The Role of Bacterial Biofilms on the Settlement and Nutrition of Mussel (*Perna canaliculus*) Larvae and Juveniles

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Attestation of Authorship

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

Signed…… **Date**……

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Abstract

The green-lipped mussel, *Perna canaliculus* is indigenous to New Zealand, and is of significant commercial importance, contributing over NZ $200 million per annum in exports. Despite the economic importance of this mussel species, very little is known about its early life. Larvae at the pediveliger stage may remain planktonic for several weeks until individuals find a suitable settlement site. A range of filamentous macro-algae provides the primary settlement site and source of nutrition for larvae until they make the transition from primary substrates on to rocky shores. The underlying mechanism for choosing specific substrates remains largely unknown, but may be related to the epiphytic bacterial biofilms present on those surfaces. Thus, the present study is the first to investigate bacterial biofilm-mediated settlement and nutrition for *P. canaliculus* larvae and juveniles through laboratory-guided experiments.

To investigate a role for specific bacterial biofilms in mussel larval settlement, experimental conditions were optimised to achieve exemplar settlement assays, and to obtain efficient and reproducible results. Settlement assays analysed pediveliger larvae between 19–21 days' old which were subjected to a range of settlement substrates and assay media. Larvae were found to settle uniformly on polystyrene plates, glass slides and noble agar media, indicating that the physicochemical properties of the settlement substrates were not important for the settlement of larvae. In addition, sterile natural seawater was preferred as a culturing environment compared with artificial seawater, which was found to be toxic for *P.*

canaliculus larvae. The 48-hour assay period resulted in minimal variation within replicate plates, hence it was found to be most suited for conducting ideal settlement experiments.

Marine bacteria were cultured from diverse marine origins, isolated through traditional culturing techniques, and their ability to induce settlement was investigated. Out of fourteen bacteria isolated, three mono-species biofilms were exclusively chosen for further study. With the aid of phylogenetic analyses, these three bacterial strains were found to be *Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1. The biofilms of the former two species of bacteria were shown to induce greater settlement (over 60%) compared with controls. Conversely, the latter species was showed to have similar settlement levels compared with sterile controls. However, this latter bacterium produced toxic molecules for mussel larvae and induced mortalities of over 70% compared with the other species of bacteria and controls.

Petri plates, glass slides and noble agar were investigated along with biofilms for their collective ability to modulate settlement of larvae. The study demonstrated that some species of bacteria were able to interact with the settlement substrate and modify their capacity to induce mussel larval settlement. However, the three key mono-species bacteria (*Macrococcus* sp., *Bacillus* sp. and *Pseudoalteromonas* sp.) continued to show consistent levels of settlement across all three substrates, indicating that the settlement cue was confined to bacteria, and was not a result of combined physicochemical cues of biofilm and their substrates.

These results led to the investigation of the origin of the settlement cues present in the biofilms of mono-species marine bacteria. The biofilm cells and the

chemicals from the biofilms (exudates) of *Macrococcus* sp. and *Bacillus* sp. resulted in similar patterns of settlement inducing properties. Conversely, washed cells of biofilms and cells grown in the planktonic form resulted in reduced settlement inducing properties. Unlike washed biofilm cells and planktonic bacteria, bacterial biofilms and biofilm exudates of *Pseudoalteromonas* sp. induced greater levels of larval mortality. These results revealed the origin of the respective settlement and toxic cues to be present in the exudates of these mono-species bacteria.

Further characterisation of the settlement cues from biofilm exudates of respective mono-species bacteria revealed their relative molecular identity. The settlement cues from exudates of *Macrococcus* sp. AMGM1 were between 1–3, 3–10 and 10–30 kDa, thermo-labile over 70°C, and contained polar proteins and non-polar lipoprotein molecules. In addition, settlement cues from *Bacillus* sp. AMGB1 were found to be between 10–30 and 30–50 kDa, were stable to up to 100°C, and contained glycolipid molecules. The bacterial biofilm toxin from *Pseudoalteromonas* sp. AMGP1 was identified as a heat labile (> 70°C) polar protein molecule of < 1kDa in size. In addition, a settlement inductive molecule was found from this bacterial species to be a heat labile non-polar lipoprotein of 30–50 kDa.

