Using Next Generation Sequencing methods to understand biofouling assemblages

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

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Abstract

Man-made infrastructure has now become a common feature of the coastal environments due to increasing human activities. However, the introduction of these structures has caused ecological and environmental impacts. They have also been considered the first point of entry for introduced species brought by shipping. The study of biofouling assemblages that develop on these hard surfaces showed that introduced species were more likely to settle on these artificial structures than native species. The development of biofouling assemblages is usually influenced by biotic and abiotic factors and the study of biofouling assemblages over time could provide an insight into the effects of anthropogenic activities on the growth of biofouling organisms. This was achieved by observing and assessing the development of biofouling assemblages at several time points in places with different levels of human activities. Biodiversity assessment has been carried out in many ecological studies using metabarcoding in recent years and settlement plates have been widely used for studies on biofouling. The main aim of this thesis was to examine the efficacy of metabarcoding in the early detection of introduced species and to assess the development of biofouling community in Gippsland Lakes, Victoria and Gulf of St Vincent, South Australia over a period of six and twelve months, respectively.

To study biofouling assemblages, Perspex settlement plates were deployed at several sampling sites in the two locations. Plates were removed at one, three and six months of deployment in Gippsland Lakes and at one, three, six, nine and twelve months of deployment in South Australia. The biofouling material collected was processed for DNA extraction. Over time, the biofouling assemblages increase, and to provide an accurate biodiversity assessment of the community, a large amount of biofouling material (>10 g) is required. However, standard DNA extraction kits (PowerMax® Soil DNA Isolation Kit) only allows up to 10 g of sample to be processed at a time. I assessed the suitability of freeze drying to process biofouling samples that weigh more than 10 g in Chapter 2. This method allowed removal of water from samples, reducing their weight and allowing them to be efficiently processed for DNA extraction. Importantly, the purity of the DNA was not compromised, and it was tested for PCR using the primers which amplify the hypervariable V4 region of the 18S ribosomal RNA. The amplicons were further used for high throughput sequencing for all chapters looking at development of biofouling assemblages. This study showed that freeze drying is an effective
In Chapter 3, I discuss the use of settlement plates and metabarcoding as a potential non-invasive approach for early detection of introduced species. To detect the presence of introduced species at a stage where the organisms were not readily identifiable, i.e. at their early life history stages, biofouling samples collected from Gippsland Lakes after one month of plate deployment were assessed. My findings showed that commonly known marine invasive species (MIS) such as Asian bag mussel *Arcuatula senhousia* were detected. A quantitative polymerase chain reaction (qPCR) analysis confirmed its detection. The introduced sea grape tunicate *Molgula manhattensis* was detected and its last reported detection in Australia was in 1976. Further molecular work using PCR-based Sanger sequencing of 28S marker and BLAST analysis confirmed the identity. Furthermore, the findings from this study showed that introduced species can be detected using settlement plates and metabarcoding when the organisms were at their early life history stages.

The ability of metabarcoding to produce a large database of DNA sequences to identify organisms makes it a robust tool for assessing biodiversity. The combined use of metabarcoding and settlement plates provided baseline information on the biodiversity in biofouling assemblages in the Gippsland Lakes, Victoria (Chapter 4) and South Australia (Chapter 5). In an estuarine system such as the Gippsland Lakes, the differences in species composition of the biofouling assemblages were attributed to the location of the sampling sites. Moreover, differences in salinity could account for the different species assemblages at the three locations. The difference in recreational boating traffic is also a possible factor in influencing the species composition in Paynesville and Metung. To assess the differences in biofouling assemblages in places of varying anthropogenic activities, I have also examined the species composition of biofouling assemblages in several locations in South Australia for twelve months. To the best of my knowledge, this is the first study that utilises metabarcoding to study development of biofouling assemblages over a period of twelve months. The findings from this chapter show that the species composition among all locations differed. The highest difference was observed between Kingscote and North Haven and the presence of introduced species had the highest effect in North Haven, which is considered to be a highly disturbed area. For example, the ascidian *Ciona intestinalis* dominated the biofouling community in North Haven over twelve months while ascidian *Asidiella aspersa* was most dominant in the
first six months in North Haven. Frequent shipping among North Haven, Wirrina Cove and Christmas Cove may have encouraged transfer of species as the species composition in the biofouling communities had high level of similarity. However, Wirrina Cove and Christmas Cove had higher similarity which could be attributed to similar environmental conditions. Findings provide an insight into understanding how environmental factors and human activity affect the growth of biofouling assemblages.

In summary, the findings from this thesis provided baseline information on the biofouling assemblages on the settlement plates in Gippsland Lakes and Gulf of St Vincent. It also allowed the detection of known and new introduced species. My research highlighted the changes in the successional stages of biofouling assemblages in places with different levels of anthropogenic activities. Moreover, the findings indicated that the presence of introduced species, especially in areas of high anthropogenic activity, may affect the biofouling assemblages. This study highlights the importance of understanding the ecological impacts of man-made structures in the environment and the need to carry out regular monitoring especially in smaller marinas and domestic ports.
Chapter 1:
General Introduction
Chapter 1: Introduction

Communities on hard surfaces are usually found on the bottom of the seafloor and in the water column and these hard surfaces are habitats to tens of thousands of organisms (Railkin, 2003; Wahl, 2009a). Three typical groups of hard surfaces can be distinguished (Railkin, 2003). The first group is composed of non-living natural substrates such as underwater rocks and stones, and the second group of hard substrates refers to artificial/man-made substrates such as metal, wood and plastic. In the third group, the surface of living animals and plants such as algae provide habitats to other marine organisms. Sessile species are usually the most dominant life forms found in these assemblages (Railkin, 2003). They are usually represented by sponges, cnidarians, bryozoans, and ascidians (Railkin, 2003; Wahl, 2009a). These sessile organisms in turn provide a new surface for other organisms creating a multilayered and complex community. For example, the jewel box clam, *Chama pellucida* provides habitat to other sessile species also known as epibionts (Vance, 1978). Borers are also typically found in hard-bottom communities. The shell boring polychaete *Polydora ciliata* survives on the periwinkle *Littorina littorea* (Thieltges and Buschbaum, 2007). Vagile species may also be found and are not restricted to hard-bottom communities only. Their ability to reside on these hard surfaces lie in the features that they possess to adhere to the surface of the substrate (Railkin, 2003). The complex community created by sessile organisms such as macroalgae also provide additional habitat for vagile species to survive in such communities. The vagile species usually include the platyhelminths, nematodes, echinoderms and vertebrates (Wahl, 2009a). Hard-bottom communities reflect the environment they are found in and are shaped by the environmental conditions governing that area, abundance of local marine organisms and their interactions in the community (Richmond and Seed, 1991; Nagabhushanam and Thompson, 1997).

According to Wahl (1989), fouling is referred to as the colonization on a solid living or non-living surface. Fouling has been mostly attributed to man-made substrates and has been associated with negative impacts (Nagabhushanam and Thompson, 1997; Yebra et al., 2004). For example, biofouling increases frictional resistance of ships leading to increased fuel consumption. In addition, deterioration, including corrosion and discolouration are more likely to occur on fouled structures such as piers and bridges. This incurs increased maintenance costs. Consequently, considerable time and resources are also spent in removal of the excess
layer of fouling organisms. Costs related to biofouling are significant. Importantly, biofouling is also a major cause of introduction of marine species into new environments and these introduced species could become marine invasive species (MIS). Introduced species may be transported as biofouling on hulls and man-made structures in coastal environments are more likely to favour introduced species over local species (Godwin, 2003; Tyrrell and Byers, 2006; Dafforn et al., 2009). In Australia, the cost to eliminate major MIS has been estimated as high as AUD$263 million (Crombie et al., 2007). The establishment of invasive species has serious impacts on biodiversity as it may also lead to species composition being homogenised over space and thus losing unique traits of specific communities (Lewis et al., 2003).

The biofouling process usually starts when the hard surface is immersed in water and the process of colonization occurs in four main stages namely the biochemical conditioning, the colonization of bacteria, colonization by unicellular eukaryotes and the colonization by multicellular eukaryote (Wahl, 1989; Maki, 1999; Railkin, 2003).

1.1 Stages of biofouling

1.1.1 Biochemical conditioning

Biochemical conditioning, also known as molecular fouling/adsorption, occurs when a solid surface is immersed in water leading to adsorption of macromolecules creating a conditioning film (Little and Zsolnay, 1985). These macromolecules include proteins, glycoproteins, proteoglycans and polysaccharides. The absorbed constituents of the molecular fouling layer have the capacity to change the environment of the solid surface, and the ability to promote or inhibit the establishment of bacteria (Fletcher, 1975). As shown in Figure 1.1, adsorption of macromolecules can occur almost immediately after the solid surface is immersed.

![Figure 1.1: Sequence of colonization. Process of colonization from the moment the solid surface is immersed into water. The biofilm is produced after one minute while microfouling occurs after an hour of immersion. This figure was taken from Wahl (1989).](image)
1.1.2 Bacterial colonization

In the microfouling process, both bacteria and unicellular prokaryotes usually colonise the surface of the substrate. After one hour, bacteria are among the first to colonise the biofilm (Zobell, 1943). During this phase, bacterial activity may be enhanced when there is adsorption of organic matter on solid surfaces (Zobell, 1943) with the rate of bacterial colonisation being strongly influenced by the characteristics of the substratum (Nagabhushanam and Thompson, 1997). Two factors that affect colonisation are surface roughness and chemistry. Net cell accumulation is higher on rough surfaces than on smooth surfaces. The chemistry has to be favourable for the bacteria to facilitate attachment to a particular surface. For example, the levels of Ca$^{2+}$ and Mg$^{2+}$ has been shown to be important for the adhesion of marine microorganisms such as *Pseudomonas mendocina* (Mangwani et al., 2014; He et al., 2016). The succession in the bacterial community is dependent on the features and the physiological requirements of the bacteria (Wahl, 1989).

1.1.3 Colonization by unicellular eukaryotes

Unicellular eukaryotes, which consist of yeasts, protozoa and diatoms, usually arrive after colonisation of bacteria in most biofouling communities (Wahl, 1989). Studies have shown that diatoms are the most dominant species to colonise the biofilm (Wahl, 1989; Maki, 2002; Lam et al., 2005). Watson et al. (2015) demonstrated that a successful bacterial colonisation provides a conducive environment for ciliates to thrive in. Interestingly, as the bacterial biofilm matured, there was a shift in ciliate communities as the colonies of sessile ciliates were gradually dominated by colonies of vagile ciliates. Many unicellular eukaryotes are carried mostly to the hard substrates through hydrodynamic means (Maki, 2002) and once they arrive on the surface, their adhesion is dependent on the nutrient gradients present on the surface (Cooksey and Wigglesworth-Cooksey, 1995). For example, adhesion and motility are dependent on Ca$^{2+}$ availability (Cooksey and Wigglesworth-Cooksey, 1995). Early settlers may change the conditions that will favour one species over others depending on the surface properties as well as the environment. Thus, no single pathway has been found to explain the mechanisms of succession (Railkin, 2003).
1.1.4 Colonisation by multicellular eukaryotes

Colonisation by multicellular eukaryotes also known as macrofouling is the last phase in which the planktonic larvae of sessile invertebrates and propagules of macroalgae will settle depending on different kinds of biochemical stimuli. For example, ammonia concentration influenced the settlement of larval oysters *Crassostrea virginica* and *Crassostrea gigas* (Fitt and Coon, 1992). Another example showed that levels of organic carbon and nitrogen promoted growth of *Phestilla* larvae which had settled to the substratum (Hadfield and Scheuer, 1985). This occurs in two stages. Firstly, larvae will look for a suitable spot where they can settle. Several studies have shown that fast growing organisms will be among the first colonisers which usually include hydroids, polychaetes and bryozoans, followed by slow-growing species such as molluscs, sponges and ascidians (Scheer, 1945; Railkin, 2003). The next stage is the metamorphosis stage where organisms such as barnacles, bryozoans and polychaetes further develop and release a permanent adhesive (Clare et al., 1992; Maki, 2002).

1.2 Models of the biofouling process

Two models have been proposed to explain how biofouling develops (Figure 1.2) (Clare et al., 1992; Terlizzi and Faimali, 2010). Firstly, the classical model implies that the stages of biofouling are sequential and there is a succession between the stages with the molecular fouling being the initial stage followed by microfouling and macrofouling, respectively (Davis et al., 1989). However, the second model, namely the dynamic model, is considered to be a more accurate description of the biofouling process. Maki (2002) argues that in the dynamic model, there is no succession in the process of fouling. Instead, the biofouling process could be caused by seasonality and stochastic factors, and the biofouling stages may be occurring simultaneously (Terlizzi and Faimali, 2010). The probabilistic view implies that factors such as amount of both molecules and organisms that settle on the surface influence biofouling while seasonality affects the recruitment of juveniles on the substrate as the life cycle of marine species is attuned to the seasonal change (Maki, 2002). Several studies indicated that settlement based on seasonality is more likely to occur in colder waters due to higher fluctuations in temperature (Osman, 1977; Richmond and Seed, 1991; Bowden, 2005; Bowden et al., 2006). However, other studies indicate that it is not always the case, and recruitment may be dependent on other factors (Scheer, 1945; Svane, 1988). Moreover, the environmental conditions and complex species interactions such as competition and predation
occurring in the biofouling community renders this process difficult to predict (Richmond and Seed, 1991; Clare et al., 1992; Terlizzi and Faimali, 2010).

Figure 1.2: Models proposed for biofouling. (a) Classical model of biofouling occurring as successive stages. (b) Dynamic model whereby the conditioning film is the primary stage and other organisms have equal opportunity to establish on the surface (Maki, 2002).

Succession occurs in the classical model as species composition and abundance change following a disturbance that has cleared space (Connell and Slatyer, 1977). There have been many studies to explain how succession occurs in marine hard-bottom communities. Experimental studies by Scheer (1945) suggested that succession occurred with the initiation of biofilm formation, followed by fast-growing organisms and slow-growing organisms and ended with a short-term climax. This succession model has also been observed in other studies (Redfield and Deevy, 1952). However, this succession theory has not always been relevant and this was the case when fast-growing organisms were absent in biofouling communities (Railkin, 2003). Connell and Slatyer (1977) put forward three models to explain the progressive pattern of species composition. The “facilitation” model describes the colonisation of an area after an ecological disturbance is dependent on the traits of the early colonizers. The second model is known as the “tolerance” model, where the environmental modifications caused by early settlers neither inhibit nor promote the growth of later colonizers. The third model is the “inhibition” model, which describes the inhibitory effect exhibited by the earlier settlers on later successional species. This model is characterised by the change in environmental conditions caused by early settlers, rendering the environment unconducive for potential settlers to colonise. Studies have supported these models of succession (Dean and Hurd, 1980). However, succession is not limited to only the models put forward. Species succession is also
affected by the temporal and spatial variability, features of substrates, growth and mortality which contributes to the complexity of the biofouling process (Jenkins and Martins, 2010).

1.3 Recruitment and settlement of marine species

There are several factors that affect the recruitment and settlement of marine species. These include among other things substratum texture, water current and light exposure (Anderson and Underwood, 1994). The substratum affects initial colonisation due to its physical and chemical composition. The substrate affects the settlement of the fouling organisms and studies have shown that fouling material is less likely to accumulate on smooth, hard, non-porous and non-fibrous surfaces (Pomerat and Weiss, 1946). For example, the oyster *Saccostrea commercialis* and barnacles *Hexaminus* spp., *Balanus amphitrite* and *Balanus variegatus* settled better on concrete and plywood surfaces compared to fiberglass or aluminium (Anderson and Underwood, 1994). A species settling on a particular surface may become abundant and influence the composition of other species on the surface as well as subsequent colonization (Anderson and Underwood, 1994). With time, the species composition tend to be less differentiated so time of submersion is an important factor where the effects of substrate is significant only at short periods of time (1-2 months) (Anderson and Underwood, 1994).

1.4 Biological invasions

Biological invasions are one of the biggest human-caused environmental threats to native biodiversity (Vitousek et al., 1997). Marine species introduced into an environment outside of their range can become invasive when they cause detrimental effects to the environment. These MIS have led to a range of biological, social and economic impacts (Vitousek et al., 1997; Bax et al., 2003; Simberloff et al., 2013). They could modify habitats upon their arrival which led to alterations in species interactions and contributed to biodiversity loss (Lodge, 1993). For example, the introduction of quagga (*Dreissena bugensis*) and zebra mussels (*Dreissena polymorpha*) have caused considerable ecological changes in the Great Lakes, USA (Wilson et al., 2006). They have changed the habitat by providing more hard surfaces, altered the nitrogen nutrient cycling and the phosphorus cycle, causing a decline in the native mussel populations (Ricciardi et al., 1998). The Northern Pacific sea star (*Asterias amurensis*) is considered one of the world’s worst invasive species and is well established in
southern Australia (ISSG, 2019; Ross et al., 2002). It is a successful opportunistic and
generalist predator feeding on a range of marine species and has led to the decline of juvenile
commercial *Fulvia tenuicostata* in fisheries in Tasmania (Ross et al., 2002). Moreover, these
biological impacts of MIS have also caused considerable economic and social impacts (Bax et
al., 2003). They negatively affect marine-based industries such as commercial fisheries,
aquaculture, tourism and marine infrastructure (Galil and Zenetos, 2002; Ross et al., 2002; Bax
et al., 2003; Nunes and Markandya, 2008; Fletcher et al., 2013). Social impacts could result in
a decrease in employment opportunities related to effects of MIS and deterioration of human
welfare caused by negative changes in the environment (Bax et al., 2003). In North European
waters, the introduction of harmful algal bloom species has caused considerable damage to the
tourism industry (Nunes and van den Bergh, 2004; Nunes and Markandya, 2008). MIS have
caused considerable economic impacts in aquaculture which is considered one of the primary
sectors in providing food resource to growing population (De Silva et al., 2009). For example,
in Canada, the non-native ascidian *C. intestinalis* has led to a decrease in the production of the
mussel *Mytilus edulis* by competing for food (Daigle and Herbinger, 2009).

The process of invasion remains the same for every MIS and is based on a simplified
model (Figure 1.3). An invasion process starts when a marine species is taken up from its native
environment and enters a pathway (Lockwood et al., 2005; Olenin et al., 2011). This pathway
involves the use of vectors which will translocate the marine organisms from one region to
another. For example, in shipping, marine species may be transported in hull and ballast water
(Rilov and Crooks, 2009). Once it arrives in a new environment, there is a certain time for
adaptation. During this time, there are several factors that will influence the establishment of a
marine organism (Williamson, 1996). The survival of the species depends on the sustainable
resources available and the absence of predators. There are some cases where there may be
vacant niches which the introduced species may occupy. Also, the presence of the native
species composition and diversity may play a role in the establishment of the introduced
species. For a successful establishment, it is crucial to not only survive in a new environment
but the rate of reproduction of an introduced species should also surpass the rate of death of
the species (Williamson, 1996). After producing recruiting juveniles and increasing its
population, a successful invader must be able to expand its geographical range. Propagule
pressure is an important and persistent factor that contributes to the success of invasion
(Lockwood et al., 2005). The greater the amount of larvae of introduced species released into
a new environment, the higher the risk that invasion will occur (Lockwood et al., 2009).
Figure 1.3: Conceptual model of the invasion process. The translocation process involves 5 stages – colonization, translocation, transfer (represented by the black arrow). The invasion process depends on the propagule pressure (white arrow) Adapted from Lockwood et al. (2005).

1.5 Vectors of invasion

Due to increasing globalisation, the spread of MIS has accelerated with the introduction of trade routes, global trade and habitat modification due to population growth and higher efficiency in shipping (Hulme, 2009; Williams et al., 2013). Marine species have been translocated from a source region by transport vectors to donor regions (Fofonoff et al., 2003; Minchin et al., 2009). There are several pathways which have been recognised in the spread of introduced species in the marine environment. The vector categories in each pathway have been classified according to Minchin et al. (2005) (Table 1.1). Translocation of MIS has been predominantly caused by shipping globally (Hewitt et al., 2004; Streftaris et al., 2005; Gollasch, 2006; Ricciardi, 2006; Gollasch, 2007; Molnar et al., 2008; Ferreira et al., 2009; Hayden et al., 2009; Mead et al., 2011; Peters et al., 2014; Schwindt et al., 2014; Ruiz et al., 2015). The second most common human-mediated pathway was aquaculture (Molnar et al., 2008).
Table 1.1: Pathways and associated vectors responsible for the translocation of introduced species. Adapted from Minchin et al. (2005)

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Vectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Ships, moveable structures (oil platforms, docks, buoys), recreational boating</td>
<td>Hull fouling</td>
</tr>
<tr>
<td></td>
<td>Ballast water and sediment</td>
</tr>
<tr>
<td></td>
<td>Aquatic cargo</td>
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<tr>
<td></td>
<td>Dredge spoil</td>
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<tr>
<td></td>
<td>Small craft trailers</td>
</tr>
<tr>
<td>2 Aquaculture activities</td>
<td>Intentional release and stock movements</td>
</tr>
<tr>
<td></td>
<td>Accidental release with related species</td>
</tr>
<tr>
<td></td>
<td>Infrastructure – movements and discarded</td>
</tr>
<tr>
<td></td>
<td>Discharge of feeds (Live, fresh, frozen)</td>
</tr>
<tr>
<td>3 Wild fisheries</td>
<td>Movement of stock with related species</td>
</tr>
<tr>
<td></td>
<td>Reestablishment of stock species and related species</td>
</tr>
<tr>
<td></td>
<td>Processing of the live and frozen food</td>
</tr>
<tr>
<td></td>
<td>Infrastructure – discarded and movement</td>
</tr>
<tr>
<td></td>
<td>Intentional release of marine species – live and frozen</td>
</tr>
<tr>
<td>4 Aquarium industry &amp; public aquaria</td>
<td>Intentional release and related species</td>
</tr>
<tr>
<td></td>
<td>Accidental release and related species</td>
</tr>
<tr>
<td></td>
<td>Species located on the accessories</td>
</tr>
<tr>
<td></td>
<td>Untreated tank and waste discharge</td>
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<tr>
<td></td>
<td>Living food</td>
</tr>
<tr>
<td>5 Marine leisure tourism</td>
<td>Live bait</td>
</tr>
<tr>
<td></td>
<td>Accidental transport &amp; release of catch</td>
</tr>
<tr>
<td></td>
<td>Intentional transport &amp; release of catch</td>
</tr>
<tr>
<td></td>
<td>Diving and fishing equipment</td>
</tr>
<tr>
<td></td>
<td>Live souvenirs and related species</td>
</tr>
<tr>
<td>6 Research Education</td>
<td>Intentional release of species and related species in field experiments</td>
</tr>
</tbody>
</table>
• Accidental release of species and related species
• Discharge of wastes
• Movement of experimental, diving and field gear

7 Others
• Biological control
• Navigation and irrigation canals
• Discharge of waste treatment

1.5.1 Aquaculture

The aquaculture industry provided 80 million tonnes of fish for human consumption in 2016. This consisted of finfish (67.6%), molluscs (21.4%), crustaceans (9.8%) and other aquatic animal species (1.2%) (FAO, 2018). The infrastructure required for production usually consists of cages, nets and ropes which provide habitat for fouling species, causing considerable economic and biological impacts (Fitridge et al., 2012). In fact, biofouling costs amount to 5-30% of final market price of cultivated marine species (Sievers et al., 2017; Adams et al., 2011). Fouling species may compete with bivalves for resources, add weight to shellfish and affect the environmental conditions in the aquaculture infrastructure (Adams et al., 2011; Fitridge et al., 2012; Lacoste and Gaertner-Mazouni, 2014). For example, fouling by invasive ascidian, Didemnum vexillum, led to a reduction in the density of small mussels Perna canaliculus (Fletcher et al., 2013).

Although some marine species have been raised for human consumption, their introduction in some regions in the world has caused ecological impacts to the local marine environment (Minchin et al., 2005). For example, the Pacific oyster, C. gigas, which originated from east Asia is an important aquaculture species (Wrange et al., 2010; FAO, 2018). However, it is considered invasive worldwide due to its ability to modify habitats and compete with native bivalve species for resources (Molnar et al., 2008; Wrange et al., 2010; Rinde et al., 2017). Moreover, the regular transportation of cultured stock by sea represents a potential risk for introduction of MIS as other marine species may be unintentionally carried with cultured stock (Minchin, 2007; Minchin et al., 2009). Their release into a foreign environment would become a threat to the local species. A possibility of interbreeding with the native species may also occur (Naylor et al., 2005).
1.5.2 Shipping

Shipping has played a major role in the translocation of introduced marine species (Fofonoff et al., 2003; Minchin, 2006). Global trade and transport have contributed largely to the spread of marine species as the rate of commercial shipping has increased (Kolar and Lodge, 2002; Hulme, 2009; Seebens et al., 2013). International shipping ports are considered hotspots for the spread of introduced species due to the heavy shipping traffic and connectivity to many other vectors (Carlton, 1996b; Floerl et al., 2009). Moreover, the establishment of introduced species is facilitated in such environments due to repeated introductions and translocation to other transport hubs (Carlton, 1996b; Minchin et al., 2006; Floerl et al., 2009; Ruiz et al., 2000). Although commercial shipping has been a major pathway of introduced species for many years, recreational boats have been recently considered a major threat as they provide a path to neighbouring domestic ports (Floerl and Inglis, 2005; Minchin, 2006; Davidson et al., 2010; Clarke Murray et al., 2011; Clarke Murray et al., 2014). In marinas, smaller boats and yachts constitute a huge majority of the shipping and travelling to neighbouring bays and estuaries provide a connecting network (Minchin et al., 2006; Davidson et al., 2010). Moreover, small craft are more likely to remain unused for weeks and months promoting biofouling growth while large ships usually remain in ports for days (Minchin et al., 2006).

Ships and boats translocate species by two main vectors, namely ballast water from ships and fouling on hulls, propellers, sea chests and anchors (Carlton, 1985; Ruiz et al., 1997; Rilov and Crooks, 2009). Ballast water is an important aspect in a ship as it balances the weight which contributes to the proper buoyancy of the ship (Walters, 1996; David, 2015). While it plays a vital role in the proper functioning of a ship, ballast water is usually loaded and discharged from one port to another, and its release represents a risk of introducing marine species into new environments (Wonham et al., 2001). Ballast water has allowed the translocation of zooplankton, phytoplankton, fish and protists across oceans (Hallegraeff and Bolch, 1991; Carlton and Geller, 1993; Galil and Hülsmann, 1997; Wonham et al., 2000). Fouling on ships represents another major of vector for spreading introduced species as it was estimated that the biofouling assemblages on the surface consisted of 100-200 species (Drake and Lodge, 2007). Although fouling organisms are usually sessile, the biofouling community also consists of mobile marine species, epibionts, bacteria, parasites and diseases (Minchin and
Gollasch, 2003). Hull fouling provide an environment that would not be suitable for marine species if they had been in ballast water (Lewis and Coutts, 2009). For example, the invasion of Asian kelp *Undaria pinnatifida* in South Australia was facilitated by vessels carrying woodchips from Tasmania to Japan (Lewis and Coutts, 2009). Although ballast water was initially regarded as a possible vector, microscopic experiments showed the spores of *U. pinnatifida* were surviving on hulls. Moreover, these spores are usually short-lived and can only tolerate temperatures up to 29°C, which is the usual temperature on a ship’s hull in the tropics. Thus, their survival would not have been possible in ballast water (Lewis and Coutts, 2009). The accumulation of bilge water within the hull has also been recently considered as a potential vector for introduced species (Fletcher et al., 2017). Both juvenile and adult life-stages of marine species have been found in bilge water.

Increase in trade opened new routes for shipping and remote places have now become accessible. The creation of canals has also enabled the transfer of organisms and removed migration barriers for marine organisms (Gollasch, 2006; Gollasch, 2007; Minchin et al., 2009). For example, the opening of the Suez Canal connected the Red Sea and Mediterranean Sea which allowed exchange of marine organisms between both seas (Gollasch, 2006). The introduction of the Panama Canal has also linked the Caribbean and the eastern Pacific side, but there have been fewer migrations as marine organisms must pass through fresh water (Cohen, 2006).

