depth, with age as a covariate. Pairwise comparisons for the depth x area interaction effect, within factor area, are included. Estimates of multivariate variation (variation %), the estimated sizes of average similarities between areas (AS), mean sums of squares (MS), *F*-statistics (F) and pairwise *t*-statistics (t) are also included.

Source of variation	*df*	MS	*F*	Variation (%)
Age	1	24058	3.95***	5
Depth	1	45716	19.35***	11
Area	2	74469	9.58***	16
Farm(Area)	70	6933	2.70***	21
Depth x Area	2	7411	3.19***	6
Long-line(Farm(Area))	52	2531	3.31***	17
Depth x Farm(Area)	70	2108	1.57***	12
Depth x Long-line(Farm(Area))	52	1346	1.76***	14
Residuals	511	766		28

Pairwise comparisons

Groups	*t*	AS (%)
Surface depth		
Inner - Middle	3.19***	41
Inner - Outer	3.06***	36
Middle - Outer	1.56***	43
Bottom depth		
Inner - Middle	3.48***	37
Inner - Outer	3.13***	33
Middle - Outer	1.82***	43

*$P<0.05$,**$P<0.01$ and *** $P<0.001$

Table A3.4. Results from the test for differences in multivariate dispersion (PERMDISP) of the depth x area interaction effect, with the associated pairwise comparisons for dispersion among and within areas, across depths. Analyses were based on Bray-Curtis dissimilarities and data were square root transformed.

Source of variation	*F*
Depth x Area	11.587***

Pairwise comparisons

Groups	*t*
BottomInner - SurfaceInner	4.44***
BottomInner - SurfaceMiddle	6.26***
BottomInner - BottomMiddle	6.28***
BottomInner - BottomOuter	2.24*
BottomInner - SurfaceOuter	1.75
SurfaceInner - SurfaceMiddle	1.84
SurfaceInner - BottomMiddle	1.70
SurfaceInner - BottomOuter	1.74

SurfaceInner - SurfaceOuter	1.94
SurfaceMiddle - BottomMiddle	0.21
SurfaceMiddle - BottomOuter	3.39***
SurfaceMiddle - SurfaceOuter	3.47***
BottomMiddle - BottomOuter	3.36***
BottomMiddle - SurfaceOuter	3.44***
BottomOuter - SurfaceOuter	0.3

*$P<0.05$;**$P<0.01$ and *** $P<0.001$

Table A3.5. Results from univariate permutational ANOVAs testing for differences in the percent cover (square root transformed) of the most prominent taxa contributing consistently to dissimilarities between areas in Pelorus Sound, across depths. Mean sums of squares (MS) and *F*-statistics (*F*) are included.

		SIMPER - Contributing taxa											
		Bryozoa		Hydroid sp-1		*M. galloprovincialis*		*Porphyra* sp.		Red algae		*U. pinnatifida*	
Source	*df*	MS	*F*	MS	*F*	MS	*F*	MS	*F*	MS	*F*	MS	*F*
Depth	1	1762.0	26.78***	96.5	0.8	9008.5	96.5***	184.6	2.5	4590.2	42.5***	1839.4	20.4***
Area	2	2206.5	33.54***	16032.8	133.3***	1051.6	11.3***	1424.4	19.3***	10828.1	100.3***	315.8	3.5*
Depth x Area	2	234.9	3.6**	440.8	3.6**	627.2	6.7**	35.6	0.5	29.4	0.3	16	0.2
Residual	756	49734.0		120.2		93.4		73.7		108		90.4	

Note: $^{**}P<0.01$, $^{***}P<0.001$.

Table A3.6. Results from univariate permutational ANOVAs testing for differences in the percent cover of the taxa (square root transformed) with a correlation >0.3 with the PCO, between areas in Pelorus Sound, across depths**.** Mean sums of squares (MS) and *F*-statistics (*F*) are included.

	Correlation taxa (>0.3)			
	Aplidium sp.		*Pomatoceros* sp.	
Source	MS	*F*	MS	*F*
Depth	203.1	11.6***	132.2	16.2***
Area	631.1	36.0***	697.4	85.5***
Depth:Area	88.7	5.1**	8.2	23.1***
Residual	17.5			

Note: $^{**}P<0.01$, $^{***}P<0.001$.

Table A3.7. Results of Mantel tests showing the slope (b), Pearson correlation coefficient (r) and *P*-values (significance) of the matrix correlation between: (1) taxonomic diversity (rarefied, *N*=6) and the distance matrix, and between (2) the biofouling community structure Bray-Curtis dissimilarity matrix and the distance matrix.

Matrices	Distance		
	b	r	Mantel *P*
Taxonomic diversity	0.03199	0.016	0.395
Structure (% cover)	0.34987	0.402	0.026

Appendix IV: Genetic protocols

A4.1 Lithium Chloride/Chloroform protocol

DNA extraction followed the protocol of Gemmell and Akiyama (1996), with the addition of an extra lithium chloride step.

Digest:

1. Add 5µl proteinkinase K (10mg/ml to final concentration of 100mg/µl)
2. Add 300 µl Isolation buffer
    a. 50mM TrisHCL
    b. 50mM EDTA
    c. 100mM NaCl
    d. 1% SDS
3. Add 2 mm^2 tissue sample, sterilised and homogenised
4. Vortex in 1.5µl tube for 15 seconds
5. Spin down samples for 1 second
6. Incubate at 50°C for 2 hours
7. Incubate at 37°C overnight

Wash:

8. Add 300µl 5M LiCl
9. Invert for 1 minute
10. Add 600µl of chloroform to supernatant
11. Place samples on rotating wheel for 30 minutes
12. Centrifuge at 12200g/rcf for 15 minutes
13. Prepare new 1.5µl tubes with 300µl chloroform – extra step due to cloudy interface
14. Add supernatant to new 1.5ml tubes with chloroform
15. Invert for 1 minute
16. Centrifuge at 12200g/rcf for 15 minutes
17. Prepare new 1.5µl tubes with 50µl LiCl
18. Pipette off supernatant and add to new tubes with LiCl
19. Invert for 1 minute
20. Centrifuge at 12200g/rcf for 10 minutes

Precipitate

21. Remove supernatant and place in new tubes, discarding the pellet
22. Add 600µl of cold 100% ethanol to supernatant
23. Invert until DNA precipitates
24. Freeze for 1 hour
25. Centrifuge at 12200g/rcf for 15 minutes
26. Carefully pipette off supernatant

Wash:

27. Add 600µl of 70% ethanol to wash pellet
28. Centrifuge at 12200g/rcf for 10 minutes

29. Carefully pipette off supernatant
30. Leave lids open for 5 minutes to dry DNA

Elute:

32. Add 100µl TE8

Store:

33. -20°C

Confirm:

34. 2µl sample on 1.5% agarose gel 0.5x TBE Buffer – electrophorese at 110 v for 20 -25 minutes
35. 1µl of sample placed on University of Canterbury NanoDrop ND-1000 Spectrophotometre to attain ng/µl

A4.2 Preliminary PCR protocol

Preliminary selection of all primers was conducted at the University of Canterbury by Sarah Redlich (Honours student, University of Canterbury). During preliminary optimisation of primers, Redlich used 9 µl PCR (polymerase chain reaction) reaction volumes comprising 1x buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.0), 1.5 mM $MgCl_2$, 20 µm dNTPs, 0.54 µm of each primer, 0.1 U *Taq* (BioLine), 5.4 µl Milli-Q water and 1 µl of template DNA (25-75ng/µl). The template DNA sample was air-dried in the PCR plate for three hours covered with a sterilised container to avoid contamination. 2 µl of PCR reaction mix was added to each sample. 5 µl of mineral oil was then added to each sample to prevent evaporation of the liquid and to enhance amplification. Loci were amplified singularly in PCRs. The thermocycling protocol involved an initial denaturation at 95°C for 15 minutes, 8 cycles at 94°C for 30 seconds, a 90 second annealing phase at 60°C, and 72°C for 60 seconds, followed by 25 cycles at 94°C for 30 seconds, 52°C for 90 seconds, 72°C for 60 seconds and a final 30 minute extension at 60°C.

A4.3 Protocol for separate PCR runs

Using the 24 primers that were recommended from Redlich's results, loci were amplified singularly in PCRs. The Type-it-kit protocol comprised a total of 2 µl reaction volumes with 2x Type-it Multiplex PCR Master Mix, 0.0216 µm forward primer (with M13-tag), 0.0864 µm reverse primer, 0.135 µm M13 label (VIC,NED,FAM,PET) and 0.96 µl RNAse-free water, added to 1 µl of air-dried (3 hours) template DNA (5.5 – 8.5ng/µl). 5 µl of mineral oil was added to samples to prevent evaporation and enhance amplification during PCR. The thermocycling protocol involved an initial denaturation at 95°C for 15 minutes, 8 cycles at 94°C for 30 seconds, a 90 second annealing phase at 52- 60°C, and 72°C for 1 minute, followed by 25 cycles at 94°C for 30 seconds, 52°C for 90 seconds, 72°C for 1 minute and a final 30 minute extension at 60°C. The annealing phase was modified (between 52- 60°C) in some PCR runs to assess the influence of a lowered annealing temperature.