Settlement assays were used to determine the responses of juvenile mussels. These assays showed that mussels at this stage of development also responded to settlement cues from mono-species bacteria. Juveniles were shown to favour low wettable polystyrene substrates and settled in response to the physicochemical properties of the settlement substrates. Addition of *Macrococcus* sp. and *Bacillus* sp. biofilms enhanced settlement on glass slides and coir substrates, whereas no effect was observed on substrates with biofilms of *Pseudoalteromonas* sp. Moreover,

retention was shown to improve during the initial 24-hour period of the assay on glass slides and ropes containing biofilms of *Macrococcus* sp. and *Bacillus* sp. These results indicated that biofilms were able to mediate settlement and early retention of juvenile mussels.

Finally, nutrition assays were conducted to identify whether mussel larvae and juveniles obtained nutrition from bacteria during the 48-hour settlement assay period. Ytterbium-labelled bacteria were fed to mussel larvae and juveniles, which were analysed for the presence of these metals after 48-hour incubation. Both mussel larvae and juveniles were shown to be bacterivorous through this assay. In addition, a sole diet containing biofilm cell suspensions of only *Macrococcus* sp. or *Bacillus* sp. and mixed diets of these bacteria together with microalgae showed similar levels of metamorphosis and survival.

This study used a multi-disciplinary approach to highlight the importance of bacterial cues for settlement of mussel larvae and juveniles and their bacterivorous nature. These specific biofilms or their chemicals have the potential for use in hatchery settings to achieve improved settlement rates and production. In addition, bacteria may a cheap alternative diet to rear mussels.

Chapter 1.

GENERAL INTRODUCTION

1. General Introduction

1.1 Background information

The following sections provide a review of the literature pertaining to the green-lipped mussel, the biofilm mode of bacterial growth and their role in the settlement and nutrition of mussel larvae and juveniles. A more detailed literature review is provided at the beginning of each chapter under the 'introduction' section.

1.1.1 The organism

The indigenous green-lipped mussel, *Perna canaliculus* (Gmelin 1791) is a common marine mollusc and has a characteristic green 'lip' along its inner shell margin. This endemic mollusc, also known by the trade name Greenshell™, is widely distributed throughout New Zealand. Yet, individuals have been frequently noticed in central and northern parts of the country where dense beds of 100 individuals m^{-2} can be found (Hickman, 1991). Adult mussels inhabit lower shores and open coasts, and are found from the mid-littoral zone down to 50 m (Jenkins, 1985). This species prefers warmer climates and can tolerate a wide range of changes in salinities (Hickman, 1991). These filter feeding bivalves can grow to over 260 mm in shell length (anterior-posterior axis) and are sexually mature within the first year (Food and Agriculture Organization, 2012). *P. canaliculus* is a dioecious broadcast spawner (Jenkins, 1985), and spawning times and duration may vary with temperatures (Alfaro, 2001). The northland (Ninety Mile Beach) mussel populations spawn between June to December (Alfaro et al., 2001, 2003), while southern populations (Marlborough Sounds) have two spawning peaks in early summer and

autumn-spring (Buchanan, 1998; Flaws, 1975; Tortell, 1976). During the reproductive season, female mussels can produce up to 100 million eggs, whereas males can produce innumerable sperm (Jenkins, 1985).