### 1.6 Factors contributing to the introduction of species in a new environment

#### 1.6.1 Propagule pressure

Propagule pressure is defined as the number of propagules released in an environment where they are not considered native. This involves both the amount of individuals released in one event (propagule size) and the number of events (propagule number) occurring (Carlton, 1996a; Johnston et al., 2009; Simberloff, 2009). It is an important factor that affects colonization and may contribute to the successful settlement of introduced species (Rilov and Crooks, 2009; Wahl, 2009b). Marine animals produce considerable amounts of larvae to ensure their survival. These propagules will be carried by currents and usually attach to the surface of ships leading to migration. Propagule pressure increases when the number of individuals in one release event increases and/or the release events increases (Lockwood et al., 2005). Once the
larvae are introduced into the environment, the introduced species must adapt to the environment to ensure its survival and be able to reproduce.

1.6.2 Spatial dispersion

Spatial dispersion is an important factor that contributes to introduction of species in a new environment. When immigration of the introduced species occurs over a wider geographical range, invasion is less likely to occur. The success of invasion also increases with the combination of a higher rate and magnitude of immigration events (Drury et al., 2007). Thus, when ships travelled to nearby ports on a frequent basis carrying introduced species, these species may eventually be translocated to the nearby environment. The transport of eggs and propagules in ballast water represents a potential risk due to the discharge of ballast water at the arriving ports (Lewis and Coutts, 2009). The spatial dispersion is further supported by the fact that dispersion of marine species is dependent on established shipping routes (Seebens et al., 2013). However, with global trade increasing, places that were considered distant are now more likely to be invaded. The new shipping routes increase the chances of higher numbers of marine species being introduced (Geburzi and McCarthy, 2018).

1.6.3 Anthropogenic activity

Anthropogenic activity at a location is an important factor that determines the likelihood of an invasion. Human-related activities have led to creation of new hard bottom habitats and loss of native habitats for marine organisms especially along coastlines (Mineur et al., 2012). In areas where hard-bottom habitats were not present, their introduction could change the ecosystem and affect the marine species composition (Caine, 1987). Also, introduced species are more likely to settle on artificial structures than native species (Connell and Glasby, 1999; Glasby and Connell, 1999; Glasby et al., 2007; Dafforn et al., 2009) and these structures usually provide the first habitat once introduced species are released into a new environment (Bax et al., 2003; Fofonoff et al., 2003). Biofouling communities on hard substrates are considered high risk as they are more likely to be invaded than other habitats such as soft substrates (Wasson et al., 2005; Jimenez et al., 2017). Fouling introduced species have a preference for artificial hard substrates, and this could be attributed to the occurrence of hard substrates in their native environment which allowed them to adapt to such surfaces.
1.7 Factors contributing to the success of invasion

1.7.1 Features of MIS

Successful MIS exhibit a high level of competitiveness with endemic species and a high rate of fertility (Branch and Steffani, 2004; Wahl, 2009b). Although there is no consistency in the lifestyle characteristics of successful MIS, Nyberg and Wallentinus (2005) demonstrated that there are correlations in the features that contribute to the effectiveness of invasion. They conducted a study on the dispersal patterns of marine macroalgae and they were ranked according to their efficiency of dispersing, rate of establishment and the high ecological impact they pose. There were 15 species which were listed as invasive among the top 20 highest rank comprising of a list of invasive and native species. Williamson and colleagues (1986; 1996) have come up with a statistical rule (the tens rule) that suggests that the probability of an introduced species becoming an invasive species would be 1 in 1000. This is explained by the invasive species succeeding 3 levels of establishment (Figure 1.4).

![Figure 1.4: Schematic diagram illustrating the tens rule. There are 3 levels before an introduced species becomes a pest and the probability in each case was 1 in 10. In the “transported” batch (blue circles), one (red circle) would become introduced and in the “introduced” batch (red circles), one (yellow circle) would become established. Finally, in the “established” batch (yellow circles), one (green circle) would be considered invasive. Adapted from Williamson and colleagues (1986; 1996).](image)

There are several factors that determine the success of invasions (Williamson, 1996). Invasion success depends on the ability of MIS to create environment that will be unsuitable for native species. Only species that have developed adaptive strategies to settle on artificial structures can survive and usually native species are not able to adapt on such surfaces (Lewis and Coutts, 2009). The frequency of MIS translocation also contributes to the success of invasion.
1.7.2 Community invasibility

There are several factors that can contribute to the invasibility of a community (Olyarnik et al., 2009). Availability of resources is important in determining the success of an invasion. Invasive species may compete with the local community for resources and amount of resources available may contribute to the invasions success if the competition for resources is reduced (Davis et al., 2000). This reduction could be caused by disturbance or consumption by competitors leading to reduced competition and increased in resource availability for the invasive species. The addition of nutrients in a rock pool invertebrate communities rendered it vulnerable to invasions due to the increasing amount of resources (Romanuk and Kolasa, 2005).

Biotic resistance is an important factor and it involves species interaction in a community to prevent invasion (Olyarnik et al., 2009; Kimbro et al., 2013). Kimbro et al. (2013) found, from a meta-analysis of marine experiments, that producers were unable to resist invasions from producers through competition if the species diversity was low. Moreover, native consumers were able to resist invasion from producers. The resistance by invasive consumers was also higher than by invasive producers. Biotic resistance was dependent on latitude, habitat and invader taxon. In a community, the resident species may have different effects on invaders as there are several mechanisms contributing to biotic resistance, such as predation, competition and disease. For example, Ruesink (2007) manipulated native competitors and predators and showed that this had an effect on the introduced species. The study showed that predators reduced survival of introduced species whereas competitors can reduce and improve survival of introduced species at wave-exposed sites. Higher species diversity has also promoted biotic resistance and reduce invasibility of a community (Stachowicz et al., 1999; Stachowicz et al., 2002). Although biotic resistance has been observed in many studies, the opposite, (i.e., biotic acceptance) has also been recorded (Stohlgren et al., 2006; Fridley et al., 2007). It suggests that both native and introduced species are able to coexist, and species richness is dependent on environmental conditions instead. This was initially observed in plant community ecology where the richness of both native and introduced plant species increased as availability of resources such as land increased (Stohlgren et al., 2006)

Facilitation by marine organisms can promote community invasibility. In some cases, native species are able to provide substrate for colonization of invasive species. For example,
the hard shells of mussels and oysters have provided a substrate for settlement of the ascidian 
*C. intestinalis* (Carver et al., 2003). There have been cases of introduced species facilitating 
the establishment of introduced species. For example, the presence of the invasive crab 
*Hemigrapsus sanguineus* reduced the native species richness while increasing the abundance 
of the tunicate *Botrylloides violaceous* (Freeman et al., 2016). Moreover, organisms in the 
community can make the area vulnerable to invasion if they can suppress the abundance of 
predators and competitors of the introduced species. These synergistic interactions would 
usually occur in areas that are already affected by anthropogenic activities, causing invasion 
meltdown (Simberloff and Von Holle, 1999).

### 1.8 Management of introduced species

Due to the adverse effects of MIS, it is important to have a proper management system 
for marine invasions (Olenin et al., 2011; Ojaveer et al., 2014). Although eradication of MIS 
would be ideal, it has not always been successful and has proven to be costly (Simberloff, 2002; 
Olenin et al., 2011). Instead, prioritising management of marine invasions require actions at 
both global and regional levels because MIS can successfully adapt to new environments 
(Gollasch et al., 2000a; Gollasch et al., 2000b; Bax et al., 2003). For example, *A. amurensis* 
which originated from the Northern Pacific has adapted to the seasons in the Southern 
hemisphere and is now well established in Australia (Byrne et al., 1997; Bax et al., 2003). The 
potential risk for marine invasions is heightened due to the increasing global shipping and 
newly opened routes (Hulme, 2009).

Managing marine invasions can be carried out at each sequential step in the invasion 
process (Figure 1.3). According to Olenin et al. (2011), there are three main stages where 
management can be implemented, namely the pre-border, border and post-border. The pre-
border refers to the stage before the marine species is taken up by a vector and arrives to its 
destination, while transport hubs such as international ports represent the border stage. In the 
post-border stage, the introduced species reach a recipient environment where they establish 
and have the potential to become invasive. Management at the pre-border stage involves 
prevention such as inspection and exclusion which would minimise the risk of spread of 
introduced species. This is possible due to the implementation of international legislations and 
agreements such as the International Convention for the Control and Management of Ships’ 
Ballast Water and Sediments and the ICES Code of Practice on the Introductions and Transfers
of Marine Organisms (Bax et al., 2003). Moreover, managing multiple vectors simultaneously allows for a cost-effective and more practical ecological approach in addressing the issues of MIS (Bax et al., 2003; Minchin, 2007; Williams et al., 2013). At the border and post-border stages, early detection is crucial for possible subsequent steps to be carried out, namely quarantine, control, mitigation and eradication depending on the situation (Myers et al., 2000; Bax et al., 2003; Olenin et al., 2011). Marine surveillance programs at transport hubs ensure that introduced species are detected at their earliest entry (Campbell et al., 2007).

1.9 Importance of taxonomic identification

Identification of taxa is important to manage the spread of introduced species as it allows the differentiation of introduced species from native taxa. Importantly, the early identification of species will allow faster removal of potential MIS. When marine species are newly introduced into an environment, they require time to adjust to the surroundings. During that time, introduced species are few in numbers and would not have any negative impacts on the native taxa (Anderson, 2005). Early detection allows for appropriate management and mitigation of eventual, detrimental effects caused by MIS. It has been demonstrated that MIS can be detected as early as 5 days post-deployment of plates into the waters (Zaiko et al., 2016). Currently, various methods are utilised to conduct MIS monitoring, and they are grouped either into the traditional methods, or molecular methods.

1.9.1 Traditional methods of identification of taxa

Prior to the development of molecular methods, identification of taxa had been conducted through baseline surveys and repeated monitoring (Bott et al., 2010b). This method requires physical sampling, followed by sorting and identification of the species by taxonomists. However, the decline in experts with taxonomic expertise poses problems (Hopkins and Freckleton, 2002; Kim and Byrne, 2006). The use of traditional methods was also labour intensive, and it could take years before there was correct identification of species (Darling and Blum, 2007; Bott et al., 2010b; Costello et al., 2013). This could be due to inability of experts to identify organisms that may be morphologically similar at the larval stage (Besansky et al., 2003; Darling and Blum, 2007). Moreover, the study of complex communities required several taxonomists, which led to increased costs (Lawton et al., 1998; Darling and Blum, 2007). Thus, the results obtained could be an underestimate of the species composition.
and diversity in a certain environment. It was also difficult to rely on this method as visual identification may sometimes be done under difficult conditions such as scuba diving at low visibility (Hayes et al., 2005; Wood et al., 2013). In order to overcome some of these problems, much research has been conducted with the use of molecular techniques.

1.9.2 Molecular techniques

The application of molecular techniques has provided a vast array of possibilities in areas where traditional methods were lacking. DNA-based techniques offer cost-savings for on-going surveillance over traditional collection and identification methods as they are accurate, rapid and do not require specialist (and expensive) taxonomic experts. Molecular techniques are robust and can detect small genetic differences between different species (Bott et al., 2010b), which will overcome the problem in distinguishing species at the larval stage. Besides that, only a small amount of DNA is required for detection which can be easily isolated from organisms. The polymerase chain reaction (PCR) (Mullis, 1990) allows the amplification of a few copies of a DNA region to obtain millions of copies of the DNA. The amplified product will be used for downstream applications. Thus, it is possible to identify an organism from a minute amount of DNA. PCR is a low cost and robust tool that is easily accessible in all laboratories. There are currently several types of PCR-based techniques such as metabarcoding.

1.9.2.1 Use of DNA metabarcoding

DNA metabarcoding (Ji et al., 2013) is seen as an effective approach for assessing biodiversity in environmental samples and providing a better understanding of the interaction of species throughout evolution and history. This is a high throughput technique which allows identification and sequencing of a huge database of marine organisms simultaneously (Chariton et al., 2010; Morgan et al., 2013). Metabarcoding has already been used in many ecological studies for biodiversity assessment (Chariton et al., 2010; Zhan et al., 2014; Pochon et al., 2015; Chain et al., 2016). It consists of using next generation sequencing (NGS) technology to sequence and identify a huge library of barcode DNA sequences from environmental samples (Pochon et al., 2013). With PCR came the advent of universal primers which revolutionised phylogenetic analyses. Universal primers are designed to bind to conserved regions positioned next to a variable region. The variable region is used to identify the taxa present while the conserved region ensures that the universal primers amplify DNA
originating from unknown taxa (Kocher et al., 1989; Taberlet et al., 1991). Some of the DNA regions widely studied for evolution include the mitochondrial DNA (Wilson et al., 1985). Two widely used universal primers amplify regions of the mitochondrial C oxidase subunit I (COI) (Folmer et al., 1994) and the ribosomal RNA (Lane et al., 1985).

1.9.2.2 Limitation of PCR-based techniques

Despite the robustness of this method, there are some limitations that can alter the PCR result. One is the presence of PCR inhibitors, which can negatively affect amplification from template DNA (Schrader et al., 2012). These PCR inhibitors include organic compounds, fat, glycogen metals, bacterial cells and non-target DNA. Another problem with PCR arises from operator error and lack of training (Bott et al., 2010b). When care is not taken while pipetting, there is also a risk of aerosol contamination which may cause the introduction of foreign DNA or irrelevant PCR products. Importantly, a PCR reaction containing all reagents, but the template DNA should be routinely included when performing an experiment. This serves as a control to monitor contamination in the PCR reaction which may lead to false positive.

1.10 Rationale

Biofouling communities on man-made structures act as a link between the marine species brought by vectors (e.g. hull fouling) and the recipient region. They can also contain more invasive species than native species (Tyrrell and Byers, 2006). These communities are dependent upon the environmental conditions and larval pool present in the waters. The assessment of biodiversity in biofouling assemblages especially on man-made structures may allow the detection of potential MIS at their earliest stages of introduction.

Settlement plates have been used extensively to study the influence of temporal and spatial development of biofouling communities. Recent studies by Marraffini et al. (2017) have also shown that the species richness, composition and MIS richness are similar to man-made structures found in coastal environments. This provides opportunities to use settlement plates as a non-invasive sampling device to understand biofouling communities formed in these environments. The combined use of settlement plates and metabarcoding could provide a potential tool to have a biodiversity assessment of the biofouling communities and to identify MIS. There have been several studies examining the use of both techniques for early detection of MIS (Pochon et al., 2015; Zaiko et al., 2016). By allowing biofouling material to grow for
a fixed period of time at different locations, it is possible to get an understanding of the development of biofouling communities.

The main aim of my research was to examine the use of metabarcoding for early detection of eukaryotic introduced species in biofouling assemblages and to study the species composition of biofouling communities. To achieve the aim of my project, I firstly examined the use of freeze-drying for processing large amount of biofouling samples to obtain DNA material of sufficient yield and quality for metabarcoding (Chapter 2). Then, I examined the efficacy of metabarcoding for the early detection of introduced species from biofouling assemblages obtained from Gippsland Lakes (Chapter 3). After validating the use of metabarcoding for species identification, I assessed the species composition of biofouling assemblages in Gippsland Lakes (Chapter 4) and South Australia (Chapter 5) to examine the effect of environmental factors and presence of introduced species on the development of biofouling communities.
Chapter 2:

Freeze drying facilitates the processing of large samples for DNA extraction
Chapter 2: Freeze drying facilitates the processing of large samples for DNA extraction

2.1 Introduction

Obtaining DNA from environmental samples (environmental DNA - eDNA) has been instrumental in the study of species composition and richness of communities (Thomsen and Willerslev, 2015) and in the detection of rare or important species (Wilcox et al., 2013) due to its presence and persistence in soils, sediments and waters (Goldberg et al., 2016). An ongoing problem with using molecular techniques in ecological studies has been obtaining sufficiently high quality nucleic acids from environmental samples such as soils and sediments (Ophel-Keller et al., 2008; Kakirde et al., 2010). A primary issue is inefficient cell lysis from limited breakdown of tough cell walls in some organisms (Maneeruttanarungroj and Incharoensakdi, 2016). If appropriate methods are not employed for sample processing and DNA extraction, there is a risk of DNA degradation. For example, proteolytic activity of DNA by temperature-sensitive enzymes is likely to occur during sample handling or when a sample is left at room temperature for a prolonged period of time (Saha et al., 1997). Samples taken from the environment have also been known to contain phenolic compounds, humic acids and heavy metals that may reduce the quality of DNA (Wilson, 1997) leading to inhibition of molecular reactions.

The use of molecular tools in ecological studies has also highlighted the importance of having sufficient sample mass for DNA extraction. In fact, this could be a limiting factor for carrying out community studies. For example, studies in soil have shown that a larger sample size allow a more complete analysis of the community structure and microhabitats present due to the high complexity and diversity of soils (Taberlet et al., 2012b; Penton et al., 2016). Moreover, for detection of soil-borne organisms in cereal fields (40-300 ha), 250-500 g of soil samples was required as starting material for DNA extraction. This was considered biologically sufficient for a representation of the field studied (Ophel-Keller et al., 2008). Currently, standard commercial DNA extraction kits such as PowerMax® Soil DNA Isolation Kit (MO BIO Laboratories, Inc) allow a maximum of 10 g of starting material which would be considered insufficient to be biologically relevant. A large sample size (>10 g) is also necessary and applicable in other types of environmental samples, such as sediment or water samples, to
be considered biologically relevant and provide an adequate representation of the species assemblage (Bott et al., 2010b). Metabarcoding has been recently introduced and used in assessment of biodiversity in various habitats such as soils and sediments as it uses NGS to identify and sequence a vast database of DNA sequences (Chariton et al., 2010). Therefore, it is becoming critical to develop sample preparation method that could process large quantities of samples (i.e., too large for standard commercial kits) to be of biological relevance for a sufficient representation of the community. In this chapter, large samples (>10 g) of marine biofouling communities that have accumulated on settlement plates over several months were collected and used as an experimental trial. Based on the results of the trial, further biofouling samples were then assessed by metabarcoding. DNA extraction from these biofouling samples can be time consuming and expensive as standard kits can process 10 g of sample at a time and this could lead to DNA degradation.

Freeze drying has been used in many studies. In studies involving faecal samples, results indicated that freeze drying improved DNA purity and recovery compared to fresh faecal samples (Ruiz and Rubio, 2009). Studies in cotton also showed that freeze drying did not affect DNA quality and could be used for downstream molecular applications (Saha et al., 1997). This chapter investigated the use of freeze drying as a method of processing biofouling samples that weigh more than 10 g prior to DNA extraction. The DNA concentration and purity of freeze-dried samples were examined and compared to the non freeze-dried samples. The relative ease of processing and handling freeze-dried samples with large mass was determined. Polymerase chain reaction (PCR) reactions were performed to test whether the quality of DNA was sufficient for downstream applications.

2.2 Material & methods

2.2.1 Sampling procedures

Sampling was conducted in two regions: Port Lincoln (South Australia, 34.7302° S, 135.8505° E) and Metung (Victoria, 37°53′21.325″S, 147°51′24.591″E). The study in Port Lincoln was only discussed in this chapter to examine the effectiveness of freeze drying. In Port Lincoln, six Perspex plates (19.8 cm × 19.8 cm) were attached to mesh panels (32 cm x 32 cm). These panels were set up at one metre and four metres depth on Southern Bluefin Tuna, Thunnus maccoyii, aquaculture cages (Marnikol Fisheries) in April 2016. Twenty-four samples were collected from Port Lincoln after one month (May) and three months (July). In Metung,
one frame with three Perspex plates (19.8 cm × 19.8 cm) was deployed vertically at one metre depth in the water in February 2018. It was removed after four months (June) of deployment. Only the biofouling material collected from one side of one plate in Metung was analysed in this chapter. The samples were first collected using clean and sterile paint scrapers into several Corning 50 mL centrifuge tubes (Sigma Aldrich Pty Ltd) and Whirl-Pak® sterilised bag (Sigma-Aldrich Pty Ltd). Sterile sponges (Whirl-Pak®, Nasco) dampened with UltraPure™ DNase/RNase-Free Distilled Water (ThermoFisher Scientific) (Pochon et al., 2015) were then used to wipe each side of the plate. The samples were kept on ice initially and stored at -20°C until further use.

2.2.2 Freeze drying and DNA extraction

From Port Lincoln, 24 samples were collected after the first month, 18 of which were freeze dried and the other 6 were stored at -20°C. All samples collected after the third month of deployment were freeze dried. The sampling material collected from the sponges was small in size (<1 g); thus, it was not necessary to freeze dry them, and they were processed according to Pochon et al. (2015). To perform pairwise comparisons of non freeze-dried (NFD) and freeze-dried (FD) samples, a fraction of the biofouling material was stored at -20°C while the remaining sample was freeze dried.

Biofouling samples collected with the paint scraper and stored in 50 ml and 120 ml tubes were sealed with parafilm that was punctured with 3-4 holes to allow evaporation of water. Each tube was filled approximately ¾ full. Batches of 3-5 tubes were stored in -80°C overnight, then lyophilised at -40°C for a period of 24-48 h (depending on the mass of material in samples) under a pressure of 0.12 mbar using the freeze dryer ALPHA 1-2 LD plus (Martin Christ). After freeze drying, the samples were further homogenised into fine particles using sterile mortar and pestle. Samples collected from Port Lincoln were weighed both prior to and post freeze drying to the nearest 0.1 g using a XS105 Analytical Balance (Mettler-Toledo) for the 16 samples of the first month of deployment. The samples from Metung were not weighed but were processed following the manufacturer’s protocol using PowerMax® Soil DNA Isolation Kit (MO BIO Laboratories, Inc) with the following modifications.

For samples from Port Lincoln, the pellets from sponge samples were added to a PowerMax® Bead Solution Tube, and samples collected with paint scrapers were added into
the same tube to a total of 10 g. For the samples that were freeze dried, only a total of 4-6 g of material was required in the tube because their dry consistency caused clumping and did not allow proper mixing of the sample with the beads in the solution. As for NFD and FD samples from Metung, the starting material used was 5 g to compare the effect of freeze drying. The tubes were vortexed for 1 minute and 1.2 ml of C1 solution was added, then vortexed for a further 40 seconds. The samples were homogenised using FastPrep®-24 instrument (MP Biomedicals) for 1 minute at 4 metres per second. DNA concentration was quantified using a NanoDrop Lite Spectrophotometer (Thermo Scientific). Qubit 2.0 High Sensitivity Assay (Life Technologies™) was also used to quantify DNA concentration for both NFD and FD samples from Metung because it is one of the recommended methods for quantifying DNA (O’Neill et al., 2011).

2.2.3 Statistical analysis

Statistical analysis was performed to determine whether there were any differences in DNA concentration and purity in FD and NFD samples from Port Lincoln and Metung using GraphPad Prism 8 (GraphPad software, CA, USA). The analysis also looked at the comparison between the use of Nanodrop and Qubit®. The Shapiro-Wilk test was first used to test normality assumptions. This was followed by the Kruskal-Wallis with post hoc Dunn’s test for samples from Port Lincoln. As for samples from Metung, two sample t-tests were carried out. Differences between the Nanodrop and Qubit® were also tested.

2.2.4 PCR amplification of the extracted DNA from samples in Gippsland Lakes

PCR was performed using DNA extracted from NFD and FD samples collected from Metung to examine the quality of the DNA for downstream processes. The PCR was performed using oligonucleotide primers which amplify the hypervariable V4 region of the 18S ribosomal RNA (Zhan et al., 2013) in a T100™ Thermal cycler (Bio-rad). PCR reactions (50 µl) were prepared in 25 µl of AmpliTaq® Gold 360 master mix (Agilent Technologies) containing 0.2 µM of each primer, 1 µl of GC Enhancer and 1 µl of template DNA was added into the reaction. The genomic DNA was firstly denatured at 95°C for 10 minutes. A further 30 cycles of denaturation (95°C, 30 seconds), annealing (56°C, 30 seconds) and elongation (72°C, 60 seconds) were performed. PCR product was analysed using a 1.5% (w/v) agarose gel containing 1 X SYBR™ Safe DNA Gel Stain (Thermo Fisher). Electrophoresis was performed for 1 hour at 100 V.
2.3 Results

2.3.1 Changes in the water content of biofouling samples from Port Lincoln

Freeze drying of samples collected from Port Lincoln after one month of deployment resulted in 60-80% water loss (Figure 2.1A). Similar results were observed for the mass of samples for three months of deployment (Figure 2.1B). In the first month, there was a mean water loss of 73%, with a range of 57.4% to 80.4%. In the third month, there was a mean water loss of 80%, with a range of 68.5% to 90.7%.

Figure 2.1: Loss of water content in samples. Samples collected from Port Lincoln after A) one month and B) three months of deployment were freeze-dried for 24-48 hours. An average of 73% and 80% of water content was lost from the initial weight at one and three months, respectively.
2.3.2 Comparison of the concentration of DNA extracted from non-freeze-dried and freeze-dried samples from Port Lincoln

After one month of deployment, the DNA concentration (ng/µl) for both non freeze-dried (NFD) and freeze-dried (FD) samples ranged between 2 and 45 ng/µl while samples collected after three months had a significantly higher range of DNA concentration between 6 and 65 ng/µl (Figure 2.2). Purity of DNA material extracted from samples in general should have an A260/A280 ratio ranging from 1.8 to 2.0, which is considered pure and suitable for molecular analysis as it has a low protein-to-DNA ratio (Kim et al., 1997; Boesenberg-Smith et al., 2012). During the first month, 9 out of 16 samples (FD) were considered within the range while in the third month, the DNA extracted from 20 out of 24 samples was pure.

Figure 2.2: DNA concentration and purity from samples collected from Port Lincoln (NFD = Non freeze-dried) (FD = Freeze-dried) * indicates p < 0.05 (Dunn’s posthoc test).

2.3.3 Measurement of the DNA concentration and purity from samples collected from Metung using Nanodrop and Qubit system

Both Nanodrop and Qubit system showed that FD samples generated a significantly higher DNA concentration (16.5-51.8 ng/µl) than NFD samples (6.3-20.1 ng/µl) (Table 2.1 & Figure 2.3A). Although, there was a wider range in DNA concentration recorded with the Nanodrop, there was no significant difference between the two quantitation methods. The DNA purity recorded by the Nanodrop showed significant differences between NFD and FD samples (Figure 2.3B). FD samples were closer to the 1.8 to 2.0 range, while NFD had a slightly higher range in DNA purity (Table 2.1).
Table 2.1: DNA concentration and purity of NFD and FD samples collected from Metung

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (ng/µl)</th>
<th>Purity A260/A280</th>
<th>Concentration (ng/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze dried</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1D</td>
<td>41.1</td>
<td>1.87</td>
<td>29.2</td>
</tr>
<tr>
<td>2D</td>
<td>51.8</td>
<td>1.87</td>
<td>44.8</td>
</tr>
<tr>
<td>3D</td>
<td>40.1</td>
<td>1.75</td>
<td>16.6</td>
</tr>
<tr>
<td>4D</td>
<td>25.5</td>
<td>1.92</td>
<td>19.3</td>
</tr>
<tr>
<td>5D</td>
<td>33.8</td>
<td>1.91</td>
<td>28.6</td>
</tr>
<tr>
<td>6D</td>
<td>33.9</td>
<td>1.83</td>
<td>16.5</td>
</tr>
<tr>
<td>Non freeze dried</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1F</td>
<td>18.2</td>
<td>1.97</td>
<td>15.6</td>
</tr>
<tr>
<td>2F</td>
<td>20.1</td>
<td>1.93</td>
<td>16.2</td>
</tr>
<tr>
<td>3F</td>
<td>17.7</td>
<td>1.99</td>
<td>11.9</td>
</tr>
<tr>
<td>4F</td>
<td>14.1</td>
<td>1.94</td>
<td>11</td>
</tr>
<tr>
<td>5F</td>
<td>9.7</td>
<td>2.03</td>
<td>6.3</td>
</tr>
<tr>
<td>6F</td>
<td>19.1</td>
<td>2</td>
<td>17.7</td>
</tr>
</tbody>
</table>

Figure 2.3: DNA concentration (A) and purity (B) from samples collected at Metung measured using Nanodrop and Qubit system. (NFD = Non freeze-dried) (FD = Freeze-dried) * indicates p < 0.05 (sample t-test).
2.3.4 Amplifying of the 18S rRNA region from DNA material obtained from samples

To determine if DNA extracted from the FD samples can be used for downstream molecular applications, PCR was performed on both FD and NFD samples from Metung for comparison. An expected band between 500 and 750 bp was observed for NFD (Figure 2.4A) and FD (Figure 2.4B) samples. The bands observed for NFD samples were consistent in brightness while amplicons from FD samples produced bands of varying intensity.

Figure 2.4: Trial PCR amplification of the DNA extracted from individual (A) NFD & (B) FD biofouling samples from Metung.