Table A4.1. Genotyping groups and the specific M13-labelled dye used for each multiplexed loci in this study. Loci allele ranges are included. Loci used within the final analysis, following genotyping results, are indicated by bold notation.

Genotype group	M13 label	Multiplexed loci	Allele Range (bp)
Group 1	FAM	Dvex26	78-142
		Dvex10	**159-201**
	PET	**Dvex01**	**74-119**
		Dvex14	165-221
	VIC	Dvex21	114-156
		Dvex23	191-223
Group 2	FAM	Dvex37	135-177
		Dvex19	**211-267**
	PET	**Dvex11**	**167-215**
	VIC	**Dvex36**	**145-187**
		Dvex30	**211-253**
	NED	**Dvex33**	**105-153**
		Dvex03	**196-238**

A4.4 Multiplex protocol

All loci were amplified using the following reagents and thermal cycle parameters, as described in Chapter III.

PCR reaction mix:

Reagents	Volume (µl per individual)	Final Concentration
Type-it kit PCR Master Mix	1	
Forward M13-labelled primer (locus specific)	0.016	0.0216µm
Reverse primer (locus specific)	0.064	0.0864µm
M13 labelled dye (FAM/PET/VIC/NED)	0.1	0.135µm
PCR H_20	0.82	
Template DNA	2	5.5 - 8.5ng/µl
Total	4	

PCR thermal cycle parameters:

Temperature (°C)	94	94	58	72	89	56	72	60	4
Time (min : sec)	15:00	0:30	1:30	1:00	0:30	1:30	1:00	30: 00	Hold
		repeat x 8				repeat x 25			

A4.5 Zooid extraction protocol

Didemnum vexillum zooids (Figure A4.1) were identified under a stereo microscope and three individual zooids were cut from a colony sample, using a flame-sterilised, single-edged razor blade. Three zooids were extracted from spatially separated areas on the sample organism to investigate allelic differences among the zooids. DNA and PCR protocols were identical to full colony methods (as per methods Chapter 3), but using only half the reaction volume. The locus Dvex01 was used to minimise time and associated costs. Alleles for this locus were scored using GENE MARKER v.1.6 (SoftGenetics LLC) and compared to allele results for the control sample across all PCR runs.

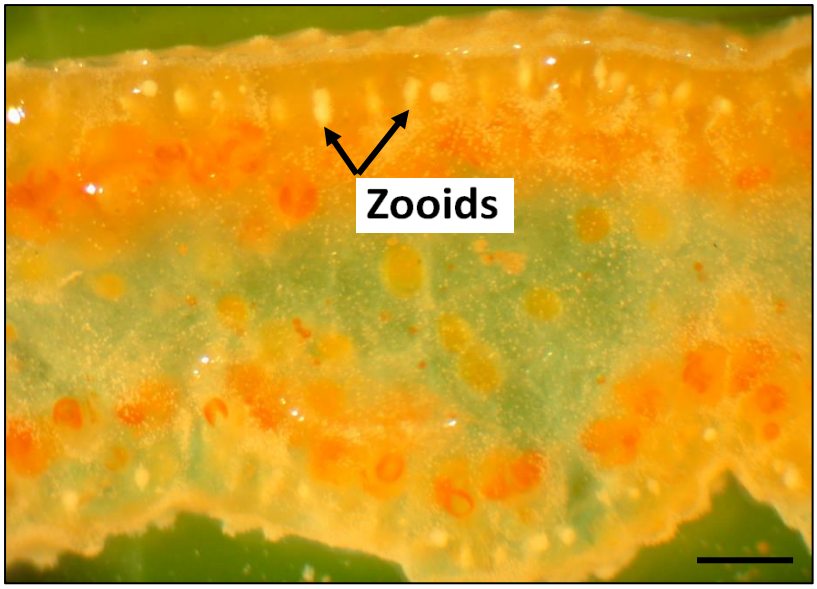

Figure A4.1. Cross section of a *Didemnum vexillum* colony (photo credit: A. Coutts). Numerous individual zooids (two indicated by arrows) are visible beneath the outer layer of the tunic. Scale bar=2 mm.

A4.6 Zooid extraction results

For all individuals, two strong allele peaks were observed, and all alleles identified within the control organism were shared among zooids (Table A4.2), potentially confirming suspected inter-colony fusion in this individual and generating variable results for three of the control runs. To be certain of these results, further genetic analyses, using the full range of loci and a larger number of potentially chimeric individuals, should be conducted.

Table A4.2. Allele peaks for the three variable results detected during PCR runs within the control sample and within three zooids taken from the control sample. Zooids were extracted from three sections of the colony sample and one locus (Dvex01) was consistent across PCR runs.

Life Stage	M13 Label	Loci	Allele Peaks (bp)		
Colony control 1	PET	Dvex01	106	112	148
Colony control 2			106	112	151
Colony control 3			112	151	
Zooid 1	PET	Dvex01	112	151	
Zooid 2			106	112	
Zooid 3			106	148	

A4.7 Model development

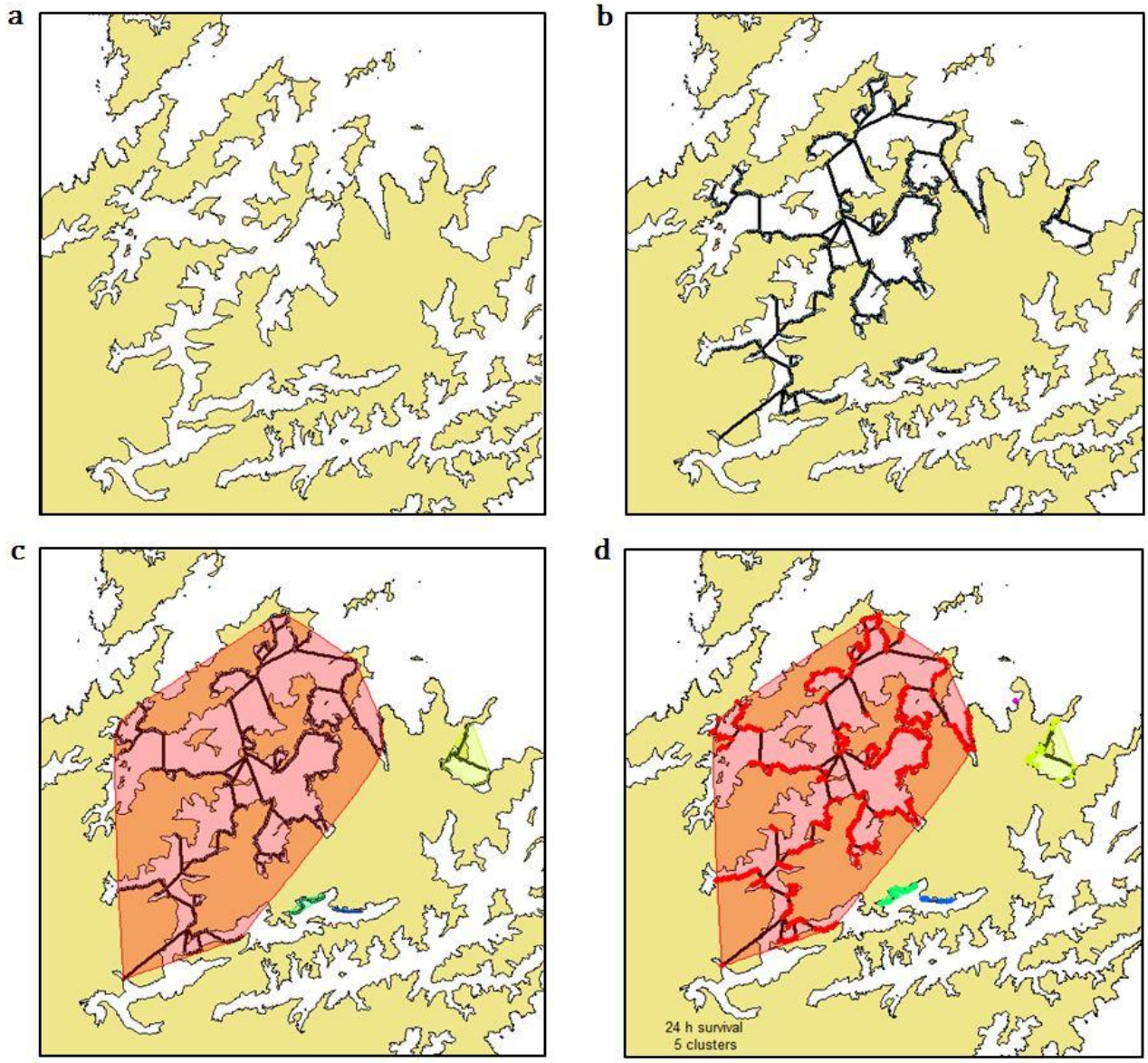

Figure A4.2. The four step process (a, b, c, d) taken in R v.3.0.2 (R Core Team 2013) to construct the connectivity matrix used to predict mussel farm connectivity in accordance to the 24 hr PLD clustering times. First, (a) the map of the Pelorus Sounds is constructed. (b) Mussel farms are plotted and 'connected' farm areas are drawn in the form of a line. (c) Areas containing farms that are connected (called 'clusters') are overlaid with a group colour. (d) The farms are outlined in a colour matching the overlaid cluster-group colour.