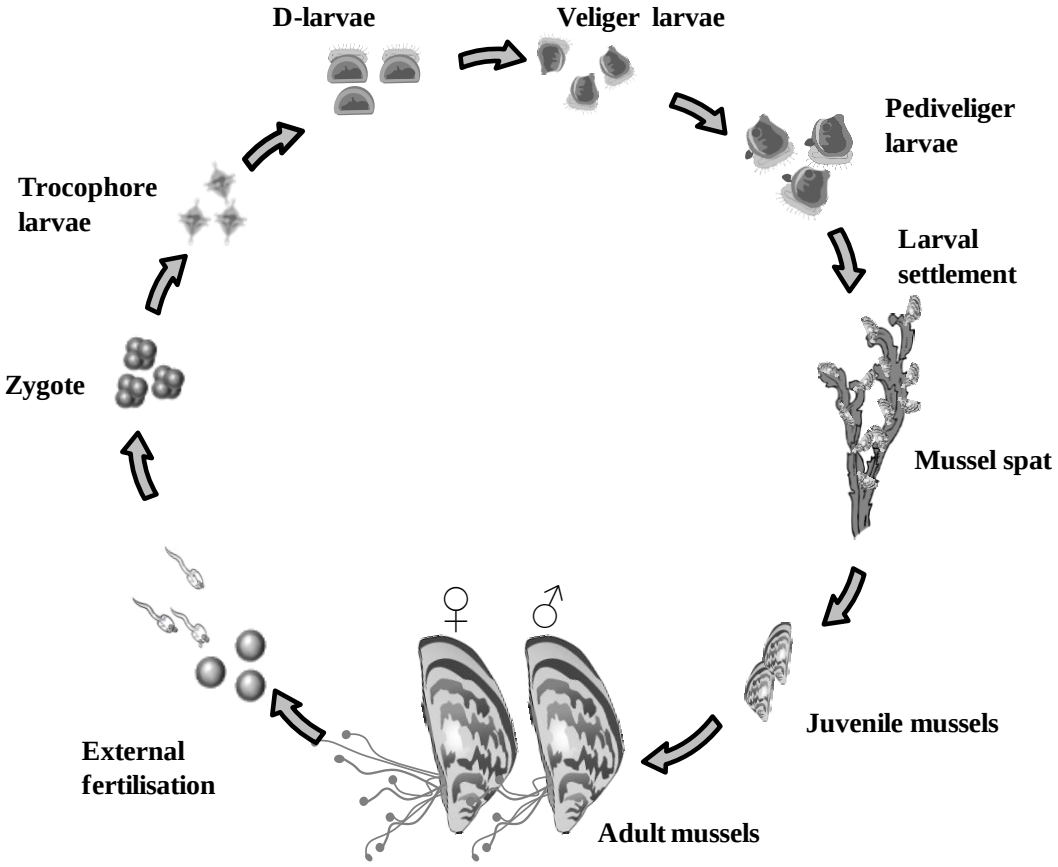

Figure 1.1: Life cycle of the green-lipped mussel, *P. canaliculus* (Young et al., 2010).

Similar to other mussel species, the green-lipped mussel has a relatively high larval fecundity, with an iteroparous (reproducing repeatedly) life cycle that can be short-lived (Fig. 1.1). The life cycle begins with the formation of zygote that undergoes mitosis to form a trocophore larva, followed by a D shaped larva within 24–48 hours post-fertilisation. At the end of this period, the veliger larvae that develop may remain swimming in the water column for up to 3–5 days, depending on water temperature, salinity, availability of food and settlement cues (Jeffs et al.,

1999; Redfearn et al., 1986). These larvae are known to be transported to great distances by ocean currents and tides (Hayden, 1995). At about 220–350 µm in size, the pediveliger larvae can remain planktonic or undergo settlement, depending on the availability of a suitable substrata (Buchanan & Babcock, 1997; Hayden, 1995). Primary and secondary settlement processes has been previously investigated for the blue mussel, *Mytilus edulis* (Bayne, 1964). This information has been the basis for understanding and the process for native mussel species, which have been confirmed through rigorous field and laboratory experiments (Alfaro et al., 2006; Alfaro & Jeffs, 2002; Buchanan & Babcock, 1997). *P. canaliculus* larvae preferentially settle on filamentous substrata, including macro-algae, debris and grass (Fig. 1.2). This occurs mostly between late winter to early summer, although settlement can be highly variable both spatially and temporally (Alfaro & Jeffs, 2002, 2003; Alfaro et al., 2004; Buchanan & Babcock, 1997; Jeffs et al., 1999). The remarkable settlement episode ends when individuals attach to the substrata *via* byssus threads and begin to metamorphose into juveniles (Buchanan & Babcock, 1997). The secondary settlement process is initiated when juveniles (spat) reach about 1–2 mm and often move to rocky substrates. These spat can sever their byssus threads in order to detach from their substrates and can 'parachute' in the water column with the help of secreted mucous. This process, termed 'byssopelagic migration' or 'mucous drifting', enables spat to selectively choose a rocky substrate several times until they reach about 6 mm, when they lose this drifting capacity (Buchanan & Babcock, 1997; Jeffs et al., 1999). Drifting seaweeds may also provide nutrition, while transporting mussel spat (Alfaro et al., 2004). The underlying rationale for the transfer of mussels from a pelagic to benthic life is believed to be an evolutionary strategy to avoid predation, intra-species competition and cannibalism by adult

mussels (Alfaro, 2006b; Alfaro et al., 2011a). This coordinated process is believed to be facilitated by bacterial biofilms present on the biotic (seaweed) and abiotic (rock) surfaces. Therefore, an in-depth understanding of biofilm mediated larval settlement and nutrition may significantly advance our knowledge on the early life cycle of this mussel species.