2.4 Discussion

In this chapter, freeze drying was tested as a method for processing large (> 10 g) quantities of biofouling material. The water loss from the samples from Port Lincoln (> 50% water loss) (Figure 2.1) was indicative of the amount of water that was evaporated in the process of freeze drying. These freeze-dried samples were homogenised, facilitating easier and faster handling and storage during DNA extraction. The removal of water also suggested that as the mass of the samples was reduced, there was an increase in the concentration of the biota used for extraction for the same weight. This was observed when the same weight of starting material was used for both NFD and FD samples from Metung, resulting in a significant increase in the DNA concentration for FD samples (Figure 2.3A).

A significant rise in DNA concentration was observed in samples collected after one (NFD & FD) and three months (FD) in Port Lincoln (Figure 2.2). It is difficult to determine what could be causing this increase between samples from one month (NFD) and three months (FD). The possible factors influencing this rise could be the difference in quantity of starting
material used, the amount of water loss and the biofouling samples collected at different sampling times. However, the comparison drawn from FD samples from both one- and three-months samples could be more relevant as the starting material used was between 4-6 g. One possible explanation for this increase could be due to the change in species composition in the biofouling assemblages where there could be a shift in species diversity. Biofouling communities are constantly changing depending on environmental conditions such as light, salinity and temperature. Also, species interactions influence the species composition in the community. For example, facilitation, tolerance and inhibition are the three patterns of succession put forward by Connell and Slatyer (1977) that could explain the changes in species composition in the biofouling community.

Quantification of DNA concentration is very important in many molecular techniques, especially in high throughput sequencing and currently, Qubit® is one of the most reliable methods for measuring DNA concentration (O’Neill et al., 2011; Hussing et al., 2015). Both the Nanodrop and Qubit® were used for determining the DNA concentration in both NFD & FD samples from Metung. Nanodrop is a UV spectrophotometer which measures concentration of nucleic acids in general (Simbolo et al., 2013). A ratio of A260/A280 of 1.8 indicates pure DNA while pure RNA would have a ratio of A260/A280 of 2.0 (Boesenberg-Smith et al., 2012). Qubit®, is a fluorometer which uses dyes emitting fluorescence when bound to molecules such as dsDNA (Simbolo et al., 2013). Both quantitation methods showed that the DNA concentration was significantly higher in FD samples than NFD samples when using the same amount of starting material. Although the DNA concentration obtained from Nanodrop had a higher range than Qubit® for NFD and FD samples (Table 2.1 & Figure 2.3A), statistical analysis indicated that there was no significant difference.

Nanodrop has been shown to sometimes provide overestimates of DNA concentration (Simbolo et al., 2013), however it has the advantage of providing DNA purity which may be crucial for PCR. The Nanodrop give an indication of presence of impurities which could be potential PCR inhibitors. This was observed in the amplification of 18S rRNA region (Figure 2.4B). One of the bands generated from FD samples (Figure 2.4B, lane 5) was very faint and the DNA purity of that sample was 1.75 indicating that there were possible impurities. Although the NFD & FD samples were processed simultaneously and the quantity of starting material and template volume were kept constant, there was a difference in the intensity of the bands generated (Figure 2.4). For NFD samples, the bands were consistent while for FD
samples, the difference in intensity could have been caused by the removal of water. This indicated that in addition to the biofouling material, any compounds present was also concentrated. This issue can be overcome by dilution of the template which would also dilute the concentration of potential PCR inhibitors (Ning et al., 2009). Although the DNA purity in NFD and FD samples was significantly different (Figure 2.3B), they were mostly found within the acceptable range of 1.8-2.0 indicating that freeze drying does not adversely affect the purity of the DNA.

The results indicate that freeze drying was an effective processing method for larger than normal (more than 10g) biological material, than typically processed by DNA extraction kits. Freeze drying reduces the mass of the samples by removing water and increases the concentration of the material used for extraction. It also did not affect the yield of PCR amplicons from the genomic DNA and was also a reliable method for processing material for downstream applications. Regardless of the amount of starting material used for DNA extraction and the number of replicates used, it allowed homogenisation of the samples which may lead to improved representation of the biodiversity in community studies including biofouling and other environmental samples. The scope of the freeze-drying method is not limited to environmental studies and could also be used to improve PCR applications in diagnostic tools for pathogens and pest monitoring.
Chapter 3:
Development of early detection system of fouling introduced species in Gippsland Lakes
Chapter 3: Development of early detection system of fouling introduced species in Gippsland Lakes

3.1 Introduction

Species introductions are a common phenomenon worldwide (Vitousek et al., 1997). Out of the numerous introduced species, only a small portion become established in their new environment and only a few become invasive when they cause ecological and economic damage in the environment (Williamson and Fitter, 1996; Boudouresque and Verlaque, 2002; Colautti and MacIsaac, 2004). It is difficult to predict which introduced species would become successful invaders once they are in the new environment (Olenin et al., 2011). MIS are considered a worldwide threat as the spread of MIS can create homogenisation of the environment and change the endemic ecological structure, trophic chains and food webs which may in turn lead to biodiversity loss (Walters, 1996; Clavel et al., 2010). They are also able to thrive in a new environment in the presence of new prey or absence of their natural predators (Rilov, 2009). MIS are usually introduced into an environment by accident or intentionally through human activities and there are many vectors that contribute to their transport and introduction into the new environment (Comtet et al., 2015). Currently, ballast water and hull fouling in ships are the most important vectors that facilitate the spread of MIS (Ruiz et al., 1997; Hewitt et al., 2009).

Introduced species have traditionally been monitored through the use of regular field surveys and morphological identification which relies heavily on taxonomic expertise (Bott et al., 2010b; Bott, 2015). Although introduced species can be detected by repeated monitoring, this usually happens when they reach the adult stage and some are practically impossible to eradicate once they become invasive (Bax et al., 2003). An appropriate stage to detect them would be at their larval stages before they are sexually mature. Identification of larval stages is often tedious and complicated as it is difficult to differentiate organisms and they may resemble native species belonging to the same taxonomic group at larval stages (Wood et al., 2013; Zaiko et al., 2016). The low population number of new organisms introduced may not be easily detected in the new environment making the task of repeated monitoring surveys even more costly. Thus, developing tools for early detection targeting larval and pre-larval stages is crucial to prevent risks of invasion (Darling and Mahon, 2011).
In order to overcome this issue, molecular methods could be used to facilitate identification of introduced species. These techniques typically require only a small amount of DNA as it can be amplified using PCR and used for other downstream applications. For example, quantitative PCR (qPCR) assays have been used in detection of early life history stage of well-known MIS (Bott et al., 2010a; Bott and Giblot-Ducray, 2011a; Bott and Giblot-Ducray, 2011b; Gillum et al., 2014; Simpson et al., 2017; Wood et al., 2017; Ardura and Zaiko, 2018). Recently, metabarcoding has been utilised in many environmental studies for biodiversity and assessment of introduced species (Chariton et al., 2010; Zhan et al., 2014; Pochon et al., 2015; Chain et al., 2016) and is viewed as having higher sensitivity than morphological identification by taxonomists (Lindeque et al., 2013; Chain et al., 2016). This technique consists of amplification of DNA using universal PCR primers and high-throughput sequencing. Due to its ability to identify cryptic species and species at low abundance (Darling and Mahon, 2011; Lindeque et al., 2013), it can be used to assess the biodiversity in early successional stages of communities and lead to identification of any potential MIS at their early life history stage (Valentini et al., 2008).

Combined knowledge of the life cycles of known MIS and the use of metabarcoding can be a powerful tool for identifying early life history stages of introduced species which would lead to early detection of incursions of MIS. Settlement plates submerged in the water can provide an alternative to studying the artificial structures in an ecosystem (Marraffini et al., 2017) and the use of metabarcoding to assess the biofouling communities may allow detection of introduced species at their early life history stages. Moreover, settlement plates provide a yield of biofouling community comparable to that of hulls (Hopkins and Forrest, 2008) which is a major vector to introduced species (Godwin, 2003) and have contributed to introduction of marine species in many marine environments (Sylvester and MacIsaac, 2010). Studies involving metabarcoding and settlement plates have been carried out in New Zealand where introduced species were successfully detected (Pochon et al., 2015; Zaiko et al., 2016). In this chapter, a field trial was carried out in the Gippsland Lakes, Victoria, to validate the effectiveness of metabarcoding and settlement plates as detection technique for early life history stages of introduced species. This was part of a larger study looking at introduced species in that region over a 6 month sampling period in 2015 (Hirst and Bott, 2016). The first aim was to analyse fouling communities that had been developing for a month for the presence of known introduced species that could also include potential MIS. The second purpose was to also examine the presence of any introduced species that were not previously reported in the
location. Molecular techniques which are currently used, namely qPCR, were also performed to validate the detection of the introduced species by metabarcoding.

### 3.2 Materials & Methods

#### 3.2.1 Sampling design and sample processing

In order to examine the presence of introduced species in the Gippsland Lakes from biofouling samples, a field setup was carried out in three sites: Lakes Entrance (37°53′6.789″S, 147°58′19.139″E), Metung (37°53′21.325″S, 147°51′24.591″E) and Paynesville (37°55′0.653″S, 147°43′41.152″E) in March 2015 (Figure 3.1).

![Figure 3.1: Map of the Gippsland lakes showing the three sites: Lakes Entrance, Metung and Paynesville. Adapted from Environment Protection Authority (2015).](image)

Three frames with three Perspex plates (19.8 cm × 19.8 cm) were deployed vertically at one metre depth in the water. Organisms were allowed to settle on those plates for a period of six months. One frame was removed from each location after one month (April), three months (June) and six months (September) of deployment and the biofouling samples were collected. The samples from each side of the plate were first collected using clean and sterile paint scrapers into several Corning 50 mL centrifuge tubes (Sigma Aldrich Pty Ltd) and 120 ml flat bottom container (Interpath Services Pty Ltd). One side of each plate was treated as one sample and from each location, six independent replicates were obtained. For the six months of deployment, one side of the plate was divided into four subsamples. Only one of the four
samples were used for further DNA extraction due to the large mass of sample collected, and the rest were stored at -20°C. This was done in accordance to the maximum recommended capacity (<10 g) of the starting material in the extraction kit used. Sterile sponges (Whirl-Pak®, Nasco) dampened with UltraPure™ DNase/RNase-Free Distilled Water (ThermoFisher Scientific) (Pochon et al., 2015) were then used to wipe each side of the plate. The samples were kept on ice initially and stored at -20°C until further use. Samples collected in Gippsland Lakes after the first month of deployment were not freeze dried, while those collected at the three and six months of deployment were freeze dried. This was due to the large amount of material (>10 g) that was generated from the latter two sampling times.

Since it is of interest to determine the presence of introduced species at their early life history stages, only the three Perspex plates that were removed from each site after one month were processed and analysed in this chapter while the biofouling samples collected for subsequent months were discussed in Chapter 4. The DNA extraction of biofouling samples was carried out as described in Chapter 2.

3.2.2 High throughput sequencing

The preparation of the library was carried out as per Illumina 16S metagenomics sequencing library preparation protocol (Illumina, 2013). A two-step PCR was carried out whereby the first step was to amplify the V4 region of the 18S rRNA (Zhan et al., 2013) followed by a second PCR using the Nextera XT index kit (Illumina Inc., San Diego, CA, USA) to attach Illumina adapters to the amplicons. In the first PCR, the 50-µl reaction were prepared in 25 µl of AmpliTaq® Gold 360 master mix (Agilent Technologies) containing 0.2 µM of each primer, 1 µl of GC Enhancer and 1-5 µl of template DNA was added into the reaction. The genomic DNA was firstly denatured at 94°C for 5 minutes. A further 30 cycles of denaturation (94°C, 30 seconds), annealing (56°C, 30 seconds) and elongation (72°C, 60 seconds) were performed. PCR products were purified using the Agencourt AMPure XP beads (Beckman Coulter) and the DNA concentration was measured using the Qubit 2.0 fluorometer (Thermo Scientific). The amplicons were purified and pooled to equimolar concentrations. Known samples of the organism Prosorhynchoides spp. (Trematoda; Bucephalidae) were included in the pooled library as a positive control for identification. The library was sequenced on the Miseq system using the Miseq reagent kit V3 (600 cycles) (Illumina) generating paired
end reads. The Miseq Reporter carried out demultiplexing and created both forward and reverse reads for each sample.

### 3.2.3 Bioinformatics analysis

As it was a pilot study, a lower limit of 10,000 reads for each sample was initially retained to remove background noise and bioinformatics analysis using the Greenfield Hybrid Amplicon Pipeline (GHAP) Amplicon pipeline (P. Greenfield, CSIRO – https://cloudstor.aarnet.edu.au/plus/index.php/s/356b7901c6fcf7a7e0401e18a49c4647) was performed on the sequences. The main steps of the pipeline include merging, trimming, de-replicating, clustering and classifying (Stephenson et al., 2018). These sequences that originate from genomic DNA of individual organisms are known as molecular operational taxonomic units (MOTUs) (Floyd et al., 2002). This pipeline uses both USEARCH and RDP classifier to analyse the data generated by Illumina platforms for metabarcoding purposes. USEARCH tools such as `fastq_mergepairs` involves merging of paired reads and those that are shorter than 410 bp are trimmed off before clustering and assigning MOTUs. The list of MOTUS is classified in reference to their nearest species match according to the SILVA database (https://www.arb-silva.de/) to identify the biodiversity present in the sample. The list of scientific names was further verified according to Encyclopedia of Life (eol.org), World Register of Marine Species (www.marinespecies.org) and past publications. MOTUs which had a % match lower than 97 and represented by sequences in one sample only were discarded in Microsoft Excel. A cut off of 76 generated from the positive control for identifications was also applied as quality control.

### 3.2.4 Molecular taxonomic detection of introduced species

In order to validate the metabarcoding results, current molecular diagnostic techniques were performed on two introduced species detected: PCR followed by Sanger sequencing, and species-specific qPCR.

#### 3.2.4.1 PCR & Sanger sequencing

A specimen of the introduced species collected from the settlement plates, the sea grape tunicate *M. manhattensis*, which had not been reported in that location was processed for molecular analysis. DNA extraction was carried out as per the manufacturer’s protocol using PowerMax® Soil DNA Isolation Kit (MO BIO Laboratories, Inc). The DNA was then
amplified using 18S primers as described in Section 3.2.2 and amplicons were purified using the ISOLATE II PCR and Gel Kit (Bioline). DNA sequencing was performed by Australian Genome Research Facility (AGRF). Samples contained 20 ng of amplicon DNA and 10 pmol of primers in a total volume of 12 µl. The Sequencher™ software (Gene Codes Corporation) was used to analyse the sequencing results.

3.2.4.2 QPCR Experiment

qPCR assays which have been developed for detection of significant MIS were used to confirm detection of one introduced species, *A. senhousia* (Bott and Giblot-Ducray, 2011b). The introduced species was initially identified based on MOTUs in the metabarcoding analysis. Reactions (20 µl) were prepared in 0.1 ml tubes using the SensiFAST™ kit (Bioline) containing 0.4 µM of the forward and reverse primers and 10 ng of DNA template. 10 µl of SensiFAST™ Probe No-ROX Kit (Bioline) was added into the reaction. Standards were prepared to a final concentration of 100 pg/µl, 10 pg/µl, 1 pg/µl of minigene (derived from *A. senhousia*

5’CGGCGGTCAGAAGCCTGTCGGAAGGTGACCCCGACCGGCCCCGGCCGGGGAGTTTATAGCTGG 3’) (Integrated DNA Technologies). The reactions were prepared in duplicates with a reaction that contains all components mentioned and a reaction with no template control. The reaction was performed using Rotor-Gene™ Q (Qiagen). The initial denaturing step was performed at 95°C for 5 minutes. A further 40 cycles of denaturation (95°C for 10 seconds), annealing and elongation (60°C for 30 seconds) were performed. Standard curve was generated from serial dilutions of the amplification of the minigene. The Ct (cycle threshold) value indicates the number of cycles which are required for the fluorescent signal to pass a fixed threshold (Bott and Giblot-Ducray, 2011b). The lower the Ct value, the higher the concentration of the DNA of the *A. senhousia*. The DNA copy number was calculated from the concentrations of the serial dilution of the standards using DNA/RNA Copy Number Calculator (http://www.endmemo.com/bio/dnacopynum.php).
3.3 Results

3.3.1 Growth profile of the biofouling community

After 1 month (Figure 3.2), the growth profile on the plates varied among the locations. In Lakes Entrance, there was very little growth visually while in Metung, barnacles covered most of the plate. In Paynesville, larvae of several organisms were present on the plates.

![Lakes Entrance](image1.png) ![Metung](image2.png) ![Paynesville](image3.png)

Figure 3.2: Growth profile of biofouling community after one month. Settlement plates were removed from Lakes Entrance, Metung and Paynesville.

3.3.2 Metabarcoding analysis of the species diversity on the settlement plates in Gippsland Lakes

A total of 641,648 raw reads was generated from the analysis by the high throughput sequencing and a total 5.4% of reads was removed (Table 3.1). The highest number of MOTUs (79 MOTUs) was found in Lakes Entrance while Metung had the lowest number of MOTUs, i.e. 46 MOTUs.

Table 3.1: The read count before and after filtering have been tabulated for each site of the three main locations in Gippsland Lakes at one month. The % removed refers to the amount that was removed after filtering the reads.

<table>
<thead>
<tr>
<th>Site</th>
<th>No. of samples</th>
<th>Raw reads</th>
<th>Filtered reads</th>
<th>Removed</th>
<th>% Removed</th>
<th>No. of MOTUs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lakes Entrance</td>
<td>6</td>
<td>196196</td>
<td>174307</td>
<td>21889</td>
<td>11.2</td>
<td>79</td>
</tr>
<tr>
<td>Metung</td>
<td>6</td>
<td>208595</td>
<td>207903</td>
<td>692</td>
<td>0.3</td>
<td>46</td>
</tr>
<tr>
<td>Paynesville</td>
<td>6</td>
<td>236857</td>
<td>224233</td>
<td>12624</td>
<td>5.3</td>
<td>48</td>
</tr>
</tbody>
</table>
The species composition in biofouling communities was compared among Lakes Entrance, Metung and Paynesville and was based on relative abundance from list of MOTUs as shown in Figure 3.3. Each location was represented by six replicates. The colours in each sample indicates the MOTUs identified via the SILVA database. From the samples collected from Lakes Entrance, the presence of 79 MOTUs indicate that there was high diversity in the samples, including notable taxa such as *Corella eumyota*, Order Podocopida and *Tisbe* spp. From the samples collected in Metung, the single colour representing the barnacles *Balanoides* spp. was the most common with more than 90% of sequences in all the six samples. As for samples collected in Paynesville, the MOTU corresponding to the *Balanoides* spp. was also the most dominant in all the samples but there was also presence of ascidian *Botryllus schlosseri*.

![Figure 3.3: Species composition for the three locations (Lakes Entrance, Metung and Paynesville) after one-month sampling. Each column represents an independent replicate at one location and the colours within each column represented the MOTUs found in the sample. The top 10 most abundant MOTUs based on sequence reads are shown in the figure legend and a red asterisk “*” represented an introduced species.](image)

3.3.3 Identification of introduced species on settlement plates in Gippsland Lakes using metabarcoding analysis

The metabarcoding analysis revealed that there was high diversity of MOTUs in the sample collected. Sequences corresponding to introduced species were determined from the list of MOTUs detected on the plates at a specific time point. From the list of the MOTUs, it was possible to determine the sequences corresponding to introduced species were detected on the plates in the Gippsland Lakes at the specific time point. According to the National
Introduced Marine Pest Information System (NIMPIS) list (http://data.daff.gov.au/marinepests/), seven species were detected as introduced in Gippsland Lakes over the period of six months as shown in Table 3.2. The introduced species are from a variety of taxonomic groups such as bivalves, ascidians, bryozoans and green algae. Four of the eight introduced species which are known as common MIS to Australia include Asian bag mussel *A. senhousia*, the ascidian *C. intestinalis* and the sea grape tunicate *M. manhattensis*.

Table 3.2: Potential introduced species recorded based on the NIMPIS list and the bioinformatics analysis for the three locations in the first month. An “x” indicates that the species was present at that location.

<table>
<thead>
<tr>
<th>Species</th>
<th>Lakes Entrance</th>
<th>Metung</th>
<th>Paynesville</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asian bag mussel <em>A. senhousia</em></td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Ascidian <em>B. schlosseri</em></td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Bryozoan <em>Bowerbankia gracilis</em></td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Bryozoan <em>Bowerbankia imbricata</em></td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Ascidian <em>C. intestinalis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sea grape tunicate <em>M. manhattensis</em></td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Sea lettuce <em>Ulva</em> species</td>
<td></td>
<td></td>
<td>x</td>
</tr>
</tbody>
</table>

3.3.4 Molecular identification of two introduced species

The metabarcoding results revealed the presence of *M. manhattensis* and *A. senhousia*. Their identity was confirmed by further experiments. *M. manhattensis* was examined for its presence on the settlement plates by using Sanger sequencing, and its identity confirmed using BLAST (Appendix, Figure 1). The top 10 search results from the BLAST analysis showed that the sequence was highly similar to *Molgula* spp., with 99% similarity to *M. manhattensis*.

As for *A. senhousia*, its identity was confirmed by using qPCR. The sensitivity to detection of MIS using metabarcoding and qPCR was compared (Table 3.3). The presence of *A. senhousia* for the qPCR result was determined by a cut-off of Ct value of 37. The results generated from the positive Ct value were used to determine the copy number. For samples collected in Lakes Entrance, the metabarcoding analysis showed that *A. senhousia* was detected in samples 1, 4 and 6. However, only sample 6 generated a copy number of 4040.7 for qPCR,
which is indicative of MIS being present. This validated the finding generated from metabarcoding which generated a read count of 4868. Interestingly, the samples collected in Metung showed that while *A. senhousia* was not present in any samples from metabarcoding results, the qPCR indicated that it was detected in samples 2 and 3 containing low copy number of 370.0 and 203.8. The samples collected in Paynesville indicated that *A. senhousia* was present in samples 2 and 3 from metabarcoding analysis while qPCR detected the organism in samples 1, 2, 4 and 5. Only sample 2 was found to be positive for both metabarcoding (Read count = 99) and qPCR analysis (Copy number = 2088.5).

Table 3.3: Comparison of presence of *A. senhousia* using either metabarcoding (Read count) or qPCR (Ct and Copy number) in all three locations in Gippsland Lakes. UD denotes that MIS was not detected from the sample. The read count in red indicates that the read count was lower than the cut-off determined by the positive control in the metabarcoding analysis and was considered not detected from the sample.

<table>
<thead>
<tr>
<th>Location</th>
<th>Sample</th>
<th>Read count</th>
<th>Detection</th>
<th>Ct</th>
<th>Copy number</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lakes Entrance</td>
<td>1</td>
<td>260</td>
<td>+</td>
<td>UD</td>
<td>UD</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>65</td>
<td>-</td>
<td>UD</td>
<td>UD</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>17</td>
<td>-</td>
<td>UD</td>
<td>UD</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>83</td>
<td>+</td>
<td>UD</td>
<td>UD</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>48</td>
<td>-</td>
<td>UD</td>
<td>UD</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4868</td>
<td>+</td>
<td>32.2</td>
<td>4040.7</td>
<td>+</td>
</tr>
<tr>
<td>Metung</td>
<td>1</td>
<td>4</td>
<td>-</td>
<td>UD</td>
<td>UD</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>-</td>
<td>35.7</td>
<td>370.0</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>60</td>
<td>-</td>
<td>36.6</td>
<td>203.8</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
<td>-</td>
<td>UD</td>
<td>UD</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
<td>-</td>
<td>UD</td>
<td>UD</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0</td>
<td>-</td>
<td>UD</td>
<td>UD</td>
<td>-</td>
</tr>
<tr>
<td>Paynesville</td>
<td>1</td>
<td>33</td>
<td>-</td>
<td>35.6</td>
<td>413.8</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>99</td>
<td>+</td>
<td>33.1</td>
<td>2088.5</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1329</td>
<td>+</td>
<td>UD</td>
<td>UD</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>37</td>
<td>-</td>
<td>35.8</td>
<td>362.3</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7</td>
<td>-</td>
<td>36.8</td>
<td>181.2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0</td>
<td>-</td>
<td>UD</td>
<td>UD</td>
<td>-</td>
</tr>
</tbody>
</table>
3.4 Discussion

For an effective management of MIS, it is crucial and optimal to carry out detection of early history stage of introduced species as it allows proper management and mitigation of possible MIS incursions. Traditional methods are not ideal for identifying early life history stages of introduced species when they first settle in a new environment. This chapter describes the use of settlement plates and metabarcoding to assess the community present and identify both native and introduced taxa from the assessment. The settlement plates have been used in the past to study biofouling communities as it is a standardised method of study for comparing species composition on the artificial structures present in the environment (Marraffini et al., 2017). These artificial structures which include piers, jetties and pontoons remain the first point of contact for incoming marine species (Bax et al., 2003; Fofonoff et al., 2003). Thus, the biofouling communities on these plates can be assessed at specific times, durations and locations to detect larval stages of introduced species before these introduced species could potentially lead to MIS incursions. Moreover, other newly introduced species, which settle in the area can be identified using this early detection approach. This is possible due to the indiscriminate nature of metabarcoding which allows identification of species present on the plates and determine whether there are any introduced species present.

In this study, an assessment of the species composition in the Gippsland Lakes was performed during the first month and a rough observation of the plates (Figure 3.2) indicated that the species assemblages in the three locations appear different. Evidently, the plates in Metung contain advanced stages of biofouling and plates in Lakes Entrance appear to have little growth on them. To confirm this observation, metabarcoding analysis was performed (Figure 3.3) and it provided information on the species diversity in the three locations. In Metung, the dominance in the MOTUs assigned to the Balanoides barnacles is evident in the read counts. In Paynesville, it also appears that MOTUs corresponding to the same barnacles is the most abundant with identification of other MOTUs such as tunicates B. schlosseri and Polycarpa pomaria as well. Strikingly, metabarcoding analysis of the samples obtained from Lakes Entrance revealed the presence of 79 MOTUs. This reflects the high species diversity which is not easily visible on the plates. It could also indicate the presence of legacy DNA from dead organisms or free-floating DNA (Pochon et al., 2017). This shows that metabarcoding has a high sensitivity to detect MOTUs that are not readily identifiable (Brown et al., 2016).
The community assessment allowed identification of both local and introduced marine organisms and eight introduced species were detected as shown in Table 3.2. Of the list found, three are of particular interest namely the Asian bag mussel *A. senhousia* (previously known as *Musculista senhousia*), the ascidian *C. intestinalis* and the sea grape tunicate *M. manhattensis*. The mussel *A. senhousia* has been known to cause habitat modification (Crooks, 1998) while *C. intestinalis* and *M. manhattensis* have an inhibitory effect on species diversity (Lambert and Lambert, 1998; Blum et al., 2007). The ascidian *C. intestinalis* is known to occur across Australia and recently, *A. senhousia* adults have been found in the Gippsland Lakes (Hirst and Bott, 2016). As for *M. manhattensis*, it was first introduced in 1976 in Australia and has not been recorded since that year (Hewitt et al., 2004; Hirst and Bott, 2016). In 1976, this species was discovered through physical sampling, and the discovery of this species at its larval stage after one-month sampling shows that metabarcoding is a sensitive tool for detection. Further molecular analysis such as DNA extraction of a specimen of *M. manhattensis* collected from plates, Sanger sequencing and BLAST analysis confirmed its identity.