Appendix V: Genetic Data

Table A5.1. Allele frequencies for each loci within each population for the diploid dataset. Bold values denote alleles with different frequencies to those attained using the polyploid dataset (polyploid dataset found in Table A5.2).

DIPLOID

Locus	Allele	Goulter	Schnapper	Hikapu	Nydia	Yncyca	Tawero	Hallam	Forsyth	Melville	Picton	Shakespeare	Onahau	Ruakaka	Te Aroha	Nelson	*Average*
DVEX10	174	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	177	**0.02**	**0.13**	**0.12**	**0.13**	**0.13**	**0.17**	**0.02**	**0.00**	**0.11**	**0.09**	**0.02**	**0.03**	**0.09**	**0.08**	**0.02**	**0.08 (± 0.01)**
	180	0.10	0.15	0.10	0.13	0.04	0.11	0.14	0.07	0.13	0.07	0.08	0.13	0.11	0.02	0.00	0.09 (± 0.01)
	183	0.33	0.21	0.26	0.22	0.29	0.22	0.31	0.41	0.20	0.20	0.26	0.32	0.29	0.25	0.43	0.28 (± 0.02)
	186	0.06	0.15	0.10	0.12	0.15	0.13	0.10	0.04	0.11	0.11	0.08	0.03	0.05	0.10	0.07	0.09 (± 0.01)
	189	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00 (± 0.00)
	192	**0.38**	**0.35**	**0.40**	**0.38**	**0.37**	**0.31**	**0.43**	**0.46**	**0.44**	**0.52**	**0.54**	**0.50**	**0.45**	**0.50**	**0.43**	**0.43 (± 0.02)**
	195	**0.10**	**0.02**	**0.02**	**0.00**	**0.02**	**0.06**	**0.00**	**0.00**	**0.00**	**0.02**	**0.02**	**0.00**	**0.02**	**0.04**	**0.02**	**0.02 (± 0.01)**
DVEX01	106	0.32	0.41	0.23	0.27	0.38	0.35	0.50	0.54	0.33	0.44	0.48	0.47	0.50	0.40	0.43	0.40 (± 0.02)
	112	0.68	0.59	0.77	0.71	0.62	0.65	0.50	0.42	0.66	0.56	0.52	0.53	0.50	0.60	0.58	0.59 (± 0.02)
	115	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	118	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
DVEX19	235	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	239	0.10	0.02	0.00	0.00	0.04	0.11	0.03	0.06	0.09	0.03	0.21	0.28	0.07	0.09	0.12	0.08 (± 0.02)
	243	0.54	0.40	0.24	0.21	0.35	0.35	0.42	0.52	0.33	0.31	0.44	0.06	0.50	0.48	0.35	0.37 (± 0.03)
	247	0.10	0.21	0.56	0.57	0.29	0.04	0.34	0.08	0.17	0.09	0.08	0.06	0.00	0.04	0.03	0.18 (± 0.05)
	251	0.08	0.08	0.15	0.19	0.04	0.13	0.13	0.19	0.17	0.13	0.19	0.50	0.27	0.22	0.29	0.18 (± 0.03)
	255	0.18	0.21	0.04	0.03	0.12	0.28	0.03	0.10	0.24	0.16	0.06	0.00	0.13	0.11	0.00	0.11 (± 0.02)
	259	0.00	0.00	0.00	0.00	0.04	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01 (± 0.00)
	263	0.00	0.04	0.02	0.00	0.13	0.04	0.05	0.02	0.00	0.28	0.02	0.11	0.04	0.02	0.21	0.07 (± 0.02)
	267	0.00	0.04	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.01 (± 0.00)
DVEX11	179	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	195	0.06	0.08	0.07	0.15	0.02	0.13	0.05	0.04	0.02	0.02	0.06	0.08	0.02	0.06	0.07	0.06 (± 0.01)
	197	**0.12**	**0.13**	**0.07**	**0.07**	**0.14**	**0.02**	**0.07**	**0.06**	**0.07**	**0.09**	**0.10**	**0.06**	**0.02**	**0.04**	**0.31**	**0.09 (± 0.02)**
	201	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)

	205	0.08	0.03	0.04	0.02	0.06	0.02	0.02	0.08	0.06	0.04	0.06	0.00	0.20	0.08	0.00	0.05 (± 0.01)
	207	**0.31**	**0.43**	**0.64**	**0.52**	**0.44**	**0.57**	**0.52**	**0.38**	**0.44**	**0.50**	**0.29**	**0.72**	**0.28**	**0.27**	**0.55**	**0.46 (± 0.04)**
	213	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	215	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.02	0.00	0.00	0.00	0.05	0.01 (± 0.01)
	217	**0.40**	**0.35**	**0.18**	**0.24**	**0.32**	**0.24**	**0.33**	**0.37**	**0.41**	**0.33**	**0.48**	**0.14**	**0.48**	**0.54**	**0.02**	**0.32 (± 0.04)**
DVEX36	142	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.01 (± 0.01)
	151	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00 (± 0.00)
	154	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.05	0.02	0.00	0.00	0.00	0.00	0.00	0.01 (± 0.00)
	163	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	166	0.25	0.31	0.34	0.31	0.19	0.37	0.21	0.20	0.24	0.21	0.29	0.18	0.13	0.26	0.23	0.25 (± 0.02)
	169	0.02	0.08	0.13	0.10	0.10	0.06	0.12	0.07	0.02	0.02	0.08	0.08	0.07	0.13	0.10	0.08 (± 0.01)
	172	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.02	0.00	0.00	0.00 (± 0.00)
	175	0.62	0.58	0.54	0.57	0.71	0.56	0.67	0.70	0.67	0.69	0.62	0.66	0.73	0.61	0.60	0.63 (± 0.02)
	178	0.12	0.02	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.06	0.00	0.00	0.04	0.00	0.06	0.02 (± 0.01)
DVEX30	211	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	214	0.00	0.05	0.00	0.08	0.00	0.00	0.06	0.00	0.02	0.00	0.06	0.00	0.00	0.00	0.00	0.02 (± 0.01)
	229	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00 (± 0.00)
	232	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	235	0.02	0.03	0.02	0.06	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.04	0.02	0.00	0.02 (± 0.01)
	238	0.13	0.29	0.30	0.23	0.13	0.30	0.09	0.16	0.14	0.00	0.10	0.06	0.13	0.12	0.02	0.15 (± 0.02)
	241	0.21	0.03	0.02	0.00	0.02	0.14	0.03	0.02	0.00	0.40	0.18	0.22	0.05	0.08	0.54	0.13 (± 0.04)
	244	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.02	0.04	0.00	0.00	0.06	0.00	0.00	0.00	0.01 (± 0.00)
	247	0.04	0.08	0.00	0.00	0.00	0.00	0.00	0.02	0.11	0.02	0.02	0.00	0.00	0.00	0.02	0.02 (± 0.01)
	250	0.54	0.42	0.52	0.54	0.56	0.54	0.78	0.78	0.39	0.58	0.54	0.67	0.77	0.75	0.40	0.58 (± 0.03)
	253	0.04	0.00	0.00	0.00	0.04	0.02	0.00	0.00	0.05	0.00	0.08	0.00	0.00	0.04	0.00	0.02 (± 0.01)
	256	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	259	0.00	0.11	0.11	0.10	0.25	0.00	0.03	0.00	0.14	0.00	0.00	0.00	0.02	0.00	0.00	0.05 (± 0.02)
DVEX33	105	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	111	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	114	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	123	**0.24**	**0.10**	**0.11**	**0.02**	**0.00**	**0.37**	**0.31**	**0.33**	**0.28**	**0.05**	**0.38**	**0.20**	**0.21**	**0.24**	**0.02**	**0.19 (± 0.03)**
	126	0.04	0.00	0.11	0.05	0.00	0.00	0.06	0.15	0.02	0.05	0.04	0.07	0.20	0.18	0.05	0.07 (± 0.02)