Figure 1.2: Attachment of juvenile mussels (spat) to filamentous macro-algae (A), debris (B) and grass (C).

1.1.2 Effect of biofilms on larval settlement

Ever since Alfaro et al., (2006) suggested the role of bacteria on macro-algae as a potentially important inducer for the settlement of *P. canaliculus* larvae, the role of biofilms on larval settlement has been a topic of interest. Background information on the relevance of multi-species and single-species bacteria on larval settlement is discussed extensively in chapters 3–7**.** The following information provides an insight into the importance of biofilms on macro-algal surfaces and their significance in settlement and nutrition of mussel larvae and juveniles.

1.1.3 Bacterial biofilm formation

Bacteria are successful ubiquitous prokaryotes, and quickly colonise on any newly submerged biotic or abiotic substrata. While bacteria often are the dominant organisms in biofilms, microalgal cells, fungal spores, ciliates and other microorganisms also form a part (Schachter, 2003). Owing to their high abundance, bacteria usually are the first colonisers on new surfaces (Dang & Lovell, 2000). Under normal conditions, the formation of bacterial biofilms involves five stages (Schachter, 2003) (Fig. 1.3).

1. Free-swimming planktonic bacteria are attracted to substrates mainly in search of nutrients (Costerton et al., 1995). The initial adhesion involves reversible and irreversible stages of attachment (Marshall, 1985; Marshall et al., 1971), and may last less than ten minutes (Wiencek & Fletcher, 1995).

2. Irreversible attachment and aggregation to form the biofilm matrix (Marshall et al., 1971; Zobell, 1943). This process is mediated by physical parameters, such as Brownian motion, electrostatic interaction, gravity, hydrophobicity (Fletcher & Loeb, 1979), and van-der-Waals forces (Wahl, 1989).

3. The maturation phase includes reproduction of bacteria and, at these minimal bacterial densities; quorum sensing begins and enables the production of extracellular polymers. (Fuqua et al., 1994).

4. Bacterial clusters embedded in the matrix reach maximum thickness and can no longer accommodate all cells within their structure.

5. Planktonic bacteria begin to escape from the matrix to colonise other surfaces and repeat the process.

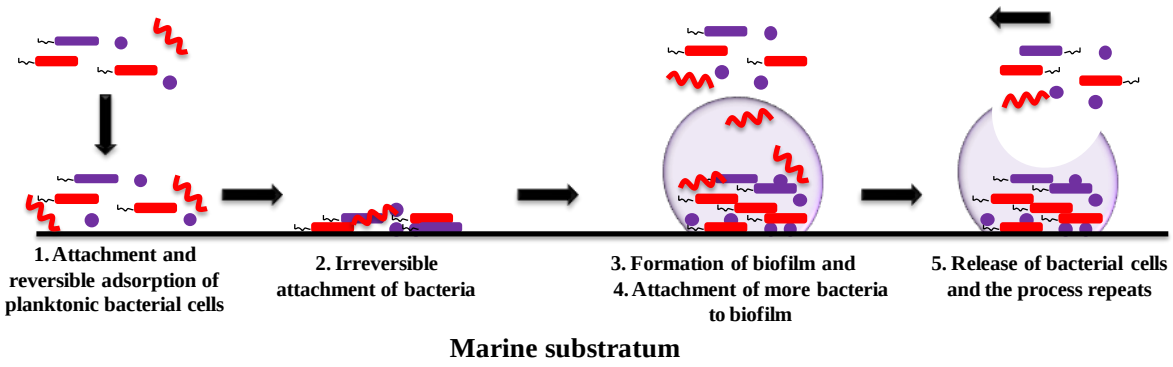

Figure 1.3: A schematic diagram of the steps involved in the biofilm formation of multi-species bacteria on any marine substrata. The bacteria are represented as rods, cocci and spirillum. Purple and red colour represents Gram-positive and negative bacteria respectively. The biofilm is a three-dimensional structure formed from micro-colonies of multi-species bacteria and their exudates. During unfavourable environmental conditions, some cells may detach and the process continues when a favourable substrata is found.