In order to validate the metabarcoding analysis, a qPCR assay (Table 3.3) was used to confirm the presence of the Asian bag mussel, as developed by Bott and Giblot-Ducray (2011b). The results (Table 3.3) indicate the presence of the mussel in all three locations which supports the findings of the metabarcoding analysis. The detection of *A. senhousia* was more efficient in qPCR than in metabarcoding and previous studies have indicated that qPCR has a higher sensitivity than metabarcoding in detection (Wood et al., 2017). qPCR is a targeted approach where species-specific primers are used to detect presence of the organism (Goldberg et al., 2016) whereas metabarcoding is regarded as another non-invasive approach which uses universal primers to provide assessment of biodiversity in a community (Taberlet et al., 2012a). Positive results for the metabarcoding analysis did not always correlate with the results generated from the qPCR. This could be caused by the lack of taxonomic resolution and databases with insufficient information on the 18S rRNA (Pochon et al., 2013; Wood et al., 2017) which might result in assigning the MOTUs to the next closest match. A study by Wood et al. (2017) showed that the qPCR could not detect *Sabella spallanzanii* which was detected in environmental samples by metabarcoding. There could have been an incorrect assignment of another *Sabella* spp. to *S. spallanzanii*. Moreover, metabarcoding may not always detect taxa which are very low in numbers (Wood et al., 2017). Murray et al. (2011) conducted a study to compare the use of qPCR and metabarcoding in detecting the presence of a target organism. The team showed that the target species could not be detected using metabarcoding.
at a Ct value of 34, which is consistent with the results (Table 3.3). The species-specific primers used in qPCR provides better detection at very low amounts as compared to metabarcoding which uses universal primers and may have difficulty in amplifying DNA with low copy number (Murray et al., 2011). Also, quantitation in qPCR has been found to be limited for marine juveniles especially due to varying cell numbers at different life stages which could lead to varying amount of DNA in the larvae (Smith et al., 2012). The different DNA amount in larvae could explain why *A. senhousia* could not be detected in sample 3 in Paynesville. The use of qPCR, a well-established method of detection, was able to validate the metabarcoding results. This gives further weight that using settlement plates and metabarcoding could be a promising addition to the early detection system. qPCR assays have been developed to target common MIS known to Australia (Bott and Giblot-Ducray, 2011a; Bott and Giblot-Ducray, 2011b; Bott et al., 2010a) and the integration of metabarcoding in an early detection monitoring system would allow the identification of unknown introduced species.

Identification of introduced species using traditional methods such as microscopy has not always been reliable because the introduced species and their endemic counterparts share similar morphology. There have been cases of misidentification of MIS at their larval and juvenile stages in the past which have had severe consequences. For example, records from the Western Australian museum showed that small juveniles of *A. senhousia* found in coastal surveys in the Swan River estuary, Western Australia, were initially identified as the local species, *Musculista glaberrima* initially (Slack-Smith and Brearly, 1987). By the time it was properly identified, *A. senhousia* had spread to many locations in Western Australia and had led to an increase in biomass of marine organisms. Another MIS misidentified was *A. amurensis*, which displayed phenotypic plasticity and was difficult to distinguish from closely related counterpart (Byrne et al., 1997; Deagle et al., 2003). In Australia, it was misidentified as a local seastar, *Uniophora granifera* and by the time it was properly ID’d, its population was too high that proper eradication could not be carried out (McEnnulty et al., 2001). Molecular analysis of *A. amurensis* and a good knowledge of its lifecycle has however facilitated its identification (Byrne et al., 1997). These past cases highlight the need for molecular techniques as they are able to overcome the limitations caused by morphological methods.

Although traditional methods such as microscopic analysis were not carried out in this study, existing studies carried out to test the efficacy of metabarcoding as an early detection
technique for introduced species have been compared with microscopy analysis where results based on morphology were lower for taxonomic diversity and resolution (Zaiko et al., 2016). Moreover, the study by Zaiko et al. (2016) showed that 1.5% of the larvae could not be identified and most of the organisms could only be classified to family, class or phylum. The difficulty in identifying early life history stages of marine organisms is usually due to their small size, low abundance and early cryptic stages of development (Chain et al., 2016; Zaiko et al., 2016). Thus, the combination of molecular tools with traditional methods has proven to be useful in overcoming this issue when carrying out monitoring surveys.

Currently, there are several studies which have tested the efficacy of metabarcoding in early detection as well as optimisation of the technique (Pochon et al., 2013; Pochon et al., 2015; Zaiko et al., 2015; Brown et al., 2016; Xiong et al., 2016; Borrell et al., 2017a; Borrell et al., 2017b). Studies have also been carried out to determine the lowest limit of detection of introduced species to demonstrate they are about as sensitive as qPCR (Pochon et al., 2013). Currently, metabarcoding can mostly be used to determine presence/absence of organisms. Although it is arguable that quantitation is still not reliable (Sun et al., 2015), other studies have shown that there is a relationship between the relative number of sequences assigned to an MOTUs and the relative abundance of that organism in a sample (Porazinska et al., 2010; Comtet et al., 2015). The results from Metung (Figures 3.2 & 3.3) also confirmed that there may be a correlation between the relative abundances in both morphological and metabarcoding analysis where in both analyses, Balanoides spp. appeared to occupy most of the surface of the plates and had the highest read counts, respectively. However, further experimental studies would be necessary before quantitation using metabarcoding can be relied upon. Further optimisation of the approach would increase its effectiveness before it can be implemented in an integrated MIS monitoring system.

In this study, only one set of universal primers which amplifies the 18S rRNA region have been used for the metabarcoding analysis and this may not cover all species present in the environment studied or allow appropriate resolution. The combined use of different markers reduces any limitation caused by any one marker and increases the chances of wider taxonomic coverage (Deagle et al., 2014). This approach would also allow the identification of marine organisms using other reference databases, which are based on other sets of genes and markers. Although the 18S rRNA has been used in several studies looking at detection of introduced species (Pochon et al., 2015; Zaiko et al., 2016), the use of this marker has its limitations when
it comes to differentiating species such as *Perna* spp. (Pochon et al., 2013). In that study, *Perna* spp. were both introduced but this would pose an issue for discriminating native and introduced organisms of the same genera. However, this issue is not applicable when the introduced species is from a genus of which there are no native species. The further use of both morphology and molecular tools can overcome the problem of distinguishing between two closely related species.

Further field trials are important to test the reproducibility of the technique and one more field trial was carried out in South Australia (Chapter 5). Observations made in the first month from Metung (Figure 3.2) reveal that *Balanoides* spp. had developed past their larval stages reaching adult stage. This suggests that *Balanoides* spp. require less than one month for maturation and during their larval stage, they would be few in numbers which would allow other organisms to also settle on the surface. Thus, the species composition may have been different and there would have been reduced competition for resources. At early successional stages of biofouling, the larval supply present in the water column could be one factor influencing the colonization of the hard substrate. As the biofouling community develops, the conditions such as environmental conditions and species interactions could have been most favourable to the settlement of the *Balanoides* spp. which could have led to decrease of other species that were present on the surface. Space is a limiting factor in a biofouling community and thus marine organisms have to compete for survival (Richmond and Seed, 1991). By allowing shorter sampling times and frequent plate set up, different biofouling assemblages may be observed on the plates before the *Balanoides* spp. increased in number and provide an insight into the composition of marine larvae present in the water at a specific time of the year. Also, this non-invasive approach would provide sufficient time to carry out measures to prevent any potential invasive incursions.

The plates used in this trial are made of Perspex and this could contribute to the ability of organisms to settle on the plate. In order to test this, other materials could be used for the plate set up. This may allow a more diverse range of species to settle on the plate and possibly lead to the detection of other introduced species present in that region. Previous studies have shown that marine organisms settle preferentially on certain types of substrates (Harriott and Fisk, 1987; Anderson and Underwood, 1994; Faimali et al., 2004; Holst and Jarms, 2007; Terlizzi and Faimali, 2010).
The combined use of metabarcoding and settlement plates has proved to be effective and allowed the overall assessment of the biofouling community. Also, it has successfully identified presence of introduced species that were both known and not recently recorded. By using this tool as the initial method of early detection of introduced species, targeted monitoring may be carried out subsequently to confirm the presence of the introduced species in a specific location and possible MIS incursions may be avoided. Thus, the use metabarcoding and settlement plates can be integrated into an early detection system that would utilise a variety of complementary tools including currently used molecular techniques such as qPCR and traditional methods for ongoing monitoring.
Chapter 4:
Study of biofouling assemblages in Gippsland Lakes over time
Chapter 4: Study of biofouling assemblages in Gippsland Lakes over time

4.1 Introduction

Coastal environments are increasingly becoming populated with man-made structures such as jetties, piers and pontoons, leading to the settlement and establishment of marine fouling species (Bulleri and Chapman, 2010; Marzinelli et al., 2012). This change is mostly observed in enclosed areas such as bays and estuaries as many international ports are located within such sheltered areas (Mineur et al., 2012). The ubiquitous presence of artificial structures has allowed introduced species to adapt to new environments and pose a risk to the local fauna and flora when they become invasive (Glasby et al., 2007). Studies have demonstrated that bays and estuaries are considered the most invaded ecosystems due to the high level of anthropogenic activity and regular transfer of introduced species by vectors (Ruiz et al., 1997; Hewitt et al., 2004; Cohen and Carlton, 1995).

Artificial structures represent the first point of contact for many incoming marine species and this could lead to an increasing number of introduced species on such surfaces (Bax et al., 2003; Fofonoff et al., 2003; Tyrrell and Byers, 2006; Marraffini et al., 2017). These artificial structures may provide habitats where there are no naturally occurring hard substrates in the new environment, and previous studies have also shown that there is a greater abundance of introduced species on artificial substrates than on neighbouring naturally occurring hard surfaces (Connell, 2001; Wasson et al., 2005; Tyrrell and Byers, 2006; Glasby et al., 2007; Ruiz et al., 2009; Marraffini et al., 2017). When these artificial hard surfaces are introduced into the marine environment, biofouling assemblages start to develop over time. The process of biofouling development is a complex process that is influenced by both biotic and abiotic factors. Species composition in early stages of colonisation is determined by both environmental conditions such as water currents, salinity and temperature as well as larval supply and recruitment (Menge et al., 1997; Fraschetti et al., 2003). As hard surfaces become populated, space becomes a limiting factor which creates competition for survival in the developing fouling community (Richmond and Seed, 1991).
Settlement plates have been used extensively in field studies in order to have a better understanding of the settlement and succession process in biofouling communities (Sutherland and Karlson, 1977; Dean and Hurd, 1980; Sutherland, 1981; Glasby, 1999; Glasby et al., 2007). Settlement plates of different shapes, sizes and materials have been used to allow the simulation of artificial structures present in the marine environment (Anderson and Underwood, 1994). This method provides a standardised way of understanding communities formed in ports, piers and marinas (Marraffini et al., 2017). Recently, there have also been studies examining the combined use of settlement plates and metabarcoding as a potential tool for early detection of MIS (Pochon et al., 2015; Zaiko et al., 2016). This method would provide information on the species composition in biofouling communities at a specific location and has been useful in surveys looking at MIS (Blum et al., 2007; Marraffini et al., 2017). The efficacy of this method has been discussed in Chapter 3.

In Australia, Victoria has a well-documented MIS community (Hirst and Bott, 2016). For example, a survey identified 100 introduced species in Port Phillip Bay which is one of the main international ports in Victoria (Hewitt et al., 2004). Port Phillip Bay is a large, sheltered and highly disturbed embayment with anthropogenic activities such as commercial fishing, channel dredging and sewage treatment. Annually, the port receives more than 150 international ship visits, leading to higher risk of marine invasions (Hewitt et al., 2004). Consequently, there is a potential risk of translocating introduced species when there are regular boating activities between Port Philip Bay and the neighbouring ports. Moreover, this risk has not been determined, as regular monitoring in other local ports has been infrequent (Hirst and Bott, 2016).

The Gippsland Lakes, located 380km east of Port Phillip Bay, consist of a network of interconnected inland waterways and span over 69 km of length and 10 km of width (Figure 4.1) (Charlton-Robb et al., 2015). They are linked to the Bass strait through a narrow man-made channel at Lakes Entrance (Davies and Martinez, 2007). This large estuarine system is composed of four lakes: Lake Wellington, Lake Reeve, Lake Victoria and Lake King, which are supplied by five river systems. These river systems are the La Trobe and Avon Rivers flowing into Lake Wellington, and the Mitchell, Nicholson and Tambo Rivers flowing into Lake King (Boon et al., 2016). Both Lakes King and Victoria are considered marine-based systems and their level of salinity varies from 20-25 ppt in autumn to 15-20 ppt in spring due to incoming fresh water from rivers (Environment Protection Authority, 2015). However, the
process of stratification creates a gradient of salinity where the surface is less saline as compared to the bottom of the lakes.

The Gippsland Lakes have high recreational, conservation and ecological value and are listed as Wetland of International Significance under the Ramsar Convention. They are well known for their recreational and fishing activities but are also used for commercial shipping (Gippsland Ports, 2015). It is a popular tourist region and harbours recreational vessels at main townships namely Lakes Entrance, Paynesville and Metung (AECOM, 2014). A major commercial boatyard is also situated in Paynesville, which services vessels based in Lakes Entrance. Commercial fishing vessels from the Bass Strait scallop fishery and Bass Strait shark fishery also operate out of Lakes Entrance, and there is regular sea traffic between the ports in Gippsland Lakes and neighbouring ports (Hirst and Bott, 2016). These include Port Welshpool, Eden, St Helens, Devonport, Hobart, Apollo Bay and Port Phillip Bay.

By deploying settlement plates in the water for a period of six months, marine organisms were allowed to settle and grow. The plates were removed at specific time points to assess the developing species diversity of the biofouling community. In this chapter, the aim was also to determine whether the introduced species that were detected after one month of plate deployment were still detectable in the biofouling community after three and six months. The effect of these introduced species on the local biofouling diversity was also assessed.

4.2 Methods & Materials

4.2.1 Sampling design and sample processing

A pilot study was set up at three locations in Gippsland Lakes; Lakes Entrance, Metung and Paynesville (see Chapter 2, section 2.2.1). In each location, three frames with three sets of Perspex plates (19.8 cm × 19.8 cm) were attached to the pilings and deployed in a vertical position at one metre depth in the water with one side of the frame facing away from the pier. One frame was removed from each location after one month (April), three months (June) and six months (September) of deployment. The biofouling samples collected were processed as described in Chapter 2, section 2.2.
4.2.2 High throughput sequencing and bioinformatics analysis

The DNA material obtained from the biofouling samples were analysed using high throughput sequencing as described in Chapter 3, section 3.2.2.

4.2.3 Statistical analysis

The raw data consisting of number of reads per molecular operational taxonomic units (MOTUs) was used to create rarefaction curves using the “Vegan” package in R (Oksanen et al., 2014). Boxplots were generated from raw data to represent the Shannon diversity. The Shapiro-Wilk test was performed for testing normality assumptions. Then, either the Kruskal-Wallis followed by Dunn’s or one-way ANOVA with post hoc Tukey test were used to determine the differences in species diversity between the time points using SPSS package (SPSS Inc. 2016) and GraphPad Prism 8 (GraphPad software, CA, USA). Statistical analysis was further performed using PRIMER-e software (Clarke and Warwick, 2001). Using this software, raw data was further transformed in 2 ways: Firstly, the data was standardised and a log(X+1) was applied. The Bray-Curtis dissimilarity was then applied to obtain dissimilarity matrices. Secondly the data was transformed using Jaccard index on the raw dataset based on the presence or absence of an MOTUs. The data obtained from both analyses was used to generate nMDS plots to determine the relationship among the species composition in the three
locations (Warwick et al., 1990). A SIMPER analysis determined the top two marine organisms contributing to the most variation among all samples in each location. Analysis of community differences was performed using the two-way PERMANOVA and the factors location and time were examined in the PERMANOVA design with 9999 permutations of residuals under a reduced model. The design was firstly based on the Bray-Curtis dissimilarity after standardising and log(X+1) was applied. It was also based on Jaccard index after applying absence or presence of MOTUs (Anderson, 2001). Monte Carlo tests were also applied where there were low permutations (<100). Pair-wise comparisons were examined where there was significant difference between the factors.

4.3 Results

4.3.1 Environmental variables

Environmental data was collected from five monitoring sites in the Gippsland Lakes namely Lake Wellington, Lake Victoria, Lake King South, Lake King North and Shaving Point (Figure 4.1, 1-5) for the period December 2014 to January 2016. Minimum and maximum temperatures were 8.5 °C in July and 23.5 °C in February respectively (Figure 4.2). During the first month of sampling, the temperature recorded was around 15 °C and according to the trend (Figure 4.2), it dropped to approximately 10 °C during the third month of sampling. The temperature increased to 15 °C during the sixth month of sampling. The turbidity was generally low (< 10 NTU) for Lake Victoria, Lake King and Shaving Point. Lake Wellington was the only site with high levels of turbidity ranging from 4.6 to 38.2 NTU.

![Temperature recorded in the Gippsland Lakes from December 2014 to January 2016. The arrows indicate the three sampling times (one, three and six months) during the field experiment.](image)
Lake Wellington recorded the lowest level of salinity with a range of 5 to 15 PSU (Figure 4.3) throughout the year. In Lake Victoria, the salinity level fluctuated from 15 to 25 PSU. Lake King and Shaving Point had wider range of salinity from 15 to 35 PSU. The field experiment was located closest to Lake King and Shaving Point. During the first month of sampling, the salinity level ranged between 25 to 35 PSU for Lake King and Shaving Point and fluctuated between 20 to 35 PSU during the third month of sampling. The salinity level remained constant during the sixth month of sampling.
4.3.2 Biofouling communities in the Gippsland Lakes

In Lakes Entrance, there was little growth on both sides of the plate compared to Metung and Paynesville after one month of plate deployment (Figure 4.4A). In Metung, barnacles were predominantly growing on both sides of the plate with an apparent larger growth on the side facing away from the pier. In Paynesville, the community was composed
of young stages of barnacles and other marine organisms. At three months of deployment (Figure 4.4B), the growth profile in Lakes Entrance appears to have considerably increased and appears dominated by bryozoans on both sides of the plate. Barnacles were abundant in Metung on both sides of the plate. In Paynesville, the growth profile differed on both sides. One side of the plate was covered with bryozoans and the other side was dominated by ascidians. For samples collected at six months (Figure 4.4C), there was relatively large amount of biofouling organisms on the plates in all locations. In Lakes Entrance, there was heavy biofouling on both sides of the plate. The same result was seen on the plates in Metung. As for Paynesville, the plate facing away from the pier appeared to be dominated by barnacles present while the other side of the plate was mainly covered by a layer of sponge and many ascidians.

![Figure 4.4: Plate deployment. The plates deployed at the 3 locations (Lakes Entrance, Metung and Paynesville) for one (A), three (B) and six (C) months after deployment. The growth of biofouling is observed in each plate facing towards and away from the pier.](image)

4.3.3 Taxonomic assignment of the molecular operational taxonomic units (MOTUs)

The samples in the three sites were processed for DNA extraction and the DNA material was amplified using PCR (See chapter 3, section 3.2.2). Amplicons were analysed by high throughput sequencing produced 2,487,824 reads and were taxonomically assigned. After
filtering the reads (as described in chapter 3), a total of 2,325,330 reads were obtained from the samples (Table 4.1). The remaining taxa included 69.6% metazoans, 14.1% Archaeplastida, 13.0% SAR (Stramenophiles, Alveolates and Rhizaria), 2.2% Fungi and 1.1% Ichthyosporea.

Table 4.1: The read count before and after filtering have been tabulated for each site of the three main locations in Gippsland Lakes at one, three and six months. The % removed refers to the amount that was removed after filtering the reads.

<table>
<thead>
<tr>
<th>Month</th>
<th>Site</th>
<th>No. of samples</th>
<th>Raw reads</th>
<th>Filtered reads</th>
<th>Removed</th>
<th>% Removed</th>
<th>No. of MOTUs</th>
</tr>
</thead>
<tbody>
<tr>
<td>One</td>
<td>Lakes Entrance</td>
<td>6</td>
<td>196196</td>
<td>174307</td>
<td>21889</td>
<td>11.2</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>Metung</td>
<td>6</td>
<td>208595</td>
<td>207903</td>
<td>692</td>
<td>0.3</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>Paynesville</td>
<td>6</td>
<td>236857</td>
<td>224233</td>
<td>12624</td>
<td>5.3</td>
<td>48</td>
</tr>
<tr>
<td>Three</td>
<td>Lakes Entrance</td>
<td>4</td>
<td>164111</td>
<td>121415</td>
<td>42696</td>
<td>26.0</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>Metung</td>
<td>6</td>
<td>291374</td>
<td>287491</td>
<td>3883</td>
<td>1.3</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Paynesville</td>
<td>2</td>
<td>142025</td>
<td>139672</td>
<td>2353</td>
<td>1.7</td>
<td>54</td>
</tr>
<tr>
<td>Six</td>
<td>Lakes Entrance</td>
<td>6</td>
<td>466464</td>
<td>421125</td>
<td>45339</td>
<td>9.7</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Metung</td>
<td>6</td>
<td>327793</td>
<td>323949</td>
<td>3844</td>
<td>1.2</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Paynesville</td>
<td>6</td>
<td>454409</td>
<td>425235</td>
<td>29174</td>
<td>6.4</td>
<td>78</td>
</tr>
</tbody>
</table>

In Figure 4.5, rarefaction curves were generated for each principal location for each time point where the graph indicated whether there has been adequate sequencing of the samples. The graphs were generated based on the number of MOTUs obtained from each sample, indicating the relative level of diversity in the samples. For each graph, one curve represented an independent replicate. The total MOTUs generated for the one-month sampling was higher in Lakes Entrance than in Metung and Paynesville (Figure 4.5A). In Lakes Entrance, most samples were starting to reach a plateau, indicating that the sequencing of samples were sufficient. In Metung, only one sample had reached saturation while the others did not reach a plateau. Similar results were observed in Paynesville. For the third month (Figure 4.5B) in Lakes Entrance, there was still high number of MOTUs indicating high diversity in that location and the total number of reads was sufficient for a good representation of the diversity. In Metung, there was a clear distinction in the read counts between the samples facing both sides of the pier, where samples collected from the plate facing away from the pier was higher than the samples from the other side. Although there were only 2 samples from
Paynesville, one of them showed good representation of the biofouling community while the other started reaching saturation. For the six-month sampling time point (Figure 4.5C), Lakes Entrance remained the most diverse with the highest number of MOTUs present compared to Metung and Paynesville.

![Rarefaction curves showing the read count per number of MOTUs. The species diversity in Lakes Entrance, Metung and Paynesville at one (A), three (B) and six (C) months of deployment.](image)

For each sampling time, a Shannon diversity analysis was computed (Figure 4.6). The species diversity at Lakes Entrance was significantly higher compared to Metung after one month of sampling. The difference in species diversity could not be tested for Paynesville after three months due to small number of replicates. Consistent with the observation on the plate where there was a large amount of *Balanoides* spp. present, the Shannon diversity analysis showed that there was low diversity in Metung. Species diversity from all three locations were significantly different after six months of sampling. Lakes Entrance had the highest species diversity.
4.3.4 Identification of introduced species on settlement plates in Gippsland Lakes using metabarcoding analysis

Using the NIMPIIS list (http://data.daff.gov.au/marinepests/) as a reference, MOTUs of eight introduced species were detected in the samples obtained from the sampling sites of Gippsland Lakes (Table 4.2). Introduced species detected in the one-month sampling event have already been discussed in Chapter 3 and only introduced species detected in the three and six month sampling event were discussed in this chapter. Two introduced species were detected only once in a location and these include pile worm *Alitta succinea*, which was detected only in Paynesville and entoproct *Barentsia benedeni*, which was detected only in Lakes Entrance after six months of deployment. The bryozoan *B. imbricata* was also only detected after six months in both Lakes Entrance and Metung. Both the Asian bag mussel *A. senhousia* and the sea grape tunicate *M. manhattensis* persisted across all sampling times in both Metung and Paynesville, but only *A. senhousia* was detected after six months in Lakes Entrance.
Table 4.2: The list of potential introduced species that were detected over six months in the Gippsland Lakes. An “x” indicates the presence of the species at that location.

<table>
<thead>
<tr>
<th></th>
<th>Lakes Entrance</th>
<th>Metung</th>
<th>Paynesville</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Three</td>
<td>Six</td>
<td>Three</td>
</tr>
<tr>
<td>Polychaete <em>A. succinea</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asian bag mussel <em>A. senhousia</em></td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Entoproct <em>B. benedeni</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascidian <em>B. schlosseri</em></td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Bryozoan <em>B. gracilis</em></td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Bryozoan <em>B. imbricata</em></td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Sea grape tunicate <em>M. manhattensis</em></td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

4.3.5 Biodiversity of the biofouling communities on the settlement plates

The species diversity in biofouling communities on the plates was compared among Lakes Entrance, Metung and Paynesville (Figure 4.7). During the first month of sampling, the presence of more than 20 MOTUs in Lakes Entrance indicated that there was high diversity in the biofouling community. As for Metung and Paynesville, the *Balanoides* spp. were most dominant in the biofouling community as they contribute to the most MOTUs in the samples. After three months of deployment (Figure 4.7B), the species composition of the biofouling community in Lakes Entrance remained highly diverse. In Metung, *Balanoides* spp. remained dominant while in Paynesville, the two samples were predominantly occupied by the sea grape tunicate *M. manhattensis* as indicated by its high % read count. After six months of deployment (Figure 4.7C), in Lakes Entrance, the species diversity of biofouling community remained constant. In Metung, although *Balanoides* spp. were dominant in all samples, *M. manhattensis* and the bay mussel *Mytilus trossolus* were the second most dominant species based on the read count. In Paynesville, *Balanoides* spp. dominated four samples while the % read count indicated that *M. manhattensis* dominated in one sample and the *Viscosia* nematode was dominant in one sample.
Figure 4.7: Species composition on the settlement plates in Gippsland Lakes. The species composition from samples collected at the three locations (Lakes Entrance, Metung and Paynesville) was represented by the total % of the read counts over one (A), three (B) and six (C) months after deployment. The figure legend represented the top 10 MOTUs present at each time point. A red asterisk “*” represented an introduced species. Each sampling site consisted of six samples except where a positive PCR result could not be obtained. One sample was represented by one column and the colours within each column represented the MOTUs found in the sample.
4.3.6 Comparison of the biofouling communities in the Gippsland Lakes

nMDS plots were generated to compare the biofouling communities on the plates in all three locations. At the first month of sampling (Figure 4.8A, left panel), the nMDS plot based on the Bray-Curtis dissimilarity showed that samples from Lakes Entrance (blue triangle) were distinctly separated from the samples of Metung (red circle) and Paynesville (green square), which shared high similarity as they were clustered together. The nMDS plot based on the Jaccard index (Figure 4.8A, right panel) showed that samples of Lakes Entrance were grouped together indicating higher degree of similarity among the samples while the samples of both Metung and Paynesville had higher variability within their samples and appeared overlapped between the two locations.

At three months (Figure 4.8B, left panel), the nMDS plot based on the Bray-Curtis dissimilarity test showed that samples from each location did not share any similarity with samples of another location. However, one sample from Lakes Entrance had higher similarity to samples from Metung than samples from its own location. The plots based on Jaccard index (Figure 4.8B, right panel) indicated that the species composition in most samples obtained from Metung were different from the composition in samples collected in Lakes Entrance and Paynesville. Samples from Lakes Entrance and Paynesville clustered together, indicating high similarity among the samples.

At six months, nMDS plots generated based on the Bray-Curtis dissimilarity test (Figure 4.8C, left panel) showed that samples of Lakes Entrance had high variability among the samples and from samples of the other two locations. All samples of Metung clustered together with four samples of Paynesville which suggested that they had high similarity in species composition. Results obtained from the plot based on Jaccard index (Figure 4.8C, right panel) showed that samples from Lakes Entrance were clustered together and shared similarity with two samples from Paynesville. Samples from Metung did not share any similarity with samples from Lakes Entrance and Paynesville. Four samples from Paynesville were grouped together and were separate from the samples of the other two locations.
Figure 4.8: Multidimensional ordination (nMDS) plots of the three locations (Lakes Entrance, Metung and Paynesville) at one (A), three (B) and six (C) months of deployment. The nMDS plot generated were based on the Bray-Curtis dissimilarity (Left column) and the Jaccard index (Right column).

The SIMPER analysis (Table 4.3) was applied to the total number of read counts based on the Bray-Curtis dissimilarity test and it displays the two taxa that contributed to the most differences between two locations over the six months. In the first month of sampling, the highest differences were observed in the species composition between Lakes Entrance and Metung (90.23%) and the differences were driven by *Balanoides* spp. in Metung and the
ascidian *C. eumyota* in Lakes Entrance. Similar results were observed for the differences in biofouling assemblages between Lakes Entrance and Paynesville (84.07%) where the differences were also driven by *Balanoides* spp. in Paynesville and *C. eumyota* in Lakes Entrance. The biofouling communities in Metung and Paynesville had the lowest dissimilarity, i.e., most similar communities (29.30%) and differences in these two communities were primarily driven by relatively higher abundances of *B. schlosseri* and *P. pomaria* reads in Paynesville and Metung, respectively.

For the samples collected after three months, the highest differences were in the assemblages sampled in Lakes Entrance and Metung (84.52%) and were driven by relatively high abundance of *Balanoides* spp. and *C. eumyota* in Metung and Lakes Entrance, respectively. The biofouling assemblages in Lakes Entrance and Paynesville had high dissimilarity (80.31%) and these differences were driven by presence of *M. manhattensis* in Paynesville and *C. eumyota* in Lakes Entrance. The biofouling assemblages in Metung and Paynesville were still considered the most similar communities (60.91%) and the differences were caused by relatively higher abundances of *M. manhattensis* in Paynesville and *Balanoides* spp. in Metung.