	129	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.02	0.00	0.00 (± 0.00)
	132	0.24	0.10	0.29	0.10	0.02	0.39	0.41	0.37	0.28	0.11	0.30	0.27	0.43	0.44	0.16	0.26 (± 0.03)
	138	0.12	0.15	0.07	0.14	0.07	0.07	0.03	0.02	0.10	0.11	0.06	0.10	0.02	0.00	0.16	0.08 (± 0.01)
	141	0.06	0.08	0.05	0.10	0.20	0.00	0.06	0.06	0.02	0.08	0.10	0.03	0.02	0.00	0.09	0.06 (± 0.01)
	144	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	147	0.22	0.17	0.21	0.31	0.20	0.13	0.00	0.06	0.14	0.34	0.08	0.13	0.13	0.02	0.36	0.17 (± 0.03)
	150	**0.08**	**0.35**	**0.16**	**0.24**	**0.43**	**0.02**	**0.13**	**0.02**	**0.10**	**0.26**	**0.04**	**0.20**	**0.00**	**0.10**	**0.16**	**0.15 (± 0.03)**
	153	0.00	0.00	0.00	0.00	0.05	0.02	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
DVEX03	187	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	214	0.02	0.07	0.03	0.03	0.02	0.04	0.07	0.00	0.00	0.05	0.00	0.08	0.00	0.06	0.02	0.03 (± 0.01)
	217	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	220	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.05	0.00 (± 0.00)
	223	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	226	0.69	0.78	0.78	0.78	0.92	0.72	0.60	0.50	0.44	0.61	0.52	0.69	0.75	0.56	0.57	0.66 (± 0.03)
	229	0.04	0.07	0.02	0.09	0.04	0.06	0.02	0.02	0.22	0.07	0.02	0.08	0.06	0.06	0.07	0.06 (± 0.01)
	232	0.19	0.04	0.17	0.10	0.02	0.17	0.31	0.48	0.31	0.27	0.42	0.14	0.19	0.29	0.27	0.23 (± 0.03)
	235	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00 (± 0.00)
	238	0.02	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00 (± 0.00)

Table A5.2. Allele frequencies for each loci, within each population for the polyploid dataset. Bold values denote alleles with different frequencies to those obtained from the diploid dataset. Bold values with no background colouration represent allele frequencies that have decreased in comparison to the diploid dataset and those with grey backgrounds represent allele frequencies that have increased. Rare alleles, not present within the diploid dataset are indicated in italics.

POLYPLOID

Locus	Allele	Goulter	Schnapper	Hikapu	Nydia	Yncyca	Tawero	Hallam	Forsyth	Melville	Picton	Shakespeare	Onahau	Ruakaka	Te Aroha	Nelson	*Average*
DVEX10	174	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	177	**0.02**	**0.09**	**0.10**	**0.08**	**0.13**	**0.12**	**0.02**	**0.00**	**0.06**	**0.06**	**0.01**	**0.01**	**0.05**	**0.05**	**0.01**	**0.05 (± 0.01)**
	180	0.10	0.16	0.10	0.14	0.04	0.10	0.13	0.06	0.13	0.08	0.08	0.13	0.11	0.02	0.01	0.09 (± 0.01)
	183	0.29	0.22	0.27	0.25	0.29	0.26	0.31	0.40	0.25	0.19	0.25	0.32	0.29	0.24	0.42	0.28 (± 0.02)
	186	0.10	0.12	0.10	0.08	0.17	0.12	0.10	0.05	0.06	0.08	0.08	0.03	0.10	0.08	0.08	0.09 (± 0.01)
	189	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00 (± 0.00)
	192	**0.33**	**0.39**	**0.41**	**0.43**	**0.36**	**0.33**	**0.43**	**0.44**	**0.50**	**0.55**	**0.54**	**0.50**	**0.40**	**0.52**	**0.43**	**0.44 (± 0.02)**
	195	**0.16**	**0.02**	**0.02**	**0.01**	**0.02**	**0.07**	**0.01**	**0.04**	**0.00**	**0.04**	**0.03**	**0.01**	**0.05**	**0.08**	**0.02**	**0.04 (± 0.01)**
DVEX01	106	0.32	0.41	0.23	0.27	0.38	0.35	0.50	0.48	0.33	0.44	0.48	0.47	0.50	0.40	0.43	0.40 (± 0.02)
	112	0.68	0.59	0.77	0.71	0.62	0.65	0.50	0.48	0.66	0.56	0.52	0.53	0.50	0.60	0.58	0.60 (± 0.02)
	115	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	118	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
DVEX19	235	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	239	0.10	0.02	0.00	0.00	0.04	0.11	0.02	0.06	0.09	0.03	0.21	0.28	0.07	0.09	0.12	0.08 (± 0.02)
	243	0.54	0.40	0.24	0.21	0.35	0.35	0.42	0.52	0.33	0.31	0.44	0.06	0.50	0.48	0.35	0.37 (± 0.03)
	247	0.10	0.21	0.56	0.57	0.29	0.04	0.33	0.08	0.17	0.09	0.08	0.06	0.00	0.04	0.03	0.18 (± 0.05)
	251	0.08	0.08	0.15	0.19	0.04	0.13	0.13	0.19	0.17	0.13	0.19	0.50	0.27	0.22	0.29	0.18 (± 0.03)
	255	0.18	0.21	0.04	0.03	0.12	0.28	0.03	0.10	0.24	0.16	0.06	0.00	0.13	0.11	0.00	0.11 (± 0.02)
	259	0.00	0.00	0.00	0.00	0.04	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01 (± 0.00)
	263	0.00	0.04	0.02	0.00	0.13	0.04	0.07	0.02	0.00	0.28	0.02	0.11	0.04	0.02	0.21	0.07 (± 0.02)
	267	0.00	0.04	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.01 (± 0.00)
DVEX11	179	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	185	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.01*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00 (± 0.00)*
	195	0.04	0.08	0.07	0.14	0.01	0.13	0.04	0.03	0.01	0.02	0.05	0.06	0.02	0.06	0.06	0.06 (± 0.01)
	197	**0.10**	**0.13**	**0.07**	**0.06**	**0.13**	**0.02**	**0.07**	**0.04**	**0.07**	**0.08**	**0.07**	**0.05**	**0.01**	**0.03**	**0.27**	**0.08 (± 0.02)**

	199	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.01*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00 (± 0.00)*
	201	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	205	0.08	0.03	0.04	0.02	0.06	0.02	0.02	0.08	0.06	0.04	0.06	0.00	0.17	0.08	0.00	0.05 (± 0.01)
	207	**0.27**	**0.43**	**0.64**	**0.50**	**0.42**	**0.57**	**0.54**	**0.36**	**0.41**	**0.49**	**0.26**	**0.66**	**0.27**	**0.26**	**0.50**	**0.44 (± 0.03)**
	213	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	215	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.02	0.00	0.00	0.00	0.05	0.01 (± 0.01)
	217	**0.48**	**0.35**	**0.18**	**0.28**	**0.36**	**0.24**	**0.33**	**0.40**	**0.44**	**0.34**	**0.54**	**0.24**	**0.53**	**0.56**	**0.12**	**0.36 (± 0.03)**
DVEX36	142	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.01 (± 0.00)
	151	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00 (± 0.00)
	154	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.04	0.01	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	163	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	166	0.25	0.31	0.34	0.31	0.19	0.37	0.21	0.20	0.24	0.21	0.29	0.20	0.14	0.26	0.23	0.25 (± 0.02)
	169	0.02	0.08	0.13	0.10	0.10	0.06	0.12	0.07	0.02	0.02	0.08	0.08	0.07	0.13	0.10	0.08 (± 0.01)
	172	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.02	0.00	0.00	0.00 (± 0.00)
	175	0.62	0.58	0.54	0.57	0.71	0.56	0.67	0.70	0.68	0.68	0.62	0.65	0.73	0.61	0.60	0.63 (± 0.02)
	178	0.12	0.02	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.06	0.00	0.00	0.04	0.00	0.06	0.02 (± 0.01)
DVEX30	211	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	214	0.00	0.05	0.00	0.08	0.00	0.00	0.06	0.00	0.02	0.00	0.06	0.00	0.00	0.00	0.00	0.02 (± 0.01)
	229	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00 (± 0.00)
	232	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	235	0.02	0.03	0.02	0.06	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.04	0.02	0.00	0.02 (± 0.01)
	238	0.12	0.28	0.29	0.22	0.13	0.28	0.09	0.13	0.14	0.00	0.09	0.06	0.12	0.12	0.02	0.14 (± 0.02)
	241	0.21	0.03	0.02	0.00	0.02	0.14	0.03	0.03	0.00	0.40	0.22	0.22	0.07	0.08	0.53	0.13 (± 0.04)
	244	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.01	0.04	0.00	0.00	0.06	0.00	0.00	0.00	0.01 (± 0.00)
	247	0.03	0.08	0.00	0.00	0.00	0.00	0.00	0.02	0.10	0.02	0.02	0.00	0.00	0.00	0.01	0.02 (± 0.01)
	250	0.56	0.44	0.54	0.53	0.56	0.55	0.78	0.81	0.39	0.58	0.53	0.67	0.76	0.75	0.41	0.59 (± 0.03)
	253	0.04	0.00	0.00	0.00	0.04	0.03	0.00	0.00	0.05	0.00	0.07	0.00	0.00	0.04	0.00	0.02 (± 0.01)
	256	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	259	0.00	0.10	0.11	0.10	0.25	0.00	0.03	0.00	0.15	0.00	0.00	0.00	0.02	0.00	0.00	0.05 (± 0.02)
DVEX33	105	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	111	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	114	0.00	0.04	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)