Results from the samples collected after six months revealed that the differences in the biofouling assemblages in Lakes Entrance and Metung remained the highest (86.89%) and were mostly driven by relatively higher abundances of *C. eumyota* in Lakes Entrance and *Balanoides* spp. in Metung. Similarly, the high differences (79.58%) in assemblages between Lakes Entrance and Paynesville were attributed to relatively higher abundance of *Balanoides* spp. in Paynesville and *C. eumyota* in Lakes Entrance. The biofouling communities in Metung and Paynesville remained the most similar, i.e., lowest dissimilarity (44.12%) caused by relatively high abundances of *M. manhattensis* and *Viscosia* spp. in Paynesville.
Table 4.3: SIMPER analysis of the differences between groups according to the % contribution of the top 2 MOTUs based on abundance for all three locations (LE= Lakes Entrance, ME= Metung, PA= Paynesville) during each time point. “Location” referred to each group in order and the % dissimilarity between two groups were in brackets. A “+” indicated that the MOTUs was dominant in that location and MOTUs in bold represented an introduced species.

<table>
<thead>
<tr>
<th>Months</th>
<th>Groups (Location 1 &amp; 2):</th>
<th>MOTUs</th>
<th>Location 1</th>
<th>Location 2</th>
<th>Contribution %</th>
</tr>
</thead>
<tbody>
<tr>
<td>One</td>
<td>LE &amp; ME (90.23%)</td>
<td><em>Balanoides</em> spp.</td>
<td>-</td>
<td>+</td>
<td>13.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>C. eumyota</em></td>
<td>+</td>
<td>-</td>
<td>13.18</td>
</tr>
<tr>
<td></td>
<td>LE &amp; PA (84.07%)</td>
<td><em>Balanoides</em> spp.</td>
<td>-</td>
<td>+</td>
<td>12.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>C. eumyota</em></td>
<td>+</td>
<td>-</td>
<td>12.35</td>
</tr>
<tr>
<td></td>
<td>ME &amp; PA (29.30%)</td>
<td><em>B. schlosseri</em></td>
<td>-</td>
<td>+</td>
<td>24.78</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. pomaria</em></td>
<td>-</td>
<td>+</td>
<td>16.63</td>
</tr>
<tr>
<td>Three</td>
<td>LE &amp; ME (84.52%)</td>
<td><em>Balanoides</em> spp.</td>
<td>-</td>
<td>+</td>
<td>11.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>C. eumyota</em></td>
<td>+</td>
<td>-</td>
<td>10.39</td>
</tr>
<tr>
<td></td>
<td>LE &amp; PA (80.31%)</td>
<td><em>M. manhattensis</em></td>
<td>-</td>
<td>+</td>
<td>14.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>C. eumyota</em></td>
<td>+</td>
<td>-</td>
<td>9.16</td>
</tr>
<tr>
<td></td>
<td>ME &amp; PA (60.91%)</td>
<td><em>M. manhattensis</em></td>
<td>-</td>
<td>+</td>
<td>37.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Balanoides</em> spp.</td>
<td>+</td>
<td>-</td>
<td>17.16</td>
</tr>
<tr>
<td>Six</td>
<td>LE &amp; ME (86.89%)</td>
<td><em>Balanoides</em> spp.</td>
<td>-</td>
<td>+</td>
<td>14.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>C. eumyota</em></td>
<td>+</td>
<td>-</td>
<td>11.12</td>
</tr>
<tr>
<td></td>
<td>LE &amp; PA (79.58%)</td>
<td><em>Balanoides</em> spp.</td>
<td>-</td>
<td>+</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>C. eumyota</em></td>
<td>+</td>
<td>-</td>
<td>10.26</td>
</tr>
<tr>
<td></td>
<td>ME &amp; PA (44.12%)</td>
<td><em>M. manhattensis</em></td>
<td>-</td>
<td>+</td>
<td>16.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Viscosia</em> spp.</td>
<td>-</td>
<td>+</td>
<td>14.53</td>
</tr>
</tbody>
</table>
nMDS plots were generated based on both Bray-Curtis dissimilarity and Jaccard index looking at the comparison over time for each location (Figure 4.9). In Lakes Entrance, the nMDS plot based on Bray-Curtis dissimilarity (Figure 4.9A, left panel) showed that the biofouling community at each sampling time had high variability and that the species composition changed over time. Results based on Jaccard index (Figure 4.9A, right panel) showed that the species composition over the six months had higher similarity.

In Metung, the nMDS plot based on Bray-Curtis dissimilarity result (Figure 4.9B, left panel) showed that most samples collected at one month had high similarity as they were grouped together. Three samples collected at three months also shared similarity with samples at one month while the remaining three samples at three months had higher variability. All the samples collected at six months were highly variable. The nMDS plot based on Jaccard index (Figure 4.9B, right panel) showed that samples from the first month had high variability. For samples collected after three months, three samples were quite separated from the other three which had higher similarity with all samples collected after six months.

In Paynesville, results based on the Bray-Curtis dissimilarity test (Figure 4.9C, left panel) show that samples from the first month were variable and also different from samples collected at three and six months. The two samples collected at three months were clustered together while samples collected at six months also were highly variable and appeared to be clustered into two main groups. The nMDS plot based on Jaccard index (Figure 4.9C, right panel) indicated the samples obtained at one month shared high variability among themselves. The two samples at three months were similar in composition while samples collected at six months had some degree of similarity among themselves.
Figure 4.9: Multidimensional ordination (nMDS) plots of the samples collected at one (blue triangle), three (red circle) and six (green square) months of deployment for each location (Lakes Entrance, Metung and Paynesville) based on Bray-Curtis dissimilarity (Left plot) and Jaccard index (Right plot).
PERMANOVA analysis based on Bray-Curtis dissimilarity test and Jaccard index (Table 4.4) showed significant differences in location, time and their interaction. Pairwise comparisons based on Bray-Curtis dissimilarity and Jaccard index were performed to determine significant differences between two locations over a period of six months (Table 4.5). After one month of sampling, multivariate analyses based on both Bray-Curtis dissimilarity and Jaccard index showed that the biofouling assemblages in all locations had significant differences. After three months sampling, the pairwise comparison between Paynesville and either Lakes Entrance or Metung had low permutations (Table 4.5, comparisons indicated by "*"). Multivariate tests based on the Bray-Curtis dissimilarity showed that the community structure was significantly different for all three locations after three months of sampling while the biofouling communities were significantly different between Lakes Entrance and either Metung or Paynesville. After six months of sampling, multivariate tests based on both Bray-Curtis dissimilarity and Jaccard index showed that the biofouling assemblages at all three locations were significantly different.

In Lakes Entrance, pairwise comparisons based on both Bray-Curtis dissimilarity and Jaccard index (Table 4.6) showed that the species composition was significantly different (p < 0.05) over the period of six months. In Metung, multivariate analyses based on Bray-Curtis dissimilarity indicate that the comparison between biofouling communities sampled at six months and either one or three months were significantly different. However, results based on Jaccard index showed that the species composition over the three timepoints. In Paynesville, the pairwise comparisons between three months and either one or six months generated low number of permutations (Table 4.6, comparisons indicated by "*"). Multivariate tests based on the Bray-Curtis dissimilarity indicated that there were significant differences in the biofouling assemblages between one month and either three or six months. However, tests based on Jaccard index detected significant variability in the biofouling communities between one and six months.
Table 4.4: PERMANOVA results for the analysis of differences in assemblage in Gippsland Lakes across the different factors (location & time) based on Bray-Curtis dissimilarity and Jaccard index. Location and time are the two fixed factors and the number of permutations to obtain p-values was 9999 permutations.

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>p</th>
<th>p (MC)</th>
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</thead>
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<tr>
<td><strong>Bray-Curtis</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Location</td>
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<td>55534</td>
<td>27767</td>
<td>41.649</td>
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<td>0.0001</td>
</tr>
<tr>
<td>Time</td>
<td>2</td>
<td>9832.3</td>
<td>4916.1</td>
<td>7.373</td>
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<td>0.0001</td>
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<tr>
<td>Location*Time</td>
<td>4</td>
<td>14009</td>
<td>3502.2</td>
<td>5.253</td>
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<td>0.0001</td>
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<tr>
<td>Residual</td>
<td>39</td>
<td>26001</td>
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<td></td>
<td></td>
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</tr>
<tr>
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<td>106140</td>
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<table>
<thead>
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<th></th>
<th>df</th>
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<th>MS</th>
<th>F</th>
<th>p</th>
<th>p (MC)</th>
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<tr>
<td><strong>Jaccard</strong></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
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<td>Residual</td>
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<td>Total</td>
<td>47</td>
<td>103880</td>
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</tr>
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</table>

Table 4.5: PERMANOVA Results obtained from Bray-Curtis dissimilarity and Jaccard index of pairwise comparisons for each location (L.E.= Lakes Entrance, ME = Metung, PA= Paynesville) at three time points (One, three and six months). Significant p values were indicated in bold (p < 0.05). * comparisons referred to in the text.

<table>
<thead>
<tr>
<th>Month</th>
<th>Location Groups</th>
<th>t-test</th>
<th>p-value</th>
<th>p (MC)</th>
<th>Location Groups</th>
<th>t-test</th>
<th>p-value</th>
<th>p (MC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>One</td>
<td>L.E., ME</td>
<td>7.89</td>
<td><strong>0.0022</strong></td>
<td><strong>0.0001</strong></td>
<td>L.E., ME</td>
<td>2.78</td>
<td><strong>0.0021</strong></td>
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<tr>
<td></td>
<td>L.E., PA</td>
<td>5.34</td>
<td><strong>0.0023</strong></td>
<td><strong>0.0001</strong></td>
<td>L.E., PA</td>
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<td>ME, PA</td>
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<td>ME, PA</td>
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<tr>
<td>Three</td>
<td>L.E., ME</td>
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<td><strong>0.0005</strong></td>
<td>L.E., ME</td>
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<td>L.E., PA*</td>
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<td>0.0627</td>
<td>0.0246</td>
<td>L.E., PA*</td>
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<td>ME, PA*</td>
<td>3.28</td>
<td><strong>0.0372</strong></td>
<td><strong>0.0031</strong></td>
<td>ME, PA*</td>
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<td>0.194</td>
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<tr>
<td>Six</td>
<td>L.E., ME</td>
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<td><strong>0.0028</strong></td>
<td><strong>0.0001</strong></td>
<td>L.E., ME</td>
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<td><strong>0.0028</strong></td>
<td><strong>0.0001</strong></td>
</tr>
<tr>
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<td>L.E., PA</td>
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<td><strong>0.0023</strong></td>
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<td>L.E., PA</td>
<td>2.82</td>
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<td><strong>0.0018</strong></td>
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<tr>
<td></td>
<td>ME, PA</td>
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<td><strong>0.0102</strong></td>
<td><strong>0.0112</strong></td>
<td>ME, PA</td>
<td>2.39</td>
<td><strong>0.0024</strong></td>
<td><strong>0.0017</strong></td>
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Table 4.6: PERMANOVA Results obtained from Bray-Curtis dissimilarity and Jaccard index of pairwise comparisons among the three time points (one, three and six months) in the three locations Lakes Entrance, Metung and Paynesville. Significant p values are indicated in bold (p < 0.05). * comparisons referred to in the text.

<table>
<thead>
<tr>
<th>Location</th>
<th>Time Groups (Months)</th>
<th>t-test</th>
<th>p-value</th>
<th>p (MC)</th>
<th>Time Groups (Months)</th>
<th>t-test</th>
<th>p-value</th>
<th>p (MC)</th>
</tr>
</thead>
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<td>Lakes Entrance</td>
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<td>2.17</td>
<td>0.0041</td>
<td>0.0081</td>
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<td>2.13</td>
<td>0.0051</td>
<td>0.006</td>
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<td>0.0024</td>
</tr>
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<td></td>
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<td>0.0108</td>
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<td>0.0045</td>
<td>0.0228</td>
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<td>Metung</td>
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<td>0.0887</td>
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<td>1.51</td>
<td>0.0345</td>
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<td>0.002</td>
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<td>Paynesville</td>
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<td>0.0339</td>
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<td>1.43</td>
<td>0.1069</td>
<td>0.1211</td>
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4.4 Discussion

This study is the first major survey assessing biofouling communities in the Gippsland Lakes. Particular attention was given to the detection of potential introduced species on the plates at each time point. The findings of Chapter 3 showed that introduced species were detected during the first month. This chapter focused on the development of biofouling communities over the period of six months and determined whether introduced species were still detectable in the subsequent months.

There was a shift in species composition on the plates over the period of six months. The biofouling community at Lakes Entrance had the highest difference in species composition among the three locations. These differences observed during the first month could be attributed to the environmental conditions influencing the sampling sites. Although salinity could not be obtained for Lakes Entrance, the trend indicated that the closer to entrance the Bass Strait the higher the level of salinity (Figures 4.1 & 4.3). Salinity is likely to be an important influence in the Gippsland Lakes, an estuarine system, where the further the location is from the entrance to the Bass Strait, the lower the level of salinity. An artificial entrance was constructed from 1870 to 1889 to provide access to the Bass Strait in the Gippsland Lakes (Wheeler et al., 2010) and this led to a change in both water and salinity level (Boon et al., 2016). Salinity levels would fluctuate due to the inflow of fresh water from river systems and incoming marine water from the entrance of the Bass Strait (Boon et al., 2016). The sampling site at Lakes Entrance was closest to the entrance to the Bass Strait, which could explain the difference in species composition at that location (Figure 4.1). Common fouling species usually have difficulties in adapting to lower salinities and those fouling organisms would usually be located further away from the opening of the estuary (Woods Hole Oceanographic Institution, 1952). This could explain the lower diversity in Paynesville and Metung compared to Lakes Entrance. Species composition was more similar for both Metung and Paynesville due to their close position to Lake King (Figure 4.8, left panel). Moreover, PERMANOVA results based on Jaccard index indicated that biofouling communities in Metung and Paynesville were not significantly different after one and three months (Table 4.5). Lake King has a salinity level of 20-25 ppt that is attributed to the incoming fresh water from both Mitchell and Tambo Rivers (Environment Protection Authority, 2015). During the field experiment, there was a wider range in the salinity level (15 to 35 PSU) in Lake King (Figure 4.3). Although Metung and Paynesville are located close to Lake King which is a deep, marine-based system, the lake
experiences stratification and the salinity is lower at the surface of the lake. The plates were deployed only 1 metre from the surface of the lake, which would suggest that the biofouling communities on the plates were affected by the low salinity levels. *Balanoides* spp. were also most abundant in Metung and Paynesville.

The samples obtained after one month indicated that the biofouling communities in both Metung and Paynesville predominantly consisted of *Balanoides* spp. based on both the morphological and metabarcoding analysis (Figures 4.4 and 4.7). In Metung, the population of *Balanoides* spp. was higher on the plate facing away from pier. Light was one of the factors that influenced the settlement of larvae of *Balanoides* spp.. Barnes et al. (1951) showed that the larvae responded to light positively which would explain the higher amount of barnacles on the side of plate receiving more sunlight. However, there was no visible difference on the plates at three-month sampling (Figure 4.4). This could be due to reduced space on the side with the most exposure to light and the *Balanoides* spp. settling on the other side of the plate as well. *Balanoides* spp. remained the most abundant in Metung over the entire six-month study.

The difference in development of biofouling communities between Metung and Paynesville may have also been influenced by the frequency in boating activities occurring in Gippsland Lakes. Both Metung and Paynesville are well-known destinations for recreational boating and fishing (AECOM, 2014). However, during the period of sampling, the sampling site at Metung was located close to the marina that was under renovation. This suggested that the boating traffic may have been reduced considerably. The change in frequency of shipping may have affected the propagule pressure in this location. Several introduced species were detected in Metung (Table 4.2). The detection of introduced species by metabarcoding analysis does not necessarily indicate live organisms and instead could be due to traces of DNA left by the organism. The development and colonization of the *M. manhattensis* in Paynesville but not in Metung could be evidence that frequent releases of propagules were necessary to ensure the settlement and colonization of the organism. The propagule pressure in Paynesville, may have been higher than in Metung. In 2015, Paynesville was considered one of the most popular spots for boating (Gippsland Ports, 2015). Propagule pressure is a crucial factor that affect the establishment of an introduced population (Lockwood et al., 2005).
The detection of introduced species in the Gippsland Lakes reveal that regular monitoring is crucial to avoid spread of MIS. Sea traffic in the ports of Gippsland Lakes usually occurs from neighbouring ports such as Port of Melbourne (Hirst and Bott, 2016). This could have been the likely route for non-native species to be introduced. The central harbour in Paynesville is a busy port and has many recreational activities. Introduced species would most likely originate from recreational boats that have travelled from neighbouring ports. The sole international ship at Lakes Entrance is the sand dredge TSHD Pelican which originates from Timaru in New Zealand and the main shipping activities are usually from recreational shipping (Hirst and Bott, 2016). Out of the eight introduced species that were detected after three and six months of sampling (Table 4.2), four introduced species namely *A. succinea*, *A. senhousia*, *B. schlosseri* and *M. manhattensis* were all identified in a survey carried out in Port Phillip Bay (Hewitt et al., 2004). There are several habitat types in Port Phillip Bay including an estuarine area and the salinity level is usually higher than 32 PSU (Hewitt et al., 2004). These two environmental factors increase the risk of marine species translocation. In fact, introduced species are more likely to survive and adapt easier from estuarine environments to another similar estuarine environment (Ruiz et al., 1997). Moreover, *A. senhousia*, *B. schlosseri* and *M. manhattensis* are well-known MIS which have caused adverse effects in estuaries, aquaculture and fisheries (Crooks, 1998; Dijkstra et al., 2007; Gittenberger, 2009). The SIMPER analysis indicated that *M. manhattensis* and *B. schlosseri* were relatively higher in abundance in Paynesville which was the main difference between Paynesville and the other two locations (Table 4.3). The high abundance of both introduced species would be characteristic of successful MIS. MIS have the ability to adapt faster, are highly competitive and have a high rate of fertility (Branch and Steffani, 2004; Wahl, 2009b).

In summary, the biofouling communities on the settlement plates appeared different at the three locations in the Gippsland Lakes. Environmental factors played a role in the differences between Lakes Entrance and either Metung or Paynesville. Although, Metung and Paynesville were both found near Lake King, their species composition differed due to the presence of introduced species and possible difference in sea traffic in both locations. The detection of several MIS in the Gippsland Lakes is consistent with the fact that estuaries are one of the most invaded marine environments in the world (Ruiz et al., 1997; Cohen and Carlton, 1998), as a result of frequent shipping and anthropogenic activities. Although metabarcoding and settlement plates have been mostly tested as promising tools for early detection systems, they also provide an insight into the development of biofouling communities.
in the presence of introduced species. This is the first field trial using settlement plates and metabarcoding to detect introduced species and characterise biofouling communities on plates in the Gippsland Lakes. The information collected could be used as a baseline for further monitoring of introduced species in the Gippsland Lakes.
Chapter 5:
Study of biofouling assemblages in South Australia
Chapter 5: Study of biofouling assemblages in South Australia

5.1 Introduction

Major international ports are considered the main recipient regions for introduced marine species due to heavy international shipping (Ruiz et al., 1997; Minchin, 2006). However, recent studies have shown that, once introduced species are established in these ports, recreational vessels actually play a significant role in facilitating secondary spread of introduced species to neighbouring areas (Wasson et al., 2001; Floerl and Inglis, 2005; Minchin et al., 2006; Zabin et al., 2014). Therefore, there is a high risk of marine invasions in recreational marinas and domestic ports. This can be particularly problematic when the ports are located in marine protected areas and undisturbed areas (Peters et al., 2017). These areas usually possess a rich local biodiversity and introduced species could have devastating impacts on endemic species composition. Several studies have been carried out to determine the potential effects of introduced species in these areas and the success of MIS has also been dependent on both biotic and abiotic factors (Gestoso et al., 2017). It is not possible to predict what the outcome would be once introduced species arrive in marine protected areas and undisturbed habitats. There have been cases of biotic resistance, i.e., where the local community have resisted marine invasions (Olyarnik et al., 2009; Kimbro et al., 2013). The rich local species diversity in marine protected areas usually suggests that marine species already occupy an ecological niche and would not allow establishment of introduced species (Ardura et al., 2016). However, the biotic acceptance hypothesis is also possible whereby the high diversity of local species would allow the establishment and coexistence of introduced species (Fridley et al., 2007).

The pervasive use of man-made infrastructure and increased shipping in highly disturbed areas such as major ports can alter both ecological and environmental conditions, which change community structure (Piola and Johnston, 2008) and contribute to marine invasions (Ruiz et al., 1997; Bulleri and Chapman, 2010). The change in local environment means that indigenous species struggle to adapt to the new conditions whereas the success of MIS is also attributed to their adaptations and success to previously similar environments. Anthropogenic disturbances cause pollution from antifouling paints (Warnken et al., 2004),
motor exhausts and hazardous material spills (Schiff et al., 2004), which affect the native habitats and lead to a decrease in native diversity (Piola and Johnston, 2008). The physical presence of artificial structures can create environments with low turbidity, reduced water flow and scouring (Mineur et al., 2012). In regions exposed to waves, the presence of breakwaters has provided shelter and could facilitate the settlement of MIS (Airoldi et al., 2005). The environmental disruption facilitates the settlement of introduced species as MIS have the potential to thrive better in disturbed areas and makes it difficult for local species to adapt. However, the high level of disturbance is not always observed in smaller marinas as there is more variation in the environment present (Ferrario et al., 2017). Previous studies have shown that this variation affected the settlement of introduced species and communities of introduced species could differ between major ports and small marinas (Ferrario et al., 2017). There are several factors, which have contributed to the difference in species composition and consist of the level of pollution, marina design, presence of neighbouring hard bottom communities and boating activities (Floerl and Inglis, 2003; Foster et al., 2016; Ferrario et al., 2017; Peters et al., 2017).

As discussed previously in Chapter 4, the combined use of settlement plates and metabarcoding provides an effective method of studying and understanding temporal and spatial changes in biofouling communities on artificial substrates. Differing from Chapter 4, Chapter 5 focusses on marine habitats associated with ecosystems used by commercial and recreational vessels in South Australia. Settlement plates were deployed at several locations in the Gulf of St Vincent, and northern coast of Kangaroo Island within the Backstairs Passage in South Australia. The Gulf of St Vincent is considered an inverse estuary as the salinity increases when it is nearer to the northern end. Salinity ranges from 39-42 ppt (Bryars et al., 2016) with sea surface temperature within the Gulf St Vincent varying from 10-15°C in winter and 20-25°C in summer (Bryars et al., 2016; Lee et al., 2018). Both ambient air and sea surface temperature have a similar seasonal pattern (Bryars et al., 2016). Each site has differing levels of anthropogenic disturbance located in a marina; North Haven, Wirrina Cove and at Christmas Cove (Kangaroo Island), and at one jetty located at Kingscote (Kangaroo Island). North Haven comprises a large recreational marina complex located 14 kilometres from the Adelaide central business district. It is also located close to Port River and Port Adelaide which is an international shipping port (Oakley, 2005) (Figure 5.1). Wirrina Cove is a frequently used recreational marina located 80 km south of Adelaide on the Fleurieu Peninsula, and is visited by yachts and motor boats coming from metropolitan Adelaide and interstate waters (Chappell...
et al., 1997; Kinloch et al., 2009). The two sites on KI (Kingscote and Christmas Cove, are locations utilised frequently by recreational boaters and commercial fishing, ecotourism and ferry transport vessels, which the latter transport tourists between the mainland and KI (McArdle et al., 2012). Both sites have visitation from vessels from mainland Adelaide and occasional interstate waters. The waters surrounding KI include four marine parks with marine habitats regarded relatively intact.

Several monitoring surveys were carried out previously within Gulf of St Vincent and on Kangaroo Island (Kinloch et al., 2009). The findings of that survey revealed the presence of two known MIS were found: *S. spallanzanii* and *A. aspersa*. They were found on recreational boats that had travelled from Port Adelaide and had spent time at marinas such as Wirrina Cove (Kinloch et al., 2009). Low numbers of *S. spallanzanii* are also collected from Kingscote jetty during routine inspection (K. Peters pers comm.). Both *S. spallanzanii* and *A. aspersa* have been reported in North Haven, Port Adelaide and Wirrina Cove marinas (Kinloch et al., 2009; Wiltshire et al., 2010; Wiltshire and Deveney, 2011). There were monitoring surveys carried out in Port Adelaide, four locations including North Haven, Outer Harbour, Inner Harbour, Upper Port in 2010 and 2011 and it revealed the presence of six known targeted MIS: *S. spallanzanii, Caulerpa racemosa* var. *cylindracea*, *Caulerpa taxifolia*, *Codium fragile ssp. fragile*, *Alexandrium catenella* and *Alexandrium minutum* (Wiltshire and Deveney, 2011). These monitoring surveys have also underlined the importance of understanding the geographical distribution of introduced species and conditions that facilitate establishment.

The aims of this study were similar to those of the field trial in Gippsland Lakes where the biofouling communities were assessed at several time points. However, in this chapter, the field trial lasted for a period of twelve months and the settlement plates were removed at five time points. To the best of my knowledge, this is the first study that utilises metabarcoding to study the development of biofouling assemblages over a period of twelve months. The detection of introduced species at early time points and their effects on the establishment of biofouling assemblages were also determined. Moreover, the field trial aimed to characterise biofouling communities in marinas and jetties with varying anthropogenic level and the possible effect of recreational boating occurring in Gulf of St Vincent.
5.2 Methods & Materials

5.2.1 Study site and sampling design

The sampling study was carried out in the Gulf of St Vincent and on Kangaroo Island, South Australia where the settlement plates were deployed and retrieved by Dr Kristian Peters (Adelaide Mount Lofty Ranges Natural Resources Management Board) in July 2015 (Winter). It included three Main locations namely North Haven, Wirrina Cove and Kangaroo Island (Figure 5.1). There were two sites of plate set up at recreational marinas at each location as shown in Table 5.1. In Kangaroo Island, the frame was deployed with one side of the plate facing east and the back facing west at Kingscote Jetty, which consists of timber cross members and pylons (Peters, pers. comm.). In all the sites except for Kingscote Jetty (Kangaroo Island), the frames were deployed below a floating arm, which consist of large interlocking plastic segments converged as network of floating pontoons secured with concrete pylon. In Christmas Cove (Kangaroo Island), a 24-berthed marina, the frame was deployed with one side of the plate facing south and the back facing north. There was differential shading in both sites. In Wirrina Cove, there were two sites of plate deployment. One side of the frame was exposed to consistent sunlight depending on ambient and water quality conditions at the 210-berthed marina. In North Haven, there were two sites of plate setup. At the Cruising Yacht Squadron South Australia, a 300-berthed marina, the frame was deployed with one side facing south while at Crown Marina, a 174-berthed marina, one side of the plate was facing west. The frames at both marinas had differential shade from berthed vessels (Peters, pers. comm.).

At each site, eighteen Perspex plates (19.8 cm × 19.8 cm) attached in one extruded hard polymer cross-framed plastic frame using nylon screws (Figure 5.2) (Peters, pers. comm.). The frame was deployed in a vertical position at approximately 60-80 cm in the water using dual-tethered galvanised chain link encased in PVC protective sleeves and three plates (position: Upper, Middle, Lower) were removed after one, three, six, nine and twelve months of deployment (Peters, pers. comm.). The biofouling material was collected using sterile scrapers.
Table 5.1: Study sites in South Australia.

<table>
<thead>
<tr>
<th>Kangaroo Island</th>
<th>Wirrina Cove</th>
<th>North Haven</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site 1: Kingscote jetty (KI)</td>
<td>Site 1: Marina St Vincent (W1)</td>
<td>Site 1: Cruising Yacht Squadron South Australia (N1)</td>
</tr>
<tr>
<td>35.66° S, 137.65° E</td>
<td>35.50° S, 138.25° E</td>
<td>34.79° S, 138.49° E</td>
</tr>
<tr>
<td>Site 2: Christmas Cove (XC)</td>
<td>Site 2: Marina St Vincent (W2)</td>
<td>Site 2: Crown Marina (N2)</td>
</tr>
<tr>
<td>35.72° S, 137.93° E</td>
<td>35.50° S, 138.25° E</td>
<td>34.78° S, 138.48° E</td>
</tr>
</tbody>
</table>

Figure 5.1: Marina and jetty locations of settlement plate setup used to assess biofouling communities and introduced species in South Australia.
Figure 5.2: Settlement plate setup in South Australia. (A) At each sampling time, three plates positioned vertically were removed and each side of the plate was divided into four sections. (B) One section was then treated as one sample.

5.2.2 Molecular & statistical analysis

Samples collected were collected in RNAlater and stored at 4°C. Because of the large amount of biofouling material on each settlement plate, only one sample of each side of a plate was randomly selected and processed for molecular analysis. The samples were centrifuged at 4,000 g for 15 minutes and the RNAlater was discarded. The pellet was processed as per the manufacturer’s protocol using PowerMax® Soil DNA Isolation Kit (MO BIO Laboratories, Inc). A PCR was carried out as described in Chapter 3 and only samples that generated positive amplicons were further analysed by high throughput sequencing. The library preparation for high-throughput sequencing and bioinformatics analysis were described in Chapter 3, and the statistical analysis was described in Chapter 4. Statistical analysis that was performed using PRIMER-e software (Clarke and Warwick, 2001) to assess differences in community structure among sites and within site variability. For this study, factors were assigned for location and timepoint (months since deployment) with four levels for the former (site: North Haven, Wirrina Cove, Kingscote and Christmas Cove) and 5 levels for the latter (month 1, 3, 6, 9, 12). The two sites in Kangaroo Island were analysed separately due to their distant locations. One-way ANOSIM tests with 999 permutations were also used to determine whether there was any significant difference among the locations over time.
5.3 Results

5.3.1 Taxonomic assignment of the MOTUs

For the twelve-month sampling event, samples from both sites at Kangaroo Island were lost due to severe weather conditions, and consequently were excluded from the study. The amplicons that were analysed across all sites by high throughput sequencing produced 4,849,800 raw sequence reads which were taxonomically assigned. These reads were filtered (as described in Chapter 3). After filtering, the reads were reduced to 3,596,303 (74.2% of the raw sequences). The number of samples, raw MOTU reads, filtered MOTU reads and percent (%) of reads removed were determined for each site in their main location over a period of twelve months (Table 5.2). Over all sites and locations, characterised sequences were grouped and comprised 71.0% metazoans, 18.2% SAR (Stramenophiles, Alveolates and Rhizaria), 10.4% archaeplastida and 0.4% cryptophyta. Five MOTUs corresponding to the family Didemnidae, genera *Diplosoma* and *Acartia* could not be identified with the SILVA database but were successfully assigned to the nearest match with BLAST (NCBI).

Table 5.2: The read count before and after filtering have been tabulated for each site of the three main locations in South Australia at one, three, six, nine and twelve months.

<table>
<thead>
<tr>
<th>Month</th>
<th>Main Location</th>
<th>Site</th>
<th>No. of samples</th>
<th>Raw reads</th>
<th>Filtered reads</th>
<th>Removed</th>
<th>% Removed</th>
<th>No. of MOTUs</th>
</tr>
</thead>
<tbody>
<tr>
<td>One</td>
<td>Kangaroo Island</td>
<td>Kingscote</td>
<td>6</td>
<td>98463</td>
<td>54435</td>
<td>44028</td>
<td>44.7</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>Christmas Cove</td>
<td>6</td>
<td>91349</td>
<td>62270</td>
<td>29079</td>
<td>31.8</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wirrina Cove</td>
<td>Site 1</td>
<td>5</td>
<td>54878</td>
<td>31168</td>
<td>23710</td>
<td>43.2</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Site 2</td>
<td>5</td>
<td>76908</td>
<td>48169</td>
<td>28739</td>
<td>37.4</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>North Haven</td>
<td>Site 1</td>
<td>6</td>
<td>208435</td>
<td>1534</td>
<td>1340</td>
<td>0.7</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Site 2</td>
<td>6</td>
<td>332307</td>
<td>246094</td>
<td>86213</td>
<td>25.9</td>
<td>68</td>
</tr>
<tr>
<td>Three</td>
<td>Kangaroo Island</td>
<td>Kingscote</td>
<td>6</td>
<td>145687</td>
<td>109490</td>
<td>36197</td>
<td>24.8</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>Christmas Cove</td>
<td>6</td>
<td>91810</td>
<td>52691</td>
<td>39119</td>
<td>42.6</td>
<td>114</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wirrina Cove</td>
<td>Site 1</td>
<td>6</td>
<td>170687</td>
<td>131837</td>
<td>38850</td>
<td>22.8</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Site 2</td>
<td>5</td>
<td>215095</td>
<td>87174</td>
<td>127921</td>
<td>59.5</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>North Haven</td>
<td>Site 1</td>
<td>6</td>
<td>209284</td>
<td>205385</td>
<td>3899</td>
<td>1.9</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Site 2</td>
<td>6</td>
<td>256040</td>
<td>253310</td>
<td>2730</td>
<td>1.1</td>
<td>57</td>
</tr>
<tr>
<td>Six</td>
<td>Kangaroo Island</td>
<td>Kingscote</td>
<td>4</td>
<td>143946</td>
<td>50155</td>
<td>93791</td>
<td>65.2</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>Christmas Cove</td>
<td>6</td>
<td>151830</td>
<td>126780</td>
<td>25050</td>
<td>16.5</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wirrina Cove</td>
<td>Site 1</td>
<td>5</td>
<td>156896</td>
<td>91771</td>
<td>65125</td>
<td>41.5</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Site 2</td>
<td>6</td>
<td>98688</td>
<td>56793</td>
<td>41895</td>
<td>42.5</td>
<td>125</td>
</tr>
</tbody>
</table>
The overall species richness for each Main location namely Kangaroo Island (Figure 5.3A), Wirrina Cove (Figure 5.3B) and North Haven (Figure 5.3C) was analysed using rarefaction curves at one, three, six, nine and twelve months. For each graph, one independent sample was represented by one curve. The number of MOTUs recorded for each location differed, with Kangaroo Island having the highest amount while North Haven had the least number of MOTUs, suggesting that Kangaroo Island was the most diverse and the species diversity was at the lowest in North Haven. Similar results were observed over the twelve months. Most curves generated from the samples in Kangaroo Island at all sampling time points were still on an incline, suggesting that fresh collections of samples could have been further sequenced as the curves have not yet reached saturation. Most curves generated for samples from Wirrina Cove were mostly on an incline for the first six months and started to reach a plateau after nine and twelve months. For North Haven, most curves were starting to plateau over the period of twelve months, suggesting that there was sufficient sequencing of the samples.
Figure 5.3: Rarefaction curves based on number of reads per MOTUs showing analysis of species diversity in South Australia. The diversity of species composition at each site of (A) Kangaroo Island (XC and KI), (B) Wirrina Cove (W1 and W2) and (C) North Haven (N1 and N2) were examined after one, three, six, nine and twelve months. For each sample, LB = lower back, UB = upper back, MB = middle back, LF = lower front, UF = upper front and MF = middle front.
For each sampling timepoint, a Shannon diversity analysis (Figure 5.4) was calculated and the values obtained had a range from 0 to 4. For the one-month sampling event, Shannon analysis showed that there was significantly lower diversity in the samples collected from North Haven compared to Kingscote, Christmas Cove and Wirrina Cove. For the three months sampling event, there were significant differences in the species diversity in the samples collected from North Haven and either Kingscote or Christmas Cove. The species diversity in North Haven was significantly lower than in Christmas Cove. Analysis of the species diversity for the six-month sampling event shows that there was significantly lower diversity in the samples collected from North Haven compared to Wirrina Cove. Species diversity in samples collected from Kingscote at nine months was significantly different compared to the samples collected from Christmas Cove and Wirrina Cove. There was no significant difference in species diversity between Wirrina Cove and North Haven for the twelve-month sampling event.

Figure 5.4: Shannon diversity index of samples sequenced over twelve months (one, three, six, nine and twelve months) in (Christmas cove (XC) (Kangaroo Island), Kingscote (KI), Kangaroo Island, Wirrina Cove and North Haven. * indicates p < 0.05 (Tukey or Dunn’s posthoc test).
5.3.2 Identification of introduced species on settlement plates in South Australia using metabarcoding analysis

MOTUs of twelve introduced species were detected in each sampling site over the five time points based on the NIMPIS list (http://data.daff.gov.au/marinepests/) (Table 5.3). Over the twelve months, Kingscote had the lowest number of introduced species detected while the highest number of introduced species detected was in site 2 of Wirrina Cove. The ascidians *C. intestinalis* and *A. aspersa* appear to be persistent at North Haven over twelve months. Their presence was also detected in most sampling times at Wirrina Cove and *A. aspersa* was also detected in Kingscote, albeit only in the sixth month. MOTUs corresponding to the ascidian *B. schlosseri* and bryozoan *Cryptosula pallasiana* were detected across all the main locations over the first six months. The bryozoans *B. gracilis, B. imbricata, Watersipora subtorquata* and *Membranipora* spp. were not reported on the NIMPIS list but reported in previous findings (Hewitt et al., 2004). They were detected in all main locations from three to twelve months sampling time. The sea lettuce *Ulva* spp. was detected at only site 2 of Wirrina Cove in the first month. Its detection was also observed in North Haven in both the first and third month. The four remaining MOTUs were found at only one site at only one sampling time. The dinoflagellate *A. catenella* was only detected at Site 2 of North Haven in the sixth month. The sandworm *A. succinea* was detected in Christmas Cove in the nine months samples while the MOTUs of sea thread hydroid *Obelia dichotoma* and European fanworm *S. spallanzanii* were detected in Wirrina Cove after twelve and nine months, respectively.
Table 5.3: The list of MOTUs of potential introduced species detected at one, three, six, nine and twelve months. An “x” symbol denotes the presence of the species in the sampling sites.

<table>
<thead>
<tr>
<th></th>
<th>Kangaroo Island</th>
<th>Wirrina Cove</th>
<th>North Haven</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kingscote</td>
<td>Christmas Cove</td>
<td>Site 1</td>
</tr>
<tr>
<td>Ascidian A. aspersa</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Ascidian B. schlosseri</td>
<td>x  x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Ascidian C. intestinalis</td>
<td>x   x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Bryozoan B. gracilis</td>
<td>x  x  x x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Bryozoan B. imbricata</td>
<td>x  x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Bryozoan C. pallasiana</td>
<td>x  x   x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Bryozoan Membranipora spp.</td>
<td>x  x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Bryozoan W. subtorquata</td>
<td>x  x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Dinoflagellate A. catenella</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Fanworm S. spallanzanii</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Green algae Ulva spp.</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Hydroid O. dichotoma</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Sandworm A. succinea</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>
5.3.3 Biodiversity of the biofouling communities on the settlement plates

The species diversity in biofouling communities from the plates was compared among North Haven, Wirrina Cove and both sites in Kangaroo Island (Kingscote and Christmas Cove) (Figure 5.5). The relative abundance of the taxa was examined and represented as %, generated from the list of MOTUs. During the first month in Kangaroo Island and Wirrina Cove (Figure 5.5A), the presence of more than 60 MOTUs at each site indicated that the community had high species diversity. In North Haven, the sampling sites were predominantly occupied (>85%) by the ascidians *C. intestinalis* and *A. aspersa*, which are introduced species. For the samples collected in the third month (Figure 5.5B), the diversity of the community remained high across the sampling times in Kangaroo Island although there was a high % of read count (>30%) of the hydrozoan *Halecium mediterraneum*. Across Sites 1 and 2 in Wirrina Cove, the introduced ascidian *B. schlosseri* and the copepod *Lichomolgus canui* were abundant collectively in six samples, while the other five samples appeared more diverse. *C. intestinalis* and *A. aspersa* remained dominant in North Haven. At six months (Figure 5.5C), there was high species diversity in Kingscote while the introduced bryozoan *B. imbricata* was dominant in Christmas Cove (>55%). There was also high species diversity in each sample collected from Wirrina Cove as observed by the presence of more than 20 MOTUs per sample. In North Haven, there was higher species diversity with higher number of read count when compared to the one- and three-month sampling event. Nevertheless, *A. aspersa* were found dominant in four samples across Sites 1 and 2 whereas *C. intestinalis* were found dominant in two samples at Site 2. Of the samples obtained from Kingscote after nine months of plate deployment, *Prostheceraeus vittatus* had the highest read count (>40%) whereas in Christmas Cove, the presence of many MOTUs suggests a high diversity in the samples (Figure 5.5D). In Site 1 and Site 2 at Wirrina Cove, the highest number of read counts was generated from MOTUs of *P. pomaria*, *Distaplia dubia* and *Didemnum* spp. In North Haven, the colour representing the MOTU of *C. intestinalis* was the most dominant at Site 1, whereas the diversity in Site 2 was higher than the former site due to presence of many MOTUs. The MOTUs of the species *P. pomeria* and *D. dubia* remained dominant at twelve months sampling in Wirrina Cove (Figure 5.5E). *C. intestinalis* also remained relatively dominant in North Haven after twelve months.
Figure 5.5: Species composition on the settlement plates in South Australia. Each site of one main location was represented by six samples except where positive PCR could not be obtained. The column represents one sample and the colours within each column reflects the species found in the sample. The species diversity is represented by % of the total read count in the (A) one, (B) three, (C) six, (D) nine and twelve months collected at the three locations (Kangaroo Island, Wirrina Cove and North Haven). The colours representing the top ten MOTUs for each time point are shown in the legend. A red asterisk “*” indicates an introduced species.
5.3.4 **Comparison of biofouling communities over twelve months**

nMDS plots were generated for each time point (one, three, six, nine and twelve months) (Figure 5.6). For each time point, two plots were generated based on Bray-Curtis dissimilarity and Jaccard index. At one month of sampling, the Bray-Curtis dissimilarity (Figure 5.6A, left panel) showed that the samples from North Haven shared high similarity in community structure as observed in the clustering of the samples. Samples from both Wirrina Cove and Kingscote were highly variable as observed in the scattering of the samples across the graph. All the samples from Christmas Cove were clustered together and shared similarity with samples from Wirrina Cove and Kingscote. The nMDS plot based on Jaccard index (Figure 5.6A, right panel) indicated that the samples collected from Kingscote were clustered together. Similarly, the samples collected from Christmas Cove were tightly clustered while samples from Wirrina Cove had high variability in species composition. Samples collected from North Haven had the highest degree of variability in species composition and two samples shared similarity with Christmas Cove and Wirrina Cove.

At three months (Figure 5.6B, left panel), the nMDS plot based on Bray-Curtis dissimilarity indicated that the samples from Wirrina Cove and Christmas Cove shared similarity but also had high variability in structure of biofouling assemblages within each location. All the samples from Kingscote were clustered together and were quite distinct from the other locations. Similarly, samples from North Haven had less variability and were mostly separated from the other locations. The plot based on Jaccard index (Figure 5.6B, right panel) showed that the species composition in the samples were generally quite diverse in each location. The highest variability was observed from samples in North Haven. Samples from Wirrina Cove also showed high variability while samples from Christmas Cove were clustered but shared similarity with some samples from both North Haven and Wirrina Cove. As for samples from Kingscote, they were clustered together and were separate from the three locations.

At six months, the plots based on Bray-Curtis dissimilarity (Figure 5.6C, left panel) showed that the samples in North Haven had the highest variability. Samples obtained from Wirrina Cove and Christmas Cove shared high degree of similarity and variability in community structure. The samples from Kingscote were clustered together and remained separated from the other locations. The nMDS plots based on Jaccard index (Figure 5.6C, right panel) showed that samples from North Haven were still highly variable in species
composition. Samples from Wirrina Cove and Christmas Cove remained in close proximity while most samples from Kingscote clustered together.

At nine months, the nMDS plot based on Bray-Curtis dissimilarity (Figure 5.6D, left panel) showed that the biofouling communities were quite distinct among the locations. However, 1 sample from both Wirrina Cove and North Haven shared high similarity in community structure. There was also high variability in biofouling assemblages within each sampling group from North Haven and Kingscote as samples were not clustered together. The nMDS plot based on Jaccard index (Figure 5.6D, right panel) showed that samples collected from Kingscote was separated from the other three locations and had high variability in the species composition. Samples from Wirrina Cove shared similarity with both Christmas Cove and North Haven as the samples were clustered together.

Based on the plots generated at twelve months sampling time point using Bray-Curtis and Jaccard analysis (Figure 5.6E), samples collected from North Haven and Wirrina Cove were highly dissimilar in the biofouling assemblages.
Figure 5.6: nMDS of the 4 locations (Kingscote, Christmas Cove, Wirrina Cove and North Haven) based on Bray-Curtis dissimilarity (Left column) and Jaccard index (Right column) during (A) one, (B) three, (C) six, (D) nine and (E) twelve months.

Bray-Curtis

Jaccard

- Christmas Cove
- Kingscote
- Wirrina Cove
- North Haven
The SIMPER analysis was used to determine the two taxa that contributed to the most differences between two locations over the six months (Table 5.4). For the one-month sampling, the analysis shows that the highest differences were for the assemblages from Kingscote and North Haven (96.13%), where the differences were driven by relatively high abundance of the introduced ascidians *A. aspersa* and *C. intestinalis* in North Haven. When the biofouling assemblages in North Haven were compared with biofouling assemblages in either Christmas Cove or Wirrina Cove, the differences were driven by relatively high abundance of the two ascidians *A. aspersa* and *C. intestinalis* in North Haven. The communities with the lowest dissimilarity were between Christmas Cove and Wirrina Cove (75.83%). Differences in these two locations were driven by *Harpacticus* spp. in Christmas Cove and Order Podocopida in Wirrina Cove. The differences in biofouling assemblages between Kingscote and Christmas Cove or Wirrina Cove was caused by Harpacticoida order and Order Podocopida in Christmas Cove and by Harpacticoida order and *Harpacticus* spp. Wirrina Cove.

The samples collected at three months indicated that the least similar communities, i.e. highest dissimilarity were between Christmas Cove and North Haven (94.94%), where the differences were driven by relatively high abundance of the organisms *A. aspersa* in North Haven and *Prophaerosyllis isabellae* in Christmas Cove. The biofouling communities in Kingscote and North Haven also had high dissimilarity (94.5%) but these communities were influenced by *H. mediterraneum* in Kingscote and *A. aspersa* in North Haven. The relative abundance of *A. aspersa* and *C. intestinalis* in North Haven led to the dissimilarity in the biofouling communities between Wirrina Cove and North Haven. The biofouling communities in Christmas Cove and Wirrina Cove had the least dissimilarity (81.75%) and was driven by *P. isabellae* in Christmas Cove and *L. canui* in Wirrina Cove. When the biofouling assemblages in Kingscote was compared to either communities in Christmas Cove and Wirrina Cove, the differences were predominantly driven by *H. mediterraneum* in Kingscote in both comparisons but species composition in Christmas Cove and Wirrina Cove were driven by *P. isabellae* and *B. schlosseri* respectively.

For the samples collected at six months, the SIMPER analysis showed that the least similar communities were between Kingscote and North Haven (93.88%) and was driven by *A. aspersa* in North Haven and *Anticomidae* spp. in Kingscote. The biofouling communities in North Haven had high % dissimilarity between either Christmas Cove (91.31%) or Wirrina Cove (91.5%). These differences were mostly driven by *A. aspersa* in North Haven for both
comparisons, *B. imbricata* in Christmas Cove and *Didemnum* spp. in Wirrina Cove. The biofouling communities which had the least dissimilarity was between Christmas Cove and Wirrina Cove (78.11%). The differences in the biofouling communities between Kingscote and Christmas Cove had high dissimilarity (91.12%) and was predominantly driven by *Anticomidae* spp. in Kingscote and *B. imbricata* in Christmas Cove. As for the assemblages sampled from Kingscote and Wirrina Cove, the differences were driven by *Anticomidae* spp. in Kingscote and *Didemnum* spp. in Wirrina Cove.

The samples collected at nine months indicated that the differences in the assemblages sampled from Kingscote and Christmas Cove were driven by the relatively higher abundance of *P. vittatus* at Kingscote and *Paramphiascella fulvofasciata* in Christmas Cove. Kingscote and Wirrina Cove had the highest dissimilarity (89.17%) with differences driven by *P. vittatus* in Kingscote and *P. pomaria* in Wirrina Cove. The differences in the sampling obtained from Christmas Cove and Wirrina Cove were primarily driven by *P. pomaria* and *D. dubia* in Wirrina Cove. The biofouling samples collected from Kingscote and North Haven, the differences were driven by *P. vittatus* in Kingscote and *C. intestinalis* in North Haven, while the presence of *C. intestinalis* in North Haven and *B. imbricata* in Christmas Cove contributed to the differences observed in both locations. Wirrina Cove and North Haven shared the highest % similarity (75.91%) and differences were driven by *C. intestinalis* in North Haven and *P. pomaria* in Wirrina Cove.

For the twelve-month sampling event, only the pairwise comparison between Wirrina Cove and North Haven was examined because samples from Kangaroo Island were lost and could not be analysed. Here, the high differences in the assemblages between Wirrina Cove and North Haven (82.98%) were primarily driven by *C. intestinalis* in North Haven and *P. pomaria* in Wirrina Cove.
Table 5.4: SIMPER analysis of the differences between groups according to the % contribution of the top 2 MOTUs based on abundance for all four locations (Ki = Kingscote, X.C. = Christmas Cove, W.C. = Wirrina Cove, N.H. = North Haven) during each time point. “Location” referred to each group in order and the % dissimilarity between two groups were in brackets. A “+” indicated that the MOTUs was dominant in that location and MOTUs in bold represented an introduced species.

<table>
<thead>
<tr>
<th>Months</th>
<th>Groups (Location 1 &amp; 2):</th>
<th>MOTUs</th>
<th>Location Mean abundance</th>
<th>Location Mean abundance</th>
<th>Contrib %</th>
</tr>
</thead>
<tbody>
<tr>
<td>One</td>
<td>Ki &amp; X.C. (83.71%)</td>
<td>Order Harpacticoida - + 9.17</td>
<td>Order Podocopida - + 5.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ki &amp; W.C. (81.77%)</td>
<td>Order Harpacticoida - + 6.18</td>
<td><em>Harpacticus</em> spp. - + 5.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>X.C. &amp; W.C. (75.83%)</td>
<td><em>Harpacticus</em> spp. - + 6.97</td>
<td>Order Podocopida + - 6.72</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ki &amp; N.H. (96.13%)</td>
<td><em>A. aspersa</em> - + 12.26</td>
<td><em>C. intestinalis</em> - + 12.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>X.C. &amp; N.H. (90.50%)</td>
<td><em>C. intestinalis</em> - + 11.75</td>
<td><em>A. aspersa</em> - + 11.71</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>W.C. &amp; N.H. (86.30%)</td>
<td><em>A. aspersa</em> - + 11.87</td>
<td><em>C. intestinalis</em> - + 11.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Three</td>
<td>Ki &amp; X.C. (88.34%)</td>
<td><em>H. mediterraneum</em> + - 7.86</td>
<td><em>P. isabellae</em> - + 5.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ki &amp; W.C. (92.47%)</td>
<td><em>H. mediterraneum</em> + - 10.20</td>
<td><em>B. schlosseri</em> - + 4.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>X.C. &amp; W.C. (81.75%)</td>
<td><em>P. isabellae</em> + - 5.63</td>
<td><em>L. canui</em> - + 5.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ki &amp; N.H.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki &amp; X.C.</td>
<td></td>
<td></td>
<td></td>
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<td>--------------------</td>
<td>-------</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Six</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(91.12%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. imbricata</td>
<td>-</td>
<td>+</td>
<td>9.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anticomidae spp.</td>
<td>+</td>
<td>-</td>
<td>5.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(86.62%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anticomidae spp.</td>
<td>+</td>
<td>-</td>
<td>4.71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Didemnum spp.</td>
<td>-</td>
<td>+</td>
<td>4.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(93.88%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. aspersa</td>
<td>-</td>
<td>+</td>
<td>6.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anticomidae spp.</td>
<td>+</td>
<td>-</td>
<td>6.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(91.31%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. imbricata</td>
<td>+</td>
<td>-</td>
<td>8.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. aspersa</td>
<td>-</td>
<td>+</td>
<td>9.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(91.50%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. aspersa</td>
<td>-</td>
<td>+</td>
<td>7.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Didemnum spp.</td>
<td>+</td>
<td>-</td>
<td>7.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(84.24%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. vittatus</td>
<td>+</td>
<td>-</td>
<td>9.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. fulvofasciata</td>
<td>-</td>
<td>+</td>
<td>5.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(89.17%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. vittatus</td>
<td>+</td>
<td>-</td>
<td>9.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. pomaria</td>
<td>-</td>
<td>+</td>
<td>7.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(84.38%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. pomaria</td>
<td>-</td>
<td>+</td>
<td>6.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. dubia</td>
<td>-</td>
<td>+</td>
<td>5.97</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The ANOSIM test was performed to support the nMDS results according to the Bray-Curtis dissimilarity and Jaccard index (as in Chapter 4). Multivariate analyses indicated that at each sampling time, there were significant differences in the biofouling assemblages based on Bray Curtis dissimilarity and Jaccard index (Table 5.5). For the samples collected at one month (Table 5.6), the pairwise comparisons based on the Bray-Curtis dissimilarity indicated that biofouling communities in North Haven were significantly different from the other three locations (Table 5.6). The lowest difference was observed for the pairwise comparisons between Kingscote and Christmas Cove ($R = 0.644$). However, results obtained from pairwise comparisons based on Jaccard index indicated that the highest differences were observed between the assemblages sampled from Kingscote and Christmas Cove ($R = 0.918$) and Wirrina Cove ($R = 0.946$). Biofouling communities from Wirrina Cove and North Haven had the least difference ($R = 0.581$). These observations were consistent with the nMDS plot (Figure 5.6A).

At three months (Table 5.6), the pairwise comparisons based on Bray-Curtis dissimilarity showed that the biofouling communities between North Haven and either Kingscote ($R = 0.985$) or Christmas Cove ($R = 0.959$) were significantly different. The pairwise comparisons between Kingscote and Christmas Cove based on the Bray-Curtis dissimilarity and Jaccard Index showed that samples obtained from these two sampling sites were also significantly different. The pairwise comparison between Christmas Cove and Wirrina Cove was not significant ($p > 0.05$). After six months, the highest differences remained between biofouling communities in Kingscote and Christmas Cove for both Bray-Curtis dissimilarity and Jaccard index. Biofouling communities from Christmas Cove and Wirrina Cove were found to be the least different ($R = 0.359$) based on the Bray-Curtis dissimilarity. The
biofouling communities in North Haven shared the highest similarity with those of Wirrina Cove ($R = 0.452$) based on Jaccard index.

At nine months (Table 5.6), the pairwise comparisons based on Bray-Curtis dissimilarity indicated that the biofouling communities in Wirrina Cove shared the highest similarity with those from North Haven ($R = 0.758$). As for the pairwise comparisons based on the Jaccard index, biofouling communities from Christmas Cove and Wirrina Cove shared the highest similarity ($R = 0.685$). These observations were consistent with the nMDS plot (Figure 5.6D). For the 12 months samples (Table 5.5), the comparison between Wirrina Cove and North Haven gave significantly similar results for both the Bray-Curtis dissimilarity ($R = 0.942$) and Jaccard index ($R = 0.926$). This finding was consistent with the nMDS plot (Figure 5.6E).

Table 5.5: Global ANOSIM Results obtained from Bray-Curtis dissimilarity and Jaccard index at one, three, six, nine and twelve months. Significant p values are indicated in bold ($p < 0.05$). The number of permutations used to obtain p-values is 999.

<table>
<thead>
<tr>
<th>Month</th>
<th>Bray-Curtis dissimilarity</th>
<th>Jaccard index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Global $R$</td>
<td>p-value</td>
</tr>
<tr>
<td>One</td>
<td>0.87</td>
<td>0.001</td>
</tr>
<tr>
<td>Three</td>
<td>0.697</td>
<td>0.001</td>
</tr>
<tr>
<td>Six</td>
<td>0.554</td>
<td>0.001</td>
</tr>
<tr>
<td>Nine</td>
<td>0.905</td>
<td>0.001</td>
</tr>
<tr>
<td>Twelve</td>
<td>0.942</td>
<td>0.001</td>
</tr>
</tbody>
</table>
Table 5.6: ANOSIM Results obtained from Bray-Curtis dissimilarity and Jaccard index of pairwise comparisons among the four locations (Ki = Kingscote, X.C. = Christmas Cove, W.C. = Wirrina Cove, N.H. = North Haven) in one, three, six and nine months. Significant p values are indicated in bold (p < 0.05). The number of permutations used to obtain p-values is 999.