	123	**0.20**	**0.10**	**0.09**	**0.02**	**0.00**	**0.34**	**0.26**	**0.31**	**0.25**	**0.05**	**0.35**	**0.18**	**0.20**	**0.23**	**0.02**	**0.17 (± 0.03)**
	126	0.03	0.00	0.11	0.05	0.00	0.00	0.05	0.15	0.01	0.04	0.03	0.04	0.18	0.17	0.05	0.06 (± 0.02)
	129	0.00	0.00	0.00	0.01	0.00	0.00	0.04	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.00	0.01 (± 0.00)
	132	0.26	0.10	0.28	0.10	0.02	0.36	0.33	0.35	0.29	0.10	0.26	0.22	0.41	0.45	0.15	0.25 (± 0.03)
	135	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.02*	*0.01*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.01*	*0.00*	*0.00 (± 0.00)*
	138	0.13	0.14	0.07	0.13	0.07	0.08	0.02	0.02	0.10	0.11	0.05	0.10	0.02	0.00	0.16	0.08 (± 0.01)
	141	0.04	0.08	0.05	0.10	0.20	0.01	0.07	0.05	0.03	0.08	0.09	0.03	0.02	0.00	0.09	0.06 (± 0.01)
	144	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	147	0.20	0.17	0.23	0.30	0.20	0.13	0.00	0.06	0.13	0.36	0.09	0.13	0.12	0.01	0.38	0.17 (± 0.03)
	150	**0.13**	**0.37**	**0.16**	**0.26**	**0.43**	**0.06**	**0.22**	**0.06**	**0.13**	**0.26**	**0.12**	**0.29**	**0.06**	**0.11**	**0.16**	**0.19 (± 0.03)**
	153	0.00	0.00	0.00	0.00	0.05	0.02	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.01 (± 0.00)
DVEX03	187	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	214	0.02	0.07	0.03	0.03	0.02	0.04	0.07	0.00	0.00	0.05	0.00	0.08	0.00	0.06	0.02	0.03 (± 0.01)
	217	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	220	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.05	0.00 (± 0.00)
	223	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00 (± 0.00)
	226	0.69	0.78	0.78	0.78	0.92	0.72	0.60	0.50	0.44	0.61	0.52	0.69	0.75	0.56	0.57	0.66 (± 0.03)
	229	0.04	0.07	0.02	0.09	0.04	0.06	0.02	0.02	0.22	0.07	0.02	0.08	0.06	0.06	0.07	0.06 (± 0.01)
	232	0.19	0.04	0.17	0.10	0.02	0.17	0.31	0.48	0.31	0.27	0.42	0.14	0.19	0.29	0.27	0.23 (± 0.03)
	235	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00 (± 0.00)
	238	0.02	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00 (± 0.00)

Table A5.3. Loci-specific observed (H_O) and expected (H_E) heterozygotes and null alleles presence (Yes or n.a) for *Didemnum vexillum* samples. Values indicating significant deviations from Hardy-Weinberg equilibrium are in boldface, at $P \leq 0.009$ after correcting for multiple tests using a False Discovery Correction.

Sites		DVEX10	DVEX01	DVEX19	DVEX11	DVEX36	DVEX30	DVEX33	DVEX03
Pelorus Sound									
Goulter	Ho	**1.00**	0.64	0.44	**0.69**	**0.54**	0.50	**1.00**	0.50
	He	0.74	0.44	0.66	0.73	0.56	0.66	0.83	0.49
	Null detected	n.a	n.a	Yes	n.a	n.a	Yes	n.a	n.a
Schnapper	Ho	**1.00**	**0.83**	0.63	0.80	0.79	**0.79**	**0.71**	0.30
	He	0.79	0.50	0.76	0.69	0.57	0.74	0.81	0.38
	Null detected	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a
Hikapu	Ho	**1.00**	0.46	**0.44**	0.57	**0.93**	**0.82**	**0.86**	0.41
	He	0.75	0.36	0.62	0.55	0.59	0.64	0.83	0.37
	Null detected	n.a	n.a	Yes	n.a	n.a	n.a	n.a	n.a
Nydia	Ho	**0.97**	0.58	0.45	0.81	0.72	**0.69**	**0.66**	0.41
	He	0.77	0.43	0.61	0.66	0.58	0.65	0.82	0.39
	Null detected	n.a	n.a	Yes	n.a	n.a	n.a	Yes	n.a
Yncyca	Ho	**1.00**	0.76	0.69	0.80	0.58	**0.50**	**0.68**	0.08
	He	0.75	0.48	0.78	0.69	0.46	0.62	0.74	0.15
	Null detected	n.a	n.a	n.a	n.a	n.a	n.a	n.a	Yes
Tawero	Ho	**1.00**	0.69	0.59	0.67	0.78	0.72	**0.96**	0.48
	He	0.81	0.46	0.78	0.61	0.56	0.61	0.70	0.45
	Null detected	n.a	n.a	Yes	n.a	n.a	n.a	n.a	n.a
Hallam	Ho	**0.90**	**1.00**	0.42	0.57	0.67	0.31	0.94	0.62
	He	0.71	0.51	0.70	0.62	0.51	0.39	0.74	0.56
	Null detected	n.a	n.a	Yes	n.a	n.a	n.a	n.a	n.a
Forsyth	Ho	**1.00**	**0.85**	**0.38**	0.85	0.57	0.32	**0.85**	0.50
	He	0.62	0.54	0.69	0.72	0.48	0.37	0.74	0.53
	Null detected	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a
Melville	Ho	**0.89**	0.69	0.59	0.56	0.62	**0.57**	**0.97**	**0.59**
	He	0.73	0.47	0.78	0.64	0.49	0.80	0.82	0.67

	Null detected	n.a	n.a	n.a	n.a	n.a	Yes	n.a	n.a
Queen Charlotte									
Picton	Ho	**0.78**	**0.88**	0.75	0.52	0.50	0.71	**0.68**	0.45
	He	0.68	0.50	0.80	0.65	0.49	0.51	0.80	0.55
	Null detected	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a
Shakespeare	Ho	**0.84**	**0.96**	0.50	0.65	0.77	0.48	**0.80**	0.44
	He	0.64	0.51	0.73	0.68	0.54	0.67	0.76	0.56
	Null detected	n.a	n.a	Yes	n.a	n.a	Yes	n.a	n.a
Ruakaka	Ho	**1.00**	**1.00**	**0.43**	0.59	0.46	0.46	**0.96**	0.33
	He	0.71	0.51	0.67	0.66	0.45	0.40	0.73	0.41
	Null detected	n.a	n.a	Yes	n.a	n.a	n.a	n.a	n.a
Te Aroha	Ho	**0.83**	**0.79**	0.57	0.54	**0.48**	0.46	**0.80**	0.58
	He	0.68	0.49	0.72	0.63	0.55	0.42	0.72	0.61
	Null detected	n.a	n.a	n.a	n.a	n.a	n.a	n.a	n.a
Onahau	Ho	**0.95**	**0.95**	**0.33**	0.33	0.68	0.50	**0.87**	0.39
	He	0.65	0.51	0.67	0.46	0.53	0.51	0.84	0.50
	Null detected	n.a	n.a	Yes	n.a	n.a	n.a	n.a	n.a
Port Nelson									
Nelson	Ho	**0.90**	**0.85**	0.59	**0.81**	0.67	**0.88**	**0.55**	0.59
	He	0.64	0.50	0.75	0.61	0.58	0.56	0.80	0.61
	Null detected	n.a	n.a	n.a	n.a	n.a	n.a	Yes	n.a

Table A5.4. Characterisation of eight primer pairs amplifying DNA microsatellites in *Didemnum vexillum*. Each locus name, the sequences for the locus-specific M13-labelled forward and reverse primers, the sequence of the M13-tails on forward primers, repeat motifs, locus-specific allele sizes and expected heterozygosity (H_E) under Hardy-Weinberg are displayed. All means ± SE.

Locus	Primer sequence (5'-3')	Repeat Motif	Allele sizes (bp)	Alleles	H_E (S.E)
DVEX10	F: M13-TTGGAAGTGCTTTGGTAGCC	(ACA)7	159-201	8	0.70 (± 0.01)
	R: TGCCAATAGTCAGGTTTGTCG				
DVEX01	F: M13-CCAACCATGAGTGTGAAGCG	(AGT)7	74-116	4	0.47 (± 0.01)
	R: GCGTTACTGTTGATTGAAGCC				
DVEX19	F: M13-CTGGTCCAAATAACGAACGATTG	(CACG)7	211-267	9	0.70 (± 0.02)
	R: TTTACGAGCAGCCAACGAAC				
DVEX11	F: M13-TCAGGGCCCCAAATACCAAG	(AC)12	167-215	9	0.63 (± 0.02)
	R: ACCATAACCCTAGAACATACCC				
DVEX36	F: M13-TGTTACTCATGCACTTGCGG	(CAA)7	145-187	9	0.52 (± 0.01)
	R: TGCATTGGTTCGACCTGTTG				
DVEX30	F: M13-TTCATCCGGGTACTCGACAG	(AGA)7	211-253	13	0.56 (± 0.03)
	R: GGGTCTTGGGCGTGTTTATG				
DVEX33	F: M13-GGAACGGATGATGATGGCTG	(GTT)8	105-153	13	0.76 (± 0.01)
	R: GCAGTCTTGTCAGGGAGGAG				
DVEX03	F: M13-TGTTTCAGGCGAGTTCATCG	(TGA)7	196-238	10	0.47 (± 0.03)
	R: CAATGAACAAACGCAACCGC				
M13	GTAAAACGACGGCCAGT				
Mean				9.38	0.60 (± 0.04)

Table A5.5. Estimates of genetic diversity for 15 *Didemnum vexillum* populations across three sites in New Zealand (Pelorus Sound, Queen Charlotte and Port Nelson) using all loci, but Dvex10 and Dvex19. Statistics are shown for: N=sample size, A_N=mean number of alleles, A_{RN}=rarefied mean allelic richness, A_{RP}=rarefied mean private allelic richness (based upon *N*=19), H_O=observed heterozygosity, H_E=expected heterozygosity under Hardy-Weinberg equilibrium, and F_{IS}=the inbreeding coefficient. All means ± SE.

Site	N	A_N	A_{RN}	A_{RP}	H_O	H_E	F_{IS}	
Pelorus Sound	240	4.93(± 0.08)	3.88 (± 0.04)	0.15 (± 0.02)	0.66 (± 0.01)	0.57 (± 0.00)	-0.20824	ns
Goulter	26	5.33(± 0.80)	4.25 (± 0.61)	0.12 (± 0.08)	0.65 (± 0.08)	0.61 (± 0.06)	-0.05656	ns
Schnapper	24	5.16 (± 0.80)	4.27 (± 0.64)	0.22 (± 0.14)	0.71 (± 0.08)	0.60 (± 0.06)	-0.24687	ns
Hikapu	29	4.50 (± 0.76)	3.76 (± 0.63)	0.01 (± 0.01)	0.68 (± 0.09)	0.55 (± 0.07)	-0.27015	ns
Nydia	30	5.00 (± 0.85)	4.03 (± 0.58)	0.16 (± 0.01)	0.68 (± 0.09)	0.58 (± 0.06)	-0.19867	ns
Yncyca	26	4.50 (± 0.76)	3.51 (± 0.54)	0.16 (± 0.12)	0.57 (± 0.12)	0.52 (± 0.09)	-0.15516	ns
Tawero	27	4.50 (± 0.62)	3.44 (± 0.54)	0.09 (± 0.06)	0.72 (± 0.06)	0.56 (± 0.04)	-0.29716	ns
Hallam	21	4.17 (± 0.60)	3.58 (± 0.45)	0.00 (± 0.00)	0.68 (± 0.10)	0.54 (± 0.05)	-0.35622	ns
Forsyth	28	4.83 (± 0.65)	3.63 (± 0.49)	0.13 (± 0.10)	0.66 (± 0.09)	0.55 (± 0.06)	-0.22754	ns
Melville	29	6.33 (± 1.36)	4.50 (± 0.81)	0.45 (± 0.15)	0.67 (± 0.06)	0.64 (± 0.06)	-0.06586	ns
Queen Charlotte	124	4.53(± 0.03)	3.74 (± 0.05)	0.11 (± 0.01)	0.56 (± 0.00)	0.63 (± 0.01)	-0.18075	ns
Picton	24	4.50 (± 0.76)	3.69 (± 0.63)	0.08 (± 0.07)	0.62 (± 0.07)	0.57 (± 0.05)	-0.15989	ns
Shakespeare	26	5.00 (± 0.82)	4.02 (± 0.61)	0.19 (± 0.10)	0.68 (± 0.08)	0.61 (± 0.04)	-0.13537	ns
Ruakaka	28	4.50 (± 0.67)	3.45 (± 0.38)	0.07 (± 0.07)	0.64 (± 0.11)	0.52 (± 0.06)	-0.26600	ns
Te Aroha	27	4.50 (± 0.67)	3.66 (± 0.41)	0.07 (± 0.05)	0.61 (± 0.06)	0.56 (± 0.04)	-0.13392	ns
Onahau	19	4.17 (± 0.65)	3.88 (± 0.59)	0.11 (± 0.10)	0.62 (± 0.104)	0.55 (± 0.06)	-0.20855	ns
Port Nelson	24							
Nelson	24	4.83 (± 0.70)	3.88 (± 0.54)	0.17 (± 0.11)	0.72 (± 0.06)	0.60 (± 0.04)	-0.28409	ns
Overall	388	4.79 (± 0.05)	3.84 (± 0.12)	0.14 (± 0.01)	0.66 (± 0.01)	0.57 (± 0.00)	-0.20300	

Note: ns = F_{IS} was not significant at $P>0.05$

Table A5.6. Analysis of molecular variance (AMOVA) results for *Didemnum vexillum* microsatellite data for all loci but locus Dvex10 and Dvex19, including *F*-statistics (FCT, FSC, and FST), associated 95% confidence intervals and percentages of explained variation. Four separate AMOVA results are presented, looking at differences across sites (Pelorus Sound, Queen Charlotte and Port Nelson) and across two different cluster groupings, according to pelagic larval duration times of 24 hours and 12 hours.

Spatial Scale		F_{CT}	F_{SC}	F_{ST}
Queen Charlotte, Pelorus Sound and Port Nelson	F-statistic	0.0163 *	0.03628 ***	0.05199 ***
	(95% CI)	(-0.0055, 0.03332)	(0.02351, 0.05529)	(0.03219, 0.07564)
	Variation (%)	1.63	3.57	94.80
Queen Charlotte and Pelorus Sound	F-statistic	0.00788	0.03629***	0.04388***
	(95% CI)	(0.00007, 0.01531)	(0.02354, 0.05574)	(0.02950, 0.06161)
	Variation (%)	0.79	3.60	95.61
Pelorus Sound Only - Cluster 24 hours	F-statistic	-0.00656 ns	0.04002 ***	0.03372 ***
	(95% CI)	(-0.02990, 0.00988)	(0.02120, 0.06944)	(0.02015, 0.05539)
	Variation (%)	-0.66	4.03	96.63
Pelorus Sound Only - Cluster 12 hours	F-statistic	-0.01428 ns	0.04496 ***	0.03132 ***
	(95% CI)	(-0.02328, 0.00693)	(0.02146, 0.07144)	(0.01995, 0.05497)
	Variation (%)	-1.43	4.56	96.87