<table>
<thead>
<tr>
<th>Month</th>
<th>Location groups</th>
<th>R statistics</th>
<th>p-value</th>
<th>Location groups</th>
<th>R statistics</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>One</td>
<td>Ki, X.C.</td>
<td>0.644</td>
<td>0.002</td>
<td>Ki, X.C.</td>
<td>0.918</td>
<td>0.002</td>
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<tr>
<td></td>
<td>Ki, W.C.</td>
<td>0.653</td>
<td>0.001</td>
<td>Ki, W.C.</td>
<td>0.946</td>
<td>0.001</td>
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<td></td>
<td>Ki, N.H.</td>
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<td>0.001</td>
<td>Ki, N.H.</td>
<td>0.77</td>
<td>0.001</td>
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<tr>
<td></td>
<td>X.C., W.C.</td>
<td>0.789</td>
<td>0.001</td>
<td>X.C., W.C.</td>
<td>0.718</td>
<td>0.001</td>
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<tr>
<td></td>
<td>X.C., N.H.</td>
<td>0.987</td>
<td>0.001</td>
<td>X.C., N.H.</td>
<td>0.642</td>
<td>0.001</td>
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<td>W.C., N.H.</td>
<td>0.913</td>
<td>0.001</td>
<td>W.C., N.H.</td>
<td>0.581</td>
<td>0.001</td>
</tr>
<tr>
<td>Three</td>
<td>Ki, X.C.</td>
<td>0.981</td>
<td>0.002</td>
<td>Ki, X.C.</td>
<td>0.959</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Ki, W.C.</td>
<td>0.422</td>
<td>0.007</td>
<td>Ki, W.C.</td>
<td>0.626</td>
<td>0.001</td>
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<td>Ki, N.H.</td>
<td>0.985</td>
<td>0.001</td>
<td>Ki, N.H.</td>
<td>0.671</td>
<td>0.001</td>
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<td></td>
<td>X.C., W.C.</td>
<td>0.132</td>
<td>0.118</td>
<td>X.C., W.C.</td>
<td>0.144</td>
<td>0.097</td>
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<td>X.C., N.H.</td>
<td>0.959</td>
<td>0.001</td>
<td>X.C., N.H.</td>
<td>0.522</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>W.C., N.H.</td>
<td>0.671</td>
<td>0.001</td>
<td>W.C., N.H.</td>
<td>0.397</td>
<td>0.001</td>
</tr>
<tr>
<td>Six</td>
<td>Ki, X.C.</td>
<td>0.988</td>
<td>0.005</td>
<td>Ki, X.C.</td>
<td>0.956</td>
<td>0.005</td>
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<tr>
<td></td>
<td>Ki, W.C.</td>
<td>0.577</td>
<td>0.001</td>
<td>Ki, W.C.</td>
<td>0.809</td>
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<td></td>
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<td>Ki, N.H.</td>
<td>0.68</td>
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<tr>
<td></td>
<td>X.C., W.C.</td>
<td>0.359</td>
<td>0.004</td>
<td>X.C., W.C.</td>
<td>0.529</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>X.C., N.H.</td>
<td>0.604</td>
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<td>X.C., N.H.</td>
<td>0.465</td>
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<tr>
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<td>W.C., N.H.</td>
<td>0.627</td>
<td>0.001</td>
<td>W.C., N.H.</td>
<td>0.452</td>
<td>0.001</td>
</tr>
<tr>
<td>Nine</td>
<td>Ki, X.C.</td>
<td>0.943</td>
<td>0.002</td>
<td>Ki, X.C.</td>
<td>0.765</td>
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<tr>
<td></td>
<td>Ki, W.C.</td>
<td>0.99</td>
<td>0.002</td>
<td>Ki, W.C.</td>
<td>0.88</td>
<td>0.001</td>
</tr>
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<td></td>
<td>Ki, N.H.</td>
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<td>0.001</td>
<td>Ki, N.H.</td>
<td>0.936</td>
<td>0.001</td>
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<tr>
<td></td>
<td>X.C., W.C.</td>
<td>0.99</td>
<td>0.001</td>
<td>X.C., W.C.</td>
<td>0.685</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>X.C., N.H.</td>
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<td>X.C., N.H.</td>
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</tr>
<tr>
<td></td>
<td>W.C., N.H.</td>
<td>0.758</td>
<td>0.001</td>
<td>W.C., N.H.</td>
<td>0.669</td>
<td>0.001</td>
</tr>
</tbody>
</table>
Figure 5.7 shows a set of nMDS plots were generated based on both Bray-Curtis dissimilarity and Jaccard index looking at change in biofouling communities over time for each location. In Kingscote, the nMDS plot based on the Bray-Curtis dissimilarity (Figure 5.7A left panel) indicated that the biofouling communities on the plates at each time point were quite distinct as samples from each other. Similarly, the results based on Jaccard index (Figure 5.7A right panel) indicate that the biofouling communities were different at each time point and were variable within each time point. The samples from three and six months shared the highest similarity as they were clustered together. There was a shift in the biofouling communities over the nine months in both plots.

In Christmas Cove, the results based on Bray-Curtis dissimilarity (Figure 5.7B left panel) showed that the biofouling communities changed over time and the samples at each time point had high variability. The samples from three to nine months shared higher similarity than with samples from one month. Based on the Jaccard index (Figure 5.7B right panel), the samples collected at three and six months were in close proximity, indicating that these samples had some degree of similarity. For both plots, the samples from one and nine months shared the highest dissimilarity in community structure and species composition.

The nMDS plot showed that there was a shift in biofouling assemblages sampled from Wirrina Cove (Figure 5.7C, left panel). Samples from three months shared similarity with samples from one month and six months. The samples were also highly variable at each time point. There was no change in community structure for samples from nine to twelve months as samples were overlapping each other. The plot based on Jaccard index (Figure 5.7C, right panel) showed a similar result where the samples obtained from three months share some similarities with the samples obtained from one and six months. There was also no change in species composition from nine to twelve months.

The plot based on the Bray-Curtis dissimilarity showed that in North Haven (Figure 5.7D, left panel), most samples collected during the first, third and sixth month were clustered together. Although samples from nine and twelve months were close to the first six months, there was higher similarity in biofouling assemblages from the latter six months. The results based on Jaccard index (Figure 5.7D, right panel) showed that samples from first six months clustered together and highly variable. There was no change in species composition from nine to twelve months.
Figure 5.7: Multidimensional ordination plots of the samples collected at one (red triangle), three (green square), six (blue cross), nine (purple circle) and twelve (blue triangle) months of deployment based on Bray-Curtis dissimilarity (Left plot) and Jaccard index (Right plot) at Kingscote (A), Christmas Cove (B), Wirrina Cove (C) and North Haven (D).
To support the results of the nMDS plot, ANOSIM tests were performed based on a Bray-Curtis dissimilarity and Jaccard index (Table 5.7 & Table 5.8). In all four locations, the multivariate analyses based on Bray-Curtis dissimilarity and Jaccard index showed that there were significant differences over the twelve months (Table 5.7).

In Kingscote (Table 5.8), pairwise comparisons based on Bray-Curtis dissimilarity show that the biofouling communities at the five sampling times were significantly different (p-value < 0.05). The highest difference was observed between the biofouling communities from three and nine months ($R = 0.976$). The pairwise comparison between the one month and six months samples had the lowest degree of separation ($R = 0.563$). The ANOSIM results based Jaccard index indicate that the biofouling communities had high degree of separation over time ($R > 0.9$).

In Christmas Cove (Table 5.8), the highest differences between the biofouling assemblages were observed based on Bray-Curtis dissimilarity between the samples obtained from one and either six or nine months ($R = 1$). There were relatively similar differences for the other four comparisons. The ANOSIM results based Jaccard index suggest that the species composition high differences for most pairwise comparisons. The comparison in biofouling communities from three and six months was the only one with high similarity ($R = 0.491$). These findings were consistent with nMDS plot (Figure 5.7B).

In Wirrina Cove (Table 5.8), biofouling communities from one and either nine or twelve months differed the most ($R = 0.99, R = 0.957$) based on Bray-Curtis dissimilarity while pairwise comparisons for samples from nine and twelve months showed the highest similarity ($R = 0.194$). Biofouling communities from three and six months also shared high similarity ($R = 0.233$). Similar results were obtained based on Jaccard index with the highest difference between samples collected from one and either nine or twelve months ($R = 1, R = 0.995$). Biofouling assemblages from nine and twelve months shared the highest similarity ($R = 0.246$). The findings were consistent with nMDS plot (Figure 5.7C).

In North Haven (Table 5.8), the community structure remained the same over the first six months based on the Bray-Curtis dissimilarity. There was also no change in the biofouling communities from nine to twelve months. However, the community structure shifted from the first six months to the latter six months. As for results based on Jaccard index, the biofouling
communities from one to three months remained the same ($R = 0.14$). There was also high similarity for samples between nine and twelve months while pairwise comparisons for samples between one and either nine or twelve months showed the highest difference ($R = 0.973, R = 0.95$). These results were consistent with the nMDS plot (Figure 5.7D).

PERMANOVA analysis based on Bray-Curtis dissimilarity and Jaccard index (Appendix, Table 1-3) also showed that there were significant differences across all locations over time and further pairwise comparisons were all significant.

Table 5.7: Global ANOSIM Results obtained from Bray-Curtis dissimilarity and Jaccard index for the four locations (Ki = Kingscote, X.C. = Christmas Cove, W.C. = Wirrina Cove, N.H. = North Haven). Significant p values are indicated in bold (p < 0.05). The number of permutations used to obtain to p-values is 999.

<table>
<thead>
<tr>
<th>Location</th>
<th>Bray-Curtis dissimilarity</th>
<th>Jaccard index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Global $R$</td>
<td>p-value</td>
</tr>
<tr>
<td>Kingscote</td>
<td>0.792</td>
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</tr>
<tr>
<td>Christmas Cove</td>
<td>0.847</td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td>Wirrina Cove</td>
<td>0.624</td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td>North Haven</td>
<td>0.406</td>
<td><strong>0.001</strong></td>
</tr>
</tbody>
</table>
Table 5.8: ANOSIM Results obtained from Bray-Curtis dissimilarity and Jaccard index of pairwise comparisons over twelve months in the four locations (Ki = Kingscote, X.C. = Christmas Cove, W.C. = Wirrina Cove, N.H. = North Haven). Significant p values are indicated in bold (p < 0.05). The number of permutations used to obtain p-values is 999.

<table>
<thead>
<tr>
<th>Location</th>
<th>Time groups (months)</th>
<th>Bray-Curtis dissimilarity</th>
<th>Jaccard index</th>
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<tbody>
<tr>
<td></td>
<td>R statistic</td>
<td>P-value</td>
<td>R statistic</td>
</tr>
<tr>
<td>Kingscote</td>
<td>one, three</td>
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<tr>
<td></td>
<td>one, six</td>
<td>0.563</td>
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<tr>
<td></td>
<td>one, nine</td>
<td>0.793</td>
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<tr>
<td></td>
<td>three, six</td>
<td>0.861</td>
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<tr>
<td></td>
<td>three, nine</td>
<td>0.976</td>
<td>0.002</td>
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<tr>
<td></td>
<td>six, nine</td>
<td>0.861</td>
<td>0.005</td>
</tr>
<tr>
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<tr>
<td></td>
<td>one, six</td>
<td>1</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>one, nine</td>
<td>1</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>three, six</td>
<td>0.726</td>
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<tr>
<td></td>
<td>three, nine</td>
<td>0.761</td>
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<tr>
<td></td>
<td>six, nine</td>
<td>0.674</td>
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<tr>
<td></td>
<td>one, twelve</td>
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<td>three, six</td>
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<td>six, nine</td>
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<tr>
<td></td>
<td>nine, twelve</td>
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<tr>
<td>North Haven</td>
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<td>one, nine</td>
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<td>one, twelve</td>
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<tr>
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<tr>
<td></td>
<td>three, twelve</td>
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<td>six, twelve</td>
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<tr>
<td></td>
<td>nine, twelve</td>
<td>0.117</td>
<td>0.079</td>
</tr>
</tbody>
</table>
5.4 Discussion

This study aimed to detect introduced species within biofouling communities and assess the composition of these communities from different sites namely North Haven, Wirrina Cove, Christmas Cove and Kingscote in South Australia using high-throughput sequencing and settlement plates. Twelve potential introduced species were detected in all locations (Table 5.3). The three bryozoans B. imbricata, B. gracilis, Membranipora spp. and W. subtorquata and the hydroid O. dichotoma were introduced to Australia but are now considered cosmopolitan (Hewitt et al., 2004; Dafforn et al., 2009). The metabarcoding results revealed the presence of Ulva spp. in the samples. However, the databases used could not provide further information on the species. Several Ulva spp. are now considered cosmopolitan while Ulva fasciata is considered as introduced (Hewitt et al., 2004). The European fanworm is an important MIS which has caused severe economic losses in scallop aquaculture and is a successful competitor for resources such as space against native organisms (Currie et al., 2000). Previous monitoring surveys that were carried out have detected the presence of the MIS in several locations in South Australia (Kinloch et al., 2009; Wiltshire and Deveney, 2011). One of the surveys revealed the presence of S. spallanzanii in Kangaroo Island.

There are many factors that influence the settlement of marine species in biofouling assemblages. For example, the introduced species B. schlosseri was dominant in three samples collected from the third month in Wirrina Cove and these samples were collected from plates found at the upper row of the frame. At the third month, larvae of B. schlosseri react positively to light upon release but they become indifferent and respond negatively before they go through metamorphosis (Grave and Woodbridge, 1924; Thorson, 1964; Brunetti, 1974). Their dominance in biofouling assemblages could be due to absence of predators although in the sixth month, it has been found to compete with other species especially the bryozoan Bugula spp. for space (Brunetti, 1974). The lifecycle of B. schlosseri is also another determining factor of its colonisation. B. schlosseri are able to grow on and cover marine fouled organisms and when they start to reduce in population, it leaves the surface of the substrate clear, allowing other marine organisms to settle on it (Terlizzi et al., 2000). The life cycle of B. schlosseri is also dependent on season and previous studies have shown that the lifespan lasts for 3 months for spring-born colonies (Chadwick-Furman and Weissman, 1995). The ascidian was detected for the first six months of the field trial which correspond to the end of winter to middle of
summer. This could explain why in the sixth month, although \textit{B. schlosseri} was still present, it was not dominant, and the species diversity was high.

It is still not entirely clear what would make a community susceptible to invasions (Stachowicz et al., 1999). The MOTUs of two introduced ascidians; \textit{C. intestinalis} and \textit{A. aspersa} were detected in both Wirrina Cove and North Haven. However, they were most abundant in North Haven (Figure 5.5 & Table 5.3). In Wirrina Cove, the presence of these ascidians had no effect on overall species diversity in all sampling times (Figure 5.5). The close proximity of North Haven to Port Adelaide which is a highly disturbed habitat could have played a role in shaping the biofouling community. Disturbances to marine environments can either reduce the availability of resources or introduce new resources which can contribute to the settlement and subsequent invasion of introduced species (Davis et al., 2000). Studies have also shown that pollution, often associated with major ports and associated heavy industry, creates a suitable environment for growth of introduced species, but can also reduce native diversity in a given location (Piola and Johnston, 2008). Moreover, the persistence of \textit{C. intestinalis} on the plates in North Haven could indicate that this location provides suitable conditions for establishment of this species. \textit{C. intestinalis} has rapid growth (Yamaguchi, 1975) and its ability to depress species richness (Blum et al., 2007). Similar to Blum et al. (2007), the findings in this chapter showed that \textit{C. intestinalis} reduced species diversity across the twelve months, which could demonstrate the capacity of long-term impacts that introduced species can have on species composition. As for \textit{A. aspersa}, it was still present in the last six months of the plate setup but had reduced in population. Previous studies showed that \textit{A. aspersa} was among the early settlers in a community when there is reduced competition (Rajbanshi and Pederson, 2007; Tatián et al., 2010). The early occurrence of \textit{A. aspersa} could also be attributed to the life cycle of \textit{A. aspersa} which usually lasts 18 months, and the decrease in these organisms at the 9 and 12 months sampling could have indicated that they have reached the end of their life cycle (Millar, 1952). The space left by \textit{A. aspersa} allowed other marine organisms to settle. The dominance of the two introduced ascidians was not observed in Wirrina Cove. This could suggest that either the habitat to colonise was unavailable due to the species diversity present at the site, or that conditions were not suitable for colonisation of these species to prosper. The species present use their resources such as space more efficiently.

Similar to other sites, as observed in the bar chart of % total of read count (Figure 5.5, see “Kingscote”), the MOTUs of introduced species detected in the species rich communities
from Kingscote (Table 5.3) did not appear to have an adverse effect on the assemblage. For example, the ascidian *B. schlosseri* was also detected but it did not appear dominant in assemblages in Christmas Cove. One possible explanation for this could be due to high diversity which could lead to invasion resistance (Stachowicz et al., 1999). Kangaroo Island is surrounded by marine parks and several studies have been carried out to determine the effect of introduced species in marine protected areas. One study showed that there was more invasive fouling on settlement plates in an unprotected area compared to presence of only one invasive species on plates in a marine reserve (Gestoso et al., 2017). Native species may have developed mechanisms to resist biological invasions in species-rich marine reserves. However, other reports have shown that there is higher or similar abundances of MIS in protected areas than in unprotected ones (Byers, 2005; Klinger et al., 2006). This is consistent with the dominance of the *B. imbricata* in Christmas Cove (Figure 5.5, see “Christmas Cove”). Although the introduced species did not influence the species diversity in Kingscote, their detection emphasises the need to carry out proactive measures, i.e. monitoring, which would reduce the risk of invasion or allow prompt management and/or eradication practices to be implemented.

There was variability in the species composition among replicate plates within sites at Kangaroo Island and Wirrina Cove. Although the plates were within a few centimetres of each other, there was significant dissimilarity in the species composition among the plates (Figure 5.5). This may be because biofouling is a complex process and studies have also showed that the variation in spatial distribution is a natural occurrence in the biofouling communities (Bloecher et al., 2013). Thus, the establishment of the biofouling community is dependent on many factors. Environmental conditions such as temperature may differ based on the depth of the water i.e. access to sunlight which may affect growth of certain organisms (Cowie, 2009). Artificial substrates have been described as ecological islands (Osman, 1982). Settlement plates are surrounded by water and can be considered isolated from the other plates. This would give rise to a community that could be unique and comparable to an island. Plates that are closer to the water surface may be more suitable to certain organisms, while deeper plates may preferentially select for others. The water current, the propagules present, and seasons would also influence the settlement of marine organisms on hard surfaces. The variability among replicates in Kangaroo Island and Wirrina Cove could also be due to lack of homogenisation. For DNA extraction, there was no initial homogenisation of the four subsamples and only one subsamples was used. Homogenisation is important to have an accurate representation of the species composition and to ensure the detection of rare species (Lohan et al., 2019).
The presence of introduced species suggests that the recreational boating among the locations may contribute largely to the translocation of marine organisms. Although the biofouling assemblages differed among locations (Figure 5.5), the samples from Wirrina Cove, Christmas Cove and North Haven showed a high degree of similarity of species composition (Figure 5.6 & Table 5.6). A trip between neighbouring locations favours transport of greater abundances of marine organisms and increases the chances of survival of marine organisms during voyage (Lavoie et al., 1999; Lewis and Coutts, 2009). Moreover, recreational boats usually stay in one location for some time before moving to the next (Mineur et al., 2008). This would give marine fouling organisms sufficient time to attach onto the surface or the boats, and eventually be translocated by fouling (Walters, 1996). Kingscote was the only site which had a jetty with no berths. Boats usually dock at jetties for a very short period of time and this may not pose as big a risk as vessels that are berthed in marinas. When these vessels remain moored in marinas for long period of time, they provide hard surfaces for colonization of fouling introduced species (Minchin et al., 2006). The risk associated with recreational vessel traffic from invaded sites to uninvaded sites has the potential to be high and to be a potentially important vector (Clarke Murray et al., 2011). The impact of recreational vessels as vectors of introduced species has been largely overlooked in favour of studies on commercial vessels (Floerl and Inglis, 2005; Davidson et al., 2010). While there is no doubt that commercial vessels play a primary vector role in transferring introduced species across vast differences, the role that recreational and other smaller vessels play in facilitating range expansion along domestic coastlines needs to be rigorously evaluated. Introduced species in marine protected areas may have detrimental effects on the biodiversity present, and monitoring is crucial to reduce their introduction. However, the regular sea traffic complicates the monitoring and management of those areas.

In locations that have different degree of anthropogenic activity, the propagule pressure may also be a significant factor that would affect settlement of biofouling communities (Lockwood et al., 2009). With both presence of artificial substrates such as pontoons, pilings and breakwaters as well as frequent shipping including recreational vessels, the propagule pressure is higher as there is an increased amount of introduced species as adults and mostly larvae in the environment. This can create different larval compositions from areas that are less disturbed. This was observed in both Kingscote and North Haven where both nMDS plots and ANOSIM showed that the samples obtained from these sampling sites were highly dissimilar.
which indicates that their respective community structure and species composition were different, and this statistical dissimilarity remained constant over time. However, the twelve months samples from Kingscote were missing, and the statistical comparison between Kingscote and North Haven could not be performed.

The change in biofouling assemblages over time varied for the four locations. In Kingscote and Christmas Cove, there was a shift in community structure and species composition at each time point and the highest dissimilarity was between one and nine months (Figure 5.7). This could be attributed to the rich native diversity present at both locations (Figure 5.3) and the marine organisms already occupying an ecological niche. The presence of introduced species had a minimal effect on the biofouling communities at both sites. However, as the level of disturbance increased, the shift was less apparent as observed in Wirrina Cove. The community structure in North Haven remained constant for the first six months regardless of the high variability in species composition. This was caused by the presence of the two introduced ascidians. However, there was a shift in the biofouling community for the last six months of the plate setup which was consistent with the decline of A. aspersa and allowing other marine organisms to settle. The presence of C. intestinalis continued to have an effect on both community structure and species composition. The natural process of biofouling development was disrupted by introduced species and the high level of disturbance.

The use of metabarcoding in assessing of biofouling communities especially in healthy habitats indicated the importance of well-documented databases. The use of two databases, i.e., SILVA and BLAST (NCBI), was more reliable. SILVA database failed to taxonomically identify 4 MOTUs, including two MOTUs which belong to the family Didemnidae. The MOTUs (Didemnum spp.) identified was in the top four MOTUs contributing to the total number of reads. It was mostly present in Wirrina Cove, and its presence had an effect on the species diversity in Wirrina Cove (Figure 5.5). Also, the high stringency of 97% match contributed to the removal of further reads. This was mostly observed in Kangaroo Island and Wirrina Cove (Table 5.2). This could be due to the possible lack of taxonomic study in that region and native diversity which has not been identified yet. Reliable and successful metabarcoding depends on the bioinformatics tools being used. The database is crucial for correct taxonomic assignment and has to be extensive as well as updated regularly to cover a wide range of organisms (Ji et al., 2013; Cristescu, 2014). In this case, biofouling communities
are being studied and identification relies heavily on the database used to properly examine the interactions among marine organisms in the community.

The use of settlement plates to study of long-term fouling communities provided an insight into how the level of disturbance and shipping can affect species diversity and change biofouling communities over time. This is the longest scale study using molecular techniques to study biofouling communities. The findings obtained from comparing settlement panels at locations with different levels of anthropogenic activities also places emphasis on the need to understand the ecological impacts of introducing man-made structures in the marine environment as this is essential for implementing management strategies (Dafforn et al., 2009). With the increase in globalisation and shipping, the impacts will become more pronounced as homogenisation and loss of native biodiversity is likely to occur in disturbed areas.
Chapter 6:
Discussion
Discussion

The increasing number of artificial structures in the marine environment has led to ecological and economic impacts such as loss of habitat for native biodiversity and introduction of potential MIS to new environments (Airoldi and Beck, 2007; Bulleri and Chapman, 2010; Dafforn et al., 2015). Biofouling communities on artificial structures have long been studied on settlement plates (Dean and Hurd, 1980; Sutherland, 1981; Glasby and Connell, 1999; Stachowicz et al., 1999), which is considered a standardised method to study these communities (Marraffini et al., 2017). To better understand the development of the biofouling community over time, experimentation on settlement plates in response to both biotic and abiotic factors has been carried out using methods such as photography and morphological identification through microscopy (Wood et al., 2013). However, the scope of identifying species, especially at early stages of biofouling, has been limited by our ability to identify cryptic species and species at low abundance (Wood et al., 2013; Zaiko et al., 2016). This thesis has demonstrated that the limitation can be overcome by using molecular methods such as metabarcoding.

My aims were firstly to validate the effectiveness of metabarcoding and settlement plates as an early detection method for introduced species in both Gippsland Lakes and South Australia. Previous studies have shown that this approach can provide passive monitoring and early detection of potential MIS in the marine environment (Pochon et al., 2015). A pilot study in Gippsland Lakes, Victoria, ran for a period of six months (Chapter 4), and a further study was carried out in Gulf of St Vincent and Kangaroo Island, South Australia looking at locations with differing degrees of anthropogenic activity for a period of twelve months (Chapter 5). The combined techniques would allow detection of introduced species known to be MIS as well as newly introduced species in the area. Settlement plates and metabarcoding have also been used for assessment of biodiversity in biofouling communities (Zaiko et al., 2016). Therefore, the second aim of this thesis was to explore the use of settlement plates and metabarcoding to assess the community structure in biofouling assemblages over time in several locations. To the best of my knowledge, the study in South Australia is the first long-scale study assessing biofouling communities using molecular methods such as metabarcoding.
6.1 Freeze-drying is an effective method of processing samples with a large mass.

To study the diversity of the biofouling assemblages over time, it is crucial to obtain a biologically relevant representative amount of samples of the community being studied, and this can only be achieved by using a sufficient amount of starting material. Long-term biofouling communities developing on hard surfaces such as settlement plates accumulate biomass, and this implies that a large amount of starting material would be required to have a relevant representation of the community for DNA extraction. However, most DNA extraction kits allow a limited amount of sample (less than 10 g) for extraction, which would be too low for biodiversity assessment due to the complexity of certain communities such as those in biofouling assemblages. Another problem that can arise from analysis of environmental samples is the presence of tough cell walls in organisms and risk of DNA degradation (Saha et al., 1997; Maneeruttanarungroj and Incharoensakdi, 2016). To address these issues, the use of freeze drying was tested as a potential processing method, and the findings indicate that it allowed removal of the water content which reduced the mass considerably as well as concentrating the biota (Chapter 2, Section 2.3.1). Freeze drying did not affect the DNA concentration and purity (Chapter 2, Figure 2.2). Moreover, measurement with both the Nanodrop and Qubit showed that the DNA concentration was higher in FD samples than NFD samples. In summary, the freeze-drying method allowed processing of a large amount of starting material (more than 10 g) and did not affect the quality of the DNA, which made it suitable for downstream molecular applications.

6.2 Settlement plates coupled with metabarcoding can be used as an early detection method for potential MIS.

Early detection of introduced species is crucial to mitigate ecological and economic impacts on the environment (Bax et al., 2003; Bott et al., 2010b; Bott, 2015). However, taxonomic identification in monitoring surveys has been complicated due to the difficulty in differentiating organisms at larval stages (Wood et al., 2013; Zaiko et al., 2016). Advances in molecular methods have improved species identification, and one such technique is metabarcoding, which provides biodiversity assessment for communities at large (Taberlet et al., 2012a). Artificial structures are usually the first point of contact for any incoming marine organisms brought by vectors in marine environments (Bax et al., 2003; Fofonoff et al., 2003), and the biofouling communities on these structures have been favoured by introduced species.
(Tyrrell and Byers, 2006). Currently, studies using metabarcoding and settlement plates have been used in early detection of introduced species (Pochon et al., 2015; Zaiko et al., 2016).

In my project, field trials in Gippsland Lakes and South Australia were carried out to validate the effectiveness of metabarcoding and settlement plates as an early detection technique. The analysis of the biofouling communities on plates that were left in the water for one month showed that potential introduced species were present by that time in both locations. In the Gippsland Lakes, the biofouling communities analysed in the three locations (Lakes Entrance, Metung and Paynesville) had different stages of biofouling on the plates. In Lakes Entrance, the biofouling was at the early settlement stages while Metung appeared to have the most advanced stages of development (Chapter 3, Section 3.3.1). Nevertheless, introduced species were successfully identified, most notably when they were not easily visible on the plate. This showed that metabarcoding had high sensitivity at detecting MOTUs that could not be easily identified (Brown et al., 2016).