Note: ns= not significant *P<0.05, **P<0.01, ***P<0.001

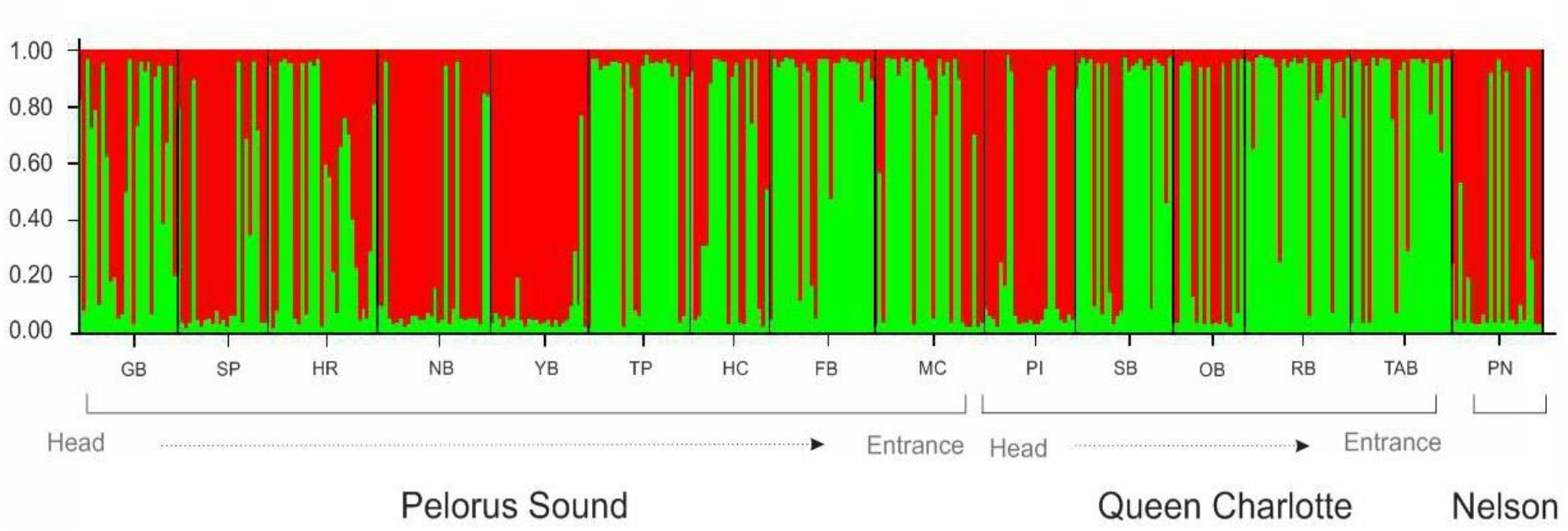

Figure A5.1. Population structure according to Bayesian clustering of *Didemnum vexillum* genotypes performed in STRUCTURE for all populations, and all loci excluding Dvex10 and Dvex19, within each of the three sites in New Zealand (Pelorus Sound, Queen Charlotte and Nelson). Populations within each sampling site, from the head to the entrance of each Sound, are indicated. Pelorus Sound sampling sites: SP=Schnapper Point, GB=Goulter Bay, HR=Hikapu Reach, NB= Nydia Bay, YB= Yncyca Bay, TP=Tawero Bay, HC=Hallam Cove, FB=Forsyth Bay, MC=Melville Cove. Queen Charlotte sampling sites: SB=Shakespeare Bay, PI=Picton Marina, OB=Opuaha Bay, RB=Ruakaka Bay, TAB= Te Aroha Bay. Nelson sampling site: PN=Port Nelson.

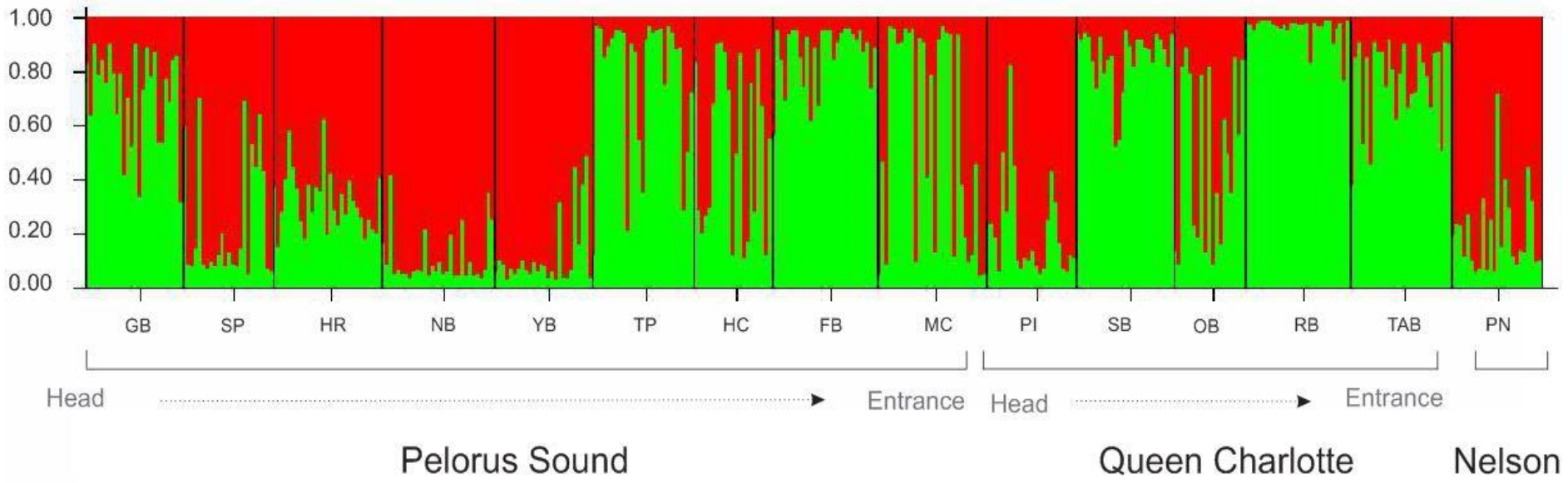

Figure A5.2. Population structure, using prior population information, according to Bayesian clustering of *Didemnum vexillum* genotypes performed in STRUCTURE for all populations, and all loci, within each of the three sites in New Zealand (Pelorus Sound, Queen Charlotte and Nelson). Populations within each sampling site, from the head to the entrance of each Sound, are indicated. Pelorus Sound sampling sites: SP=Schnapper Point, GB=Goulter Bay, HR=Hikapu Reach, NB= Nydia Bay, YB= Yncyca Bay, TP=Tawero Bay, HC=Hallam Cove, FB=Forsyth Bay, MC=Melville Cove. Queen Charlotte sampling sites: SB=Shakespeare Bay, PI=Picton Marina, OB=Opuaha Bay, RB=Ruakaka Bay, TAB= Te Aroha Bay. Nelson sampling site: PN=Port Nelson.

Table A5.7. Isolation-by-distance at three hierarchical spatial scales (all populations, Pelorus Sound and Queen Charlotte Sound) using all loci and without Dvex10 and Dvex19 (6 loci) for Nei's genetic distance vs. Euclidean geographical distances (kilometres). The correlations (r_{xy}), associated *p*-values, corrected *p*-values (*CPV*) for significance and the number of tests used for the *p*-value corrections (*N*) are indicated.

All Loci

Sites	# Sites	Distance (km)			
		r_{xy}	*p*-value	*CPV*	*N*
Pelorus Sound and Queen Charlotte	2	0.132	0.260	0.008	*N*=91
Pelorus Sound	9	0.220	0.109	0.010	*N*=36
Queen Charlotte Sound	5	0.204	0.206	0.020	*N*=10
Overall	16	0.556	0.575		

6 Loci

Sites	# Sites	Distance (km)			
		r_{xy}	*p*-value	*CPV*	*N*
Pelorus Sound and Queen Charlotte	2	0.128	0.154	0.008	*N*=91
Pelorus Sound	9	0.282	0.033	0.010	*N*=36
Queen Charlotte Sound	5	0.375	0.150	0.020	*N*=10
Overall	16	0.785	0.337		

Table A5.8. Pairwise FST (below diagonal) and Jost's *D* (above diagonal) matrix (including all loci) for each cluster grouping, from 24 hours (a), to 12 hours (b) and 2 hours (c). Significant pairwise combinations after False Discovery Rate corrections for multiple tests (N=36) are indicated in bold, $P \leq 0.01$. Pelorus Sound populations include: GOUL=Goulter Bay, SCHN=Schnapper Point, HIKA=Hikapu Reach, NYDA=Nydia Bay, YNCY=Yncyca Bay, TAWO=Tawero Point, HALM= Hallam Cove, FORS=Forsyth Bay and MELV=Melville Cove.

a. 24 HOUR PLD

	Clust 1	*Clust 2*							*Clust 3*
	GOUL	SCHN	HIKA	NYDA	YNCY	TAWO	HALM	FORS	MELV
GOUL	-	0.05	**0.09**	**0.11**	**0.10**	0.03	0.05	**0.05**	**0.03**
SCHN	0.01	-	**0.06**	**0.03**	0.00	**0.07**	**0.08**	**0.14**	**0.05**
HIKA	**0.05**	**0.02**	-	0.00	**0.08**	**0.09**	**0.06**	**0.15**	**0.08**
NYDA	**0.05**	**0.01**	0.00	-	**0.04**	**0.14**	**0.11**	**0.20**	**0.11**
YNCY	**0.04**	0.00	**0.04**	**0.02**	-	**0.15**	**0.11**	**0.18**	**0.12**
TAWO	0.01	**0.03**	**0.04**	**0.06**	**0.06**	-	**0.06**	**0.08**	**0.04**
HALM	0.01	**0.02**	**0.02**	**0.04**	**0.04**	**0.02**	-	0.01	0.05
FORS	**0.03**	**0.07**	**0.09**	**0.10**	**0.10**	**0.05**	0.00	-	**0.06**
MELV	**0.02**	**0.02**	**0.04**	**0.05**	**0.06**	**0.02**	0.01	**0.03**	-
							-		

b. 12 HOUR PLD

	Clust 1	*Clust 2*						*Clust 3*	*Clust 4*
	GOUL	SCHN	HIKA	NYDA	YNCY	TAWO	FORS	HALM	MELV
GOUL	-	0.05	**0.09**	**0.11**	**0.10**	0.03	0.05	0.05	**0.03**
SCHN	0.01	-	**0.06**	**0.03**	0.00	**0.07**	**0.14**	**0.08**	**0.05**
HIKA	**0.05**	**0.02**	-	0.00	**0.08**	**0.09**	**0.15**	**0.06**	**0.08**
NYDA	**0.05**	**0.01**	0.00	-	**0.04**	**0.14**	**0.20**	**0.11**	**0.11**
YNCY	**0.04**	0.00	**0.04**	**0.02**	-	**0.15**	**0.18**	**0.11**	**0.12**
TAWO	0.01	**0.03**	**0.04**	**0.06**	**0.06**	-	**0.08**	**0.06**	**0.04**
FORS	0.03	**0.07**	**0.09**	**0.10**	**0.10**	**0.05**	-	0.00	0.05
HALM	**0.01**	**0.02**	**0.02**	**0.04**	**0.04**	**0.02**	0.01	-	**0.06**
MELV	**0.02**	**0.02**	**0.04**	**0.05**	**0.06**	**0.02**	0.03	0.01	-

c. 2 HOUR PLD

	Clust 1	*Clust 2*	*Clust 3*	*Clust 4*	*Cluster 5*	*Clust 6*	*Clust 7*	*Clust 8*	*Clust 9*
	GOUL	SCHN	HIKA	NYDA	YNCY	TAWO	FORS	HALM	MELV
GOUL	-	0.05	**0.09**	**0.11**	**0.10**	0.03	**0.05**	0.05	**0.03**
SCHN	0.01	-	**0.06**	**0.03**	0.00	**0.07**	**0.14**	**0.08**	**0.05**
HIKA	**0.05**	**0.02**	-	0.00	**0.08**	**0.09**	**0.15**	**0.06**	**0.08**
NYDA	**0.05**	**0.01**	0.00	-	**0.04**	**0.14**	**0.20**	**0.11**	**0.11**
YNCY	**0.04**	0.00	**0.04**	**0.02**	-	**0.15**	**0.18**	**0.11**	**0.12**
TAWO	0.01	**0.03**	**0.04**	**0.06**	**0.06**	-	**0.08**	**0.06**	**0.04**
FORS	**0.03**	**0.07**	**0.09**	**0.10**	**0.10**	**0.05**	-	0.00	0.05
HALM	0.01	**0.02**	**0.02**	**0.04**	**0.04**	**0.02**	0.01	-	**0.06**
MELV	**0.02**	**0.02**	**0.04**	**0.05**	**0.06**	**0.02**	**0.03**	0.01	-

Table A5.9. Pairwise FST (below diagonal) and Jost's *D* (above diagonal) matrix (including all loci, except Dvex10 and Dvex19) for each cluster grouping, from 24 hours (a), to 12 hours (b) and 2 hours (c). Significant pairwise combinations after False Discovery Rate corrections for multiple tests (N=35) are indicated in bold, $P \leq 0.01$. Pelorus Sound populations include: GOUL=Goulter Bay, SCHN=Schnapper Point, HIKA=Hikapu Reach, NYDA=Nydia Bay, YNCY=Yncyca Bay, TAWO=Tawero Point, HALM=Hallam Cove, FORS=Forsyth Bay and MELV=Melville Cove.

a. 24 HOUR PLD

	Clust 1	*Clust 2*							*Clust 3*
	GOUL	SCHN	HIKA	NYDA	YNCY	TAWO	HALM	FORS	MELV
GOUL	-	0.05	**0.05**	**0.06**	**0.11**	0.03	**0.05**	**0.06**	0.03
SCHN	0.01	-	0.04	0.00	0.01	**0.09**	**0.11**	**0.16**	**0.07**
HIKA	**0.03**	**0.02**	-	0.01	**0.09**	0.03	**0.07**	**0.12**	**0.07**
NYDA	**0.02**	0.00	0.00	-	0.03	**0.09**	**0.13**	**0.18**	**0.10**
YNCY	**0.02**	0.00	**0.05**	0.02	-	**0.17**	**0.15**	**0.21**	**0.15**
TAWO	0.05	**0.04**	0.01	**0.04**	**0.08**	-	0.03	**0.07**	**0.05**
HALM	0.02	**0.04**	**0.03**	**0.04**	**0.06**	0.01	-	-0.01	0.05
FORS	**0.01**	**0.09**	**0.08**	**0.10**	**0.12**	**0.05**	-0.01	-	**0.06**
MELV	0.04	**0.03**	**0.04**	**0.04**	**0.08**	**0.03**	0.00	**0.04**	-

b. 12 HOUR PLD

	Clust 1	*Clust 2*						*Clust 3*	*Clust 4*
	GOUL	SCHN	HIKA	NYDA	YNCY	TAWO	FORS	HALM	MELV
GOUL	-	0.05	**0.05**	**0.06**	**0.11**	0.03	**0.06**	**0.05**	0.03
SCHN	0.01	-	0.04	0.00	0.01	**0.09**	**0.16**	**0.11**	**0.07**
HIKA	**0.03**	**0.02**	-	0.01	**0.09**	0.03	**0.12**	**0.07**	**0.07**
NYDA	**0.02**	0.00	0.00	-	0.03	**0.09**	**0.18**	**0.13**	**0.10**
YNCY	**0.02**	0.00	**0.05**	0.02	-	**0.17**	**0.21**	**0.15**	**0.15**
TAWO	0.05	**0.04**	0.01	**0.04**	**0.08**	-	**0.07**	0.03	**0.05**
FORS	**0.01**	**0.09**	**0.08**	**0.10**	**0.12**	**0.05**	-	-0.01	0.05
HALM	0.02	**0.04**	**0.03**	**0.04**	**0.06**	0.01	-0.01	-	**0.06**
MELV	0.04	**0.03**	**0.04**	**0.04**	**0.08**	**0.03**	**0.04**	0.00	-

c. 2 HOUR PLD

	Clust 1	*Clust 2*	*Clust 3*	*Clust 4*	*Cluster 5*	*Clust 6*	*Clust 7*	*Clust 8*	*Clust 9*
	GOUL	SCHN	HIKA	NYDA	YNCY	TAWO	FORS	HALM	MELV
GOUL	-	0.05	**0.05**	**0.06**	**0.11**	0.03	**0.06**	**0.05**	0.03
SCHN	0.01	-	0.04	0.00	0.01	**0.09**	**0.16**	**0.11**	**0.07**
HIKA	**0.03**	**0.02**	-	0.01	**0.09**	0.03	**0.12**	**0.07**	**0.07**
NYDA	**0.02**	0.00	0.00	-	0.03	**0.09**	**0.18**	**0.13**	**0.10**
YNCY	**0.02**	0.00	**0.05**	0.02	-	**0.17**	**0.21**	**0.15**	**0.15**
TAWO	0.05	**0.04**	0.01	**0.04**	**0.08**	-	**0.07**	0.03	**0.05**
HALM	**0.01**	**0.09**	**0.08**	**0.10**	**0.12**	**0.05**	-	-0.01	0.05
FORS	0.02	**0.04**	**0.03**	**0.04**	**0.06**	0.01	-0.01	-	**0.06**
MELV	0.04	**0.03**	**0.04**	**0.04**	**0.08**	**0.03**	**0.04**	0.00	-