Due to the indiscriminate nature of metabarcoding, this method of monitoring furthermore allowed detection of introduced species not previously recorded in the region such as *M. manhattensis*. Seven introduced species were detected in Gippsland Lakes and three were of particular interest; *A. senhousia*, *C. intestinalis* and *M. manhattensis* as they have been shown to have negative ecological impacts elsewhere (Crooks, 1998; Lambert and Lambert, 1998; Blum et al., 2007). *M. manhattensis* has not been recorded since 1976 (Hewitt et al., 2004; Hirst and Bott, 2016), and further molecular analysis confirmed its identity (Chapter 3, Section 3.3.5). In South Australia, five introduced species were also successfully detected during the first month after deployment, namely *C. intestinalis*, *A. aspersa*, *Ulva* spp., *B. schlosseri* and *C. pallasiana* (Chapter 5, Table 5.3). These two field trials indicate that the use of metabarcoding and settlement plates can be replicated and used in any location for providing an assessment of the community and allowing detection of potential introduced species.

The use of metabarcoding also has its limitations and before it can be implemented as an early detection tool, further research has to be conducted to address these pitfalls such as quantitation and lack of certain taxonomic groups in reference databases. Several studies have been conducted to test its efficacy in early detection and optimisation (Pochon et al., 2013; Pochon et al., 2015; Zaiko et al., 2015; Brown et al., 2016; Xiong et al., 2016; Borrell et al., 2017b; Borrell et al., 2017a). Currently, metabarcoding cannot be reliably used for quantitation
(Amend et al., 2010; Sun et al., 2015) because the mismatch of primers and templates in species could decrease the amplification efficiency (von Wintzingerode et al., 1997). Also, identification of taxa may be impaired by incomplete coverage of taxonomy in reference databases (Pochon et al., 2015). Detection of taxa using a single marker may lead to false positive results, especially at a low taxonomic level (Zaiko et al., 2016). 18S rRNA has been used in studies looking at detection of introduced species, and one problem encountered by Pochon et al. (2013) was the difficulty in discerning among congeners, such as *Perna* spp. This is not an issue when all the undiscernible species are considered introduced to the study location. However, in other cases, the ability to identify to the species level would be essential to differentiate between native and introduced species of the same genus. This issue was also observed in the field trial in South Australia, where one of the introduced species was the *Ulva* spp. (Chapter 5, Table 5.3) and the database used could not identify at the species level. These problems can be reduced by using several databases and by applying multiple markers, which will help reduce the risk of incorrect taxa identification. In order to validate the presence of organisms in a sample, it is advisable to use more than one set of universal primers as they provide a better coverage (Briand et al., 2018; Zhang et al., 2018). In fact, the 18S primers have previously been able to identify only 73-86% of the community. However, the combined use of COI and 18S provided 89-93% coverage in the same study and detection rates of species increased by 14% to 35% (Zhang et al., 2018).

The use of qPCR to validate the metabarcoding analysis showed that detection of the introduced species *A. senhousia* in the three locations by qPCR correlated with the metabarcoding analysis (Chapter 3, Table 3.3). However, it was not always consistent in some samples where qPCR and metabarcoding could not both detect *A. senhousia* in the same samples. Wood et al. (2017) showed that qPCR has a higher sensitivity compared to metabarcoding in detecting introduced species such as *S. spallanzanii*. The difference in detection could be attributed to the primers used in both qPCR and metabarcoding. While qPCR requires the use of primers that are specific to the species and allow detection at very low quantities (Goldberg et al., 2016), metabarcoding uses universal primers to provide biodiversity assessment within a community (Taberlet et al., 2012a). Moreover, Murray et al. (2011) showed that when detection by qPCR was positive in samples which had a Ct value > 34, metabarcoding could not detect the target species, and this observation was consistent with my research (Chapter 3, Table 3.3). However, *A. senhousia* could not be detected by qPCR in sample 3 in Paynesville. This could be caused by the limitation of qPCR in quantifying marine
juveniles which have different cell numbers at different life stages and would have varying amount of DNA at larval stages (Smith et al., 2012).

Use of extensive reference databases and more than one set of universal primers are essential for carrying out biodiversity assessment in an environment. In the field study from South Australia, a high % of reads was removed after filtering (Chapter 5, Table 5.2). These could refer to a large amount of reads that could not be identified due to the lack of reference databases for these marine organisms. Also, this gives an indication of the lack of taxonomic study in that region, and the unidentified taxa may be native only to Australia and also signify the rich diversity which has not been uncovered. Moreover, the high quantity of reads removed could also be due to the high stringency in the quality control in the bioinformatics analysis. Indeed, presence of *A. aspersa* and *A. senhousia* in samples from Gippsland Lakes and S. Australia, respectively was found in samples that had less than 10,000 reads and could not be analysed. Metabarcoding requires sufficient quality control such as removal of sequencing errors, but would also allow rare species to be identified (Zhan and MacIsaac, 2015). The rarefaction curves (Chapter 5, Figure 5.3) also indicate that further sequencing effort would have been required, as the curves were still in sharp incline for many samples.

6.3 Biofouling communities are influenced by both external and intrinsic factors.

6.3.1 Biofouling communities in Gippsland Lakes (Chapter 4)

The biodiversity in biofouling communities in the Gippsland Lakes was assessed, and the findings indicated that the development of the communities was influenced by a number of factors, namely environmental conditions and frequency of boating activities. Salinity was an important factor that affected the development of biofouling, especially in an estuarine system, as the effect of salinity was observed in the difference in the species composition among the three locations (Chapter 4, Figure 4.3). In an estuarine system, salinity levels usually vary across a wide range. In Gippsland Lakes, the salinity levels were regulated by freshwater inflow from the river systems and from the entrance to the Bass Strait. Lakes Entrance had the most differences in species composition among the three locations due to its close location to the entrance of the Bass Strait (Chapter 4, Figure 4.7), whereas both Paynesville and Metung are situated by Lake King. The one-month samples collected from Paynesville and Metung were mostly dominated by *Balanoides* spp. (Chapter 4, Figure 4.4 and Figure 4.7). However, the analysis from three and six-month samples showed that their species composition started
diverging. In Metung, both sides of the plates were mostly dominated by *Balanoides* spp., while the results from Paynesville indicated that *Balanoides* spp. were dominant only on one side of the plate.

The difference in species composition in biofouling communities between Metung and Paynesville could also be attributed to the frequency of boating activities. Both Metung and Paynesville are very popular places for boating activities (Gippsland Ports, 2015). At the time of sampling, the jetty at Metung was being renovated which would reduce the frequency of boating activities there. Small boats have been considered important vectors of introduced species, and the usual traffic in the harbour at Paynesville would have facilitated the introduction of *M. manhattensis*. Constant propagule pressure brought by shipping and colonization pressure play a role in successful invasion (Lockwood et al., 2009). When marine species are introduced in a new environment, only a small number will establish, and as the frequency of the introductions increases, the more likely the introduced species will establish.

Shipping and disturbance have contributed mostly to marine invasions in estuaries as they are considered one of the most invaded marine environments (Cohen and Carlton, 1998; Ruiz et al., 1997). One well-known invaded estuary is the San Francisco Bay (Cohen and Carlton, 1998). Although there is very limited number of international vessels in Gippsland Lakes, the secondary spread from neighbouring ports such as Port of Melbourne and Port Phillip Bay pose a risk as comparable as any major ports face. This was further supported by the detection of several introduced species in Gippsland Lakes which had been previously recorded in Port Phillip Bay. My study revealed that the frequent recreational activities and numerous marinas contribute to the high level of anthropogenic activities in the Gippsland Lakes.

6.3.2 Biofouling communities in South Australia (Chapter 5)

In Gulf of St Vincent and Kangaroo Island, South Australia, the species composition in biofouling communities assessed for twelve months suggested that differences in the level of anthropogenic activity between locations could contribute to the variations in species composition over time. This is the first long-scale study assessing biofouling communities using molecular methods such as metabarcoding. The combined effect of vectors such as shipping and release of individuals into an environment outside of their range affect the
propagule pressure and determine the establishment of the population of the individual in that environment (Lockwood et al., 2005).

The presence of the introduced species *C. intestinalis* and *A. aspersa* in North Haven had a profound effect on the biofouling community, where they remained dominant for the first six months. *C. intestinalis* was also dominating the biofouling community in the last six months of the field trial, whereas *A. aspersa* was still present but had reduced in population size. Previous studies showed that *A. aspersa* was among the early settlers in a community when there is reduced competition (Rajbanshi and Pederson, 2007; Tatián et al., 2010). The early occurrence of *A. aspersa* could also be attributed to the life cycle of *A. aspersa* which usually lasts 18 months, and the decrease in these organisms at the 9 and 12 months sampling could have indicated that they have reached the end of their life cycle (Millar, 1952). The space left by *A. aspersa* allowed other marine organisms to settle. The conditions present in North Haven may have been favourable to the settlement of the introduced ascidians.

The similarity in the species composition in the biofouling communities over the twelve months could be attributed to their similar habitats and the domestic shipping occurring between North Haven, Wirrina Cove and Christmas Cove (Kinloch et al., 2009). Recreational boating represent a potential pathway for the secondary spread of marine species, especially introduced species, to smaller coastal marinas (Wasson et al., 2001). The risk of introduced species in Wirrina Cove, which is a recreational marina, was considered low. However, frequent sea traffic from North Haven would have increased the likelihood of introducing marine species to Wirrina Cove. Indeed, introduced species such as *C. intestinalis* and *A. aspersa* detected in North Haven have been also detected in Wirrina Cove (Chapter 5, Table 5.3). Moreover, the voyages between neighbouring locations promoted transfer of marine species among the three locations and allowed them to adapt better (Lavoie et al., 1999; Lewis and Coutts, 2009).

Biofouling communities in Wirrina Cove and Christmas Cove shared the highest degree of similarity among all locations (Chapter 5, Figure 5.6). In previous studies comparing commercial ports and marinas (Ferrario et al., 2017), species diversity was also found to differ between ports and marinas, and this was due to the difference in levels of disturbance. Commercial ports usually have high levels of pollution and anthropogenic activities which would alter the natural habitat and make it difficult for native species diversity to adapt (Piola
and Johnston, 2008). The disturbed environment favours introduced species as they may have encountered similar environments when they were taken up by a vector (Carlton and Geller, 1993). Although *C. intestinalis* was detected in Wirrina Cove over the twelve months, its population was not dominating the species composition in that location. Stachowicz et al. (1999) hypothesised that a high species diversity would resist invasion, which is consistent with the large amount of native species present in Wirrina Cove (Chapter 5, Figure 5.5).

Propagule pressure also could have played a role in shaping the biofouling communities in all locations. Propagule pressure would be higher in disturbed areas due to the presence of artificial structures and shipping, as there would be frequent supply of larvae brought by vectors (Lockwood et al., 2005). This could have led to different larval composition on the hard substrates and given rise to different biofouling assemblages. The highest difference was observed in the species composition between Kingscote and North Haven (Chapter 5, Figure 5.6). In Kingscote, the variability among replicate plates indicate that there was high native species diversity and the biofouling communities remained highly diverse over the nine months. It is difficult to predict the species composition at specific timepoints as high species diversity may create complex interactions in the biofouling communities (Kuriyan, 1953; Richmond and Seed, 1991).

The comparison of locations with differing levels of anthropogenic activity has provided an insight into how disturbance and domestic shipping can affect the species composition in the biofouling assemblages over time. The low species diversity and dominance of introduced species in disturbed areas such as North Haven are cause for concern as they could signify loss of native species diversity and encourage the likelihood of marine invasions.

### 6.3.3 Comparison of biofouling communities in Gippsland Lakes, Victoria and South Australia

The biofouling communities analysed in Gippsland Lakes, Victoria and South Australia provided baseline information on fouling species present on hard surfaces. The study of biofouling communities over time in various sites also gave an insight into the possible drivers of establishment on artificial substrates. Based on the findings from both field experiments, the development of biofouling communities is an intricate process and governed by both biotic and abiotic factors including environmental conditions, anthropogenic level and species interactions.
In Gippsland Lakes, the two main factors influencing the biofouling communities were environmental conditions and frequency of boating activities. Estuarine environments usually have to face both natural and anthropogenic stressors which would make them areas of high disturbance and mostly invaded ecosystems (Mayer-Pinto et al., 2018; Ruiz et al., 1997). The varying influence of the factors largely contributed to the differences in the biofouling communities in the three sites (Chapter 4, Figure 4.8 & Table 4.4). Lakes Entrance had the highest level of salinity while Paynesville and Metung had similar levels of salinity. However, boating activities were higher in Paynesville than in Metung during the field experiment, leading to higher propagule pressure in Paynesville. As for South Australia, the biofouling communities located in the marinas and jetties with varying anthropogenic level were assessed and the anthropogenic level was certainly an important factor contributing to the differences in biofouling communities. The higher level of disturbance and propagule pressure facilitated the settlement and colonisation of introduced species. For example, studies in *Sargassum muticum* have revealed that the interaction of disturbance and propagule pressure was crucial in determining invasion success in both the short term and long term (Britton-Simmons and Abbott, 2008). However, the similarity in biofouling assemblages over time among North Haven, Wirrina Cove and Christmas Cove indicated that regular boating activities were also shaping the species diversity on the settlement plates (Chapter 5, Figure 5.6). The complexity of biofouling process was further enhanced by the species interactions present in the communities. In fact, the process of invasional “meltdown” caused by *C. intestinalis* and *A. aspersa* was observed in North Haven (Chapter 5, Table 5.3 & Figure 5.5) (Simberloff and Von Holle, 1999).

The two field trials were the first plate setup carried out in their respective locations analysed using metabarcoding. In both cases, several introduced species were detected in Gippsland Lakes and South Australia. There were three introduced species in common to both field experiments, namely *B. schloesser*, *B. imbricata* and *B. gracilis*. Several introduced species identified in both field trials were also well-known MIS and had also been detected in neighbouring major ports. For example, *A. senhousia*, which was detected in Gippsland Lakes, had been previously identified in a survey in Port Phillip Bay (Hewitt et al., 2004). As for South Australia, both *C. intestinalis* and *S. spallanzanii* were also found in surveys in Port Adelaide (Wiltshire and Deveney, 2011). Moreover, the regular sea traffic may be a contributing factor and ensured a constant supply of larvae introduced. This may have allowed introduced species
such as *M. manhattensis* in Paynesville and *C. intestinalis* and *A. aspersa* in North Haven to colonize and become dominant on the hard substrates. The low species diversity at early stages of biofouling in Paynesville may have facilitated the colonization of *M. manhattensis*, while *C. intestinalis* and *A. aspersa* were among the first colonizers on the substrate. The presence of introduced ascidians indicated that they have been introduced by human activities as they are usually short-lived and have poor dispersal rates (Lambert and Lambert, 1998).

The species composition in the biofouling communities at both locations consisted mainly of commonly known sessile marine species that colonize the biofouling community in ports and marinas, including ascidians, sponges, bryozoans and barnacles (Woods Hole Oceanographic Institution, 1952; Mineur et al., 2012). However, further analysis of the biofouling communities revealed that not only are these communities shaped by the factors affecting the environment, but they also reveal further information about the environment studied. Although factors influencing the biofouling process have been well studied, the combined effect of several factors cannot be determined which makes it more difficult to predict the settlement of marine species. It is also complicated to predict the impacts of introduced species as these factors affect the biofouling communities to different degrees. Moreover, the study of biofouling communities was carried out in marinas which in general consist of man-made structures. But marinas are also located in close proximity to natural hard bottom communities (Epstein and Smale, 2018). Although introduced species usually have a preference for artificial substrates, the risk for introduced species to possibly settle on natural hard substrates is always present in marinas (Connell, 2001; Glasby et al., 2007). For example, *U. pinnatifida* is a well-known MIS which has been recorded on natural surfaces found close to marinas (Epstein and Smale, 2018). The spillover of *U. pinnatifida* occurred due to the increase in population of the MIS over time.

Regional shipping including recreational vessels and commercial fishing vessels represented the main pathway for translocation of introduced species in both Gippsland Lakes and South Australia. By identifying the possible pathways occurring in Gippsland Lakes and South Australia, potential vectors could then be identified. The sites that were studied are considered smaller marinas where no major international ships travel to, and the results from both field experiments indicated that the detection of those introduced species could mostly come from nearby major ports. In the case of Gippsland Lakes, there were no major international shipping except for the sand dredge TSHD Pelican and commercial fishing
vessels as well as recreational vessels. While in South Australia, the marinas where the field trial was carried out were smaller marinas and recreational vessels are known to frequent these marinas. Previous studies have highlighted the consequence of regional shipping and contribution of secondary spread to smaller ports and marinas (Wasson et al., 2001; Floerl and Inglis, 2005; Minchin et al., 2006; Zabin et al., 2014). In Australia, the impacts caused by secondary spread could become serious as there is an increase in the number of recreational vessels. Indeed, there has been a 36% increase in the number of registered vessels from 1999 to 2009 (Burgin and Hardiman, 2011; Lane et al., 2018). However, there are currently guidelines provided for boat owners on good maintenance and treatment of biofouling (Department of Agriculture and Water Resources, 2019). Managing the vectors would reduce the risk of translocating introduced species to other neighbouring ports and marinas.

Although vector management contributes largely to preventing the translocation of introduced species, the findings in both field trials highlight the importance of including regular monitoring surveys in smaller and neighbouring ports and marinas. It is not possible to determine what new and known introduced species could be present in these areas and still going undetected. In Gippsland Lakes, this was the first trial characterising biofouling communities on settlement plates and was part of the larger field trial looking at introduced species present (Hirst and Bott, 2016). The detection of introduced species such as M. manhattensis in the biofouling communities (Chapter 3, Table 3.2) indicates that, although its introduction in Australia was in 1976 and was the only known record since that year, this introduced species has become well-established in Gippsland Lakes (Hewitt et al., 2004; Hirst and Bott, 2016). Due to the lack of monitoring in smaller ports along the coastline, its presence was undetected until this field trial. In contrast, monitoring has already been carried out in South Australia, and the results from metabarcoding also support the detection of already known introduced species in the area such as S. spallanzanii, C. intestinalis and A. aspersa (Kinloch et al., 2009; Wiltshire et al., 2010; Wiltshire and Deveney, 2011). It also showed other introduced species that were not previously detected, such as O. dichotoma (Chapter 5, Table 5.3). Further monitoring and experiments would be required to confirm the presence of this introduced species. The baseline information obtained could also be used for the implementation of regular monitoring in these locations, especially in areas vulnerable to invasions.
The study of biofouling communities over several months using metabarcoding indicates that a sufficient amount of biofouling material for DNA extraction is important to provide a good representation of the community. In the field trial of Gippsland Lakes, freeze drying was tested and showed promising results for processing a large quantity of starting material (more than 10 g). In South Australia, another method was used where biofouling material on the plate was divided into 4 samples and stored in individual tubes in RNAlater. This thesis showed that there are several ways to processing biofouling material. However, obtaining DNA of sufficient quality that would allow for a good community level assessment is the most important factor. For this reason, homogenisation is also considered a crucial step (Lohan et al., 2019). Studies in zooplankton have revealed that homogenisation increased the detection of MOTUs with low abundance, and this is particularly important when the aim is to detect introduced species early (Lohan et al., 2019). Homogenisation could have played a role in the species diversity in Kangaroo Island and Wirrina Cove, South Australia (Chapter 5, Figure 5.5). The variation among replicates within sites could have been caused by using only one subsample for DNA extraction without prior homogenisation.

PCR inhibitors were present in the DNA extracted in both field trials. Indeed, the presence of inhibitors in environmental samples such as humic acids may interfere with the PCR reaction (Wilson, 1997; Schrader et al., 2012; Wood et al., 2017). Thus, it is important to dilute the concentration of inhibitors while avoiding compromising detection limit of the DNA (Tsai and Olson, 1992a; Tsai and Olson, 1992b). A range of template volumes (1:5 dilution, 1-5 µl) was used on the biofouling samples to optimise the PCR reactions. In many cases, the use of a lower volume of DNA template led to a successful amplification. However, the use of varying template volumes could not produce positive results in a few samples. The PCR reaction may be further optimised by addition of BSA to prevent PCR inhibitors from binding to the Taq polymerase (Farell and Alexandre, 2012; Lekang et al., 2015). Optimising the extraction of genomic DNA is also crucial for a successful amplification. Most samples that could not be amplified were from two sites (Lakes Entrance & Paynesville) in Gippsland Lakes and one site (Wirrina Cove) from South Australia. The presence of specific marine organisms could indicate that DNA extraction was inefficient and further steps in the protocol may be required to ensure the recovery of high-quality DNA. For example, DNA extraction from plants and algae is difficult and may work for only one type of plant due to the difference in composition of the cell wall (Doyle and Doyle, 1990; Maneeruttanarungroj and Incharoensakdi, 2016). Studies comparing several DNA extraction methods for sand sediments.
and sediments with high content of silt and clay showed that the MoBio PowerSoil DNA extraction kit generated the best results in DNA yield, purity and diversity (Lekang et al., 2015). The kit used in this thesis was the MoBio PowerMax® Soil DNA Isolation Kit, which follows the same protocol but allows for a greater quantity of starting material. However, in this case, biofouling samples, which may have different content from the sediments studied were processed with modified steps in the DNA extraction.

6.4 Limitations and future work

In my research, the use of Perspex plates was explored in the early detection of introduced species and study of biofouling communities over time. Man-made infrastructure is made of different materials and shapes which would provide habitats for many marine species (Mineur et al., 2012). Anderson and Underwood (1994) showed that the properties of the hard surface could influence the early settlement of marine species as well as the development of the biofouling community over time. Importantly, the use of these Perspex plates did not compromise the early detection of introduced species. To further study the influence of surface type on the settlement and growth of biofouling organisms, plates that are made of materials such as fibreglass, aluminium or glass can be used. Different orientations of the substrates also contribute to the biofouling assemblages. Glasby and Connell (2001) showed that serpulids were more likely to favour horizontal undersides than plates positioned vertically on reefs. Therefore, deploying plates in varying orientations in both Gippsland Lakes and South Australia would provide valuable information to understanding the development of biofouling assemblages.

The first aim of my thesis was to validate the potential use of settlement plates and metabarcoding for early detection of introduced species. This proved successful as introduced species were detected in the first month of sampling at all locations. My research also showed that metabarcoding had high sensitivity and could detect introduced species when they were not readily identifiable. The study of biofouling communities using molecular methods required the processing of large mass (> 10 g) of sampling material and was possible by using the freeze-drying method. This method did not affect the quality of DNA which was used for metabarcoding. The second aim was to study biofouling communities in locations with varying levels of anthropogenic activities using molecular methods over long periods of time. My findings showed that biofouling communities are complex and influenced by many factors.
simultaneously. There was also evidence that the presence of introduced species in disturbed areas could affect the species composition on the biofouling communities. Overall, my research contributes to understanding biofouling communities on hard surfaces in various marine environments. As these findings provide preliminary data on the biofouling communities, there is more work required to understand the development of these communities. The analysis of biofouling communities was initially performed after one month and subsequently for every two or three months. As adult stages of fouling species were already observed in the biofouling assemblages at one month (e.g. in Metung), it would be interesting to characterise biofouling communities at earlier sampling times and shorter time intervals. Moreover, further field trials in neighbouring ports along the coastline would be important to understand the similar and particular factors influencing the development of biofouling communities.

Metabarcoding is a powerful tool for studying biofouling communities. However, for a successful management of MIS, it is important to analyse species diversity present in soft-bottom communities or the water column. There are currently DNA-based tools used in the early detection of specific introduced species (Bott et al., 2010b). However, it is not always possible to determine whether there are new introduced species in other types of communities. Metabarcoding is advantageous because it provides assessment of the entire species diversity. Studies using metabarcoding have already been done in zooplankton and bilge water (Lindeque et al., 2013; Zhan et al., 2014; Bucklin et al., 2016; Chain et al., 2016; Fletcher et al., 2017). This approach could be applied to Gippsland Lakes & South Australia to detect introduced species not associated with hard bottom surfaces. Also, certain life-stages of introduced species could occur in the water column before they settle on hard surfaces (Hirst and Bott, 2016). Therefore, the results obtained from analysing different sample types combined with the findings in my thesis would allow a full assessment of both native and introduced taxa in both locations (Holman et al., 2018).

The implementation of a successful early detection system requires the use of several tools simultaneously. In fact, for a successful management of introduced species, some of the requirements to consider include taxonomic expertise, early detection, use of molecular tools (Ojaveer et al., 2014). Settlement plates and metabarcoding did provide early detection of introduced species and an assessment of the biofouling communities at any given time. However, once a potential MIS has been detected, further tools should be applied to confirm the identity of the introduced species. For example, once *A. senhousia* was detected in
Gippsland Lakes, qPCR test was further carried out to confirm its presence. This underlined the importance of using several techniques to support the findings of one technique and to compensate for the limitations of one method of detection. Other techniques that should be considered for early detection of potential MIS include point-of-care (POC) diagnostics, and there has been major advances of these technologies in the field of medicine (Niemz et al., 2011; Darling et al., 2017). These POC diagnostics would be advantageous and beneficial in the early detection of targeted introduced species as they are low-cost, sensitive, fast, portable and user-friendly (Song et al., 2014). In fact, studies by Wang et al. (2014) demonstrated the potential use of a microfluidic chip in the detection of microalgae in ballast water. Other POC methods have focused on the use of isothermal nucleic acid amplification (Niemz et al., 2011). These techniques consist of three stages which include preparation of sample, amplification of the target DNA sequence using specific primers and detection of the target species (Niemz et al., 2011). The POC diagnostics would be useful for early detection of potential MIS that are known to a specific area or considered of sufficient economic or ecological importance to warrant monitoring. Furthermore, an early detection system should also incorporate monitoring surveys in neighbouring marinas and ports. Marinas differ from major ports with regards to pollution disturbance and presence of natural habitats, which reiterates the importance of including them in baseline monitoring programmes (Ferrario et al., 2017). The findings in this thesis highlight how recreational marinas and areas with limited exposure to sea traffic are vulnerable to marine invasions, and they provide crucial information into understanding the effect of introduced species in the species diversity present.
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Figure 4: BLAST result confirming the identity of *M. manhattensis*. The sequence alignment between the nucleotide sequence obtained from Sanger sequencing (blue) was compared to the nucleotide sequence of the 18S rRNA gene from *M. manhattensis* (black). The underlined region depicts the nucleotides that are not similar between the two sequences.

| Table 1: PERMANOVA results for the analysis of differences in assemblage in South Australia across the different factors (location & time) based on Bray-Curtis dissimilarity and Jaccard index. Location and time are the two fixed factors and the number of permutations to obtain p-values was 9999 permutations. |
|---|---|---|---|---|---|
| | df | SS | MS | F | p |
| **Bray-Curtis** | | | | | |
| Location | 4 | 87669 | 21917 | 11.263 | **0.0001** |
| Time | 3 | 128450 | 42815 | 22.003 | **0.0001** |
| Location*Time | 10 | 97579 | 9757.9 | 5.0147 | **0.0001** |
| Residual | 136 | 264640 | 1945.9 | | |
| Total | 153 | 593870 | | | |
| **Jaccard** | | | | | |
| Location | 4 | 114960 | 28740 | 15.995 | **0.0001** |
| Time | 3 | 59249 | 19750 | 10.992 | **0.0001** |
| Location*Time | 10 | 66689 | 6668.9 | 3.7117 | **0.0001** |
| Residual | 136 | 244360 | 1796.7 | | |
| Total | 153 | 500720 | | | |
Table 2: PERMANOVA Results obtained from Bray-Curtis dissimilarity and Jaccard index of pairwise comparisons for each location (Ki = Kingscote, X.C. = Christmas Cove, W.C. = Wirrina Cove, N.H. = North Haven) at three time points (One, three and six months). Significant p values were indicated in bold (p < 0.05).

<table>
<thead>
<tr>
<th>Month</th>
<th>Location Groups</th>
<th>Bray-Curtis dissimilarity</th>
<th>Jaccard index</th>
</tr>
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<td>Ki, N.H.</td>
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<td>W.C., N.H.</td>
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<tr>
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Table 3: PERMANOVA Results obtained from Bray-Curtis dissimilarity and Jaccard index of pairwise comparisons among the five time points (one, three, six, nine and twelve months) in the four locations Kingscote, Christmas Cove, Wirrina Cove and North Haven. Significant p values are indicated in bold (p < 0.05).

<table>
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<th>Location</th>
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<th>Jaccard index</th>
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