1.1.4 Biofilm chemistry

The chemical makeup of biofilms is determined by the extracellular polymeric substances secreted by the biofilms. Otherwise known as extracellular matrix or exudates, these molecular films 'house' bacterial cells, and primarily function to aid attachment to a variety of substrata and protect the cells against environmental stress and dehydration (Flemming & Wingender, 2010; Vu et al., 2009). Unlike their planktonic counterparts, biofilm cells are resistant to bacteriophages, chemical biocides, antibiotics, protozoan grazers and host immune responses (Decho, 1990; Flemming & Wingender, 2010). These characteristics allow biofilms to cover any moist environment where a suitable substrata and sufficient flow of nutrients is available (Singh et al., 2006).

In addition to bacterial cells and water, these biofilms contain polymers from sequestered nutrients, products from cell-lysis, particulate matter, and detritus from their immediate environment, which enable them to display a high degree of micro-

heterogeneity (Flemming, 1995). Besides peptidoglycan, lipids, phospholipids and other cell components, all major bio-macromolecules (polysaccharides, lipids, proteins, DNA and RNA) are present within a biofilm (Donlan, 2002; Flemming & Wingender, 2010; Schachter, 2003). In addition, these mucilaginous structures are believed to be predominantly neutral in Gram-negative bacteria (Sutherland, 1977) and cationic in Gram-positive bacteria (Donlan, 2002). Thus, the composition and quantity of biofilm exudates may vary depending on the type of microbes, their age and external environmental conditions (Sutherland, 2001).

The production of exudates and growth of the biofilm are controlled and regulated by density-dependent regulatory mechanisms (Vu et al., 2009). As the name suggests, when a certain population density (quorum) is achieved, the genes involved in the biofilm differentiation and maturation are initiated by the quorum sensing (QS) signals (Diggle et al., 2008; Donlan, 2002). The QS systems allow bacteria to interact collectively with each other to function efficiently as a coordinated group, similar to a multi-cellular organism (Diggle et al., 2008; Waters & Bassler, 2005). These systems encompass production and release of low molecular weight signalling molecules that regulate bacterial genes. The genetic induction results in communication between individual bacterial cells and facilitates the production of enzymes, toxins, and other secondary metabolites (Diggle et al., 2008; Waters & Bassler, 2005; Williams et al., 2007). Thus far, two QS processes namely, the autoinducer type 1 (AI-1) and type 2 (AI-2), have been described for bacteria that are mainly involved in intra-species and inter-species communication, respectively (Waters & Bassler, 2005; Williams et al., 2007). The auto-inducers in Gram-negative bacteria are acyl homoserine lactones with different subunits depending on the

bacterial species, and for Gram-positive bacteria are oligo-peptides (Fuqua et al., 2001; March & Bentley, 2004). The paradigm for such a signalling pathway was first described by Fuqua et al., (1994) for the bioluminescent marine bacteria, *Vibrio fischeri* and their symbionts (marine fishes and squids). However, the role of QS system interactions with the settlement of marine invertebrate larvae remains largely unknown.

In addition to being an integral part of the marine food web (Azam & Malfatti, 2007), biofilms function effectively as communication tools for micro- and macro-organisms within a marine habitat (Decho, 1990; Donlan, 2002). The QS systems of the biofilms enable communication of microbes across genera, family and kingdoms (de Nys et al., 1999; Diggle et al., 2008; Waters & Bassler, 2005; Williams et al., 2007). This cross-kingdom signalling mediates the biofouling processes (Fig. 1.4). While, biofouling is a nuisance for marine, aquaculture and shipping industries (Yebra et al., 2004), the underlying dynamics provide useful information on the enigmatic settlement processes of prokaryotes and eukaryotes. This knowledge can then be extrapolated to understand how biofilms on substrates such as macro-algae may initiate settlement of marine invertebrate larvae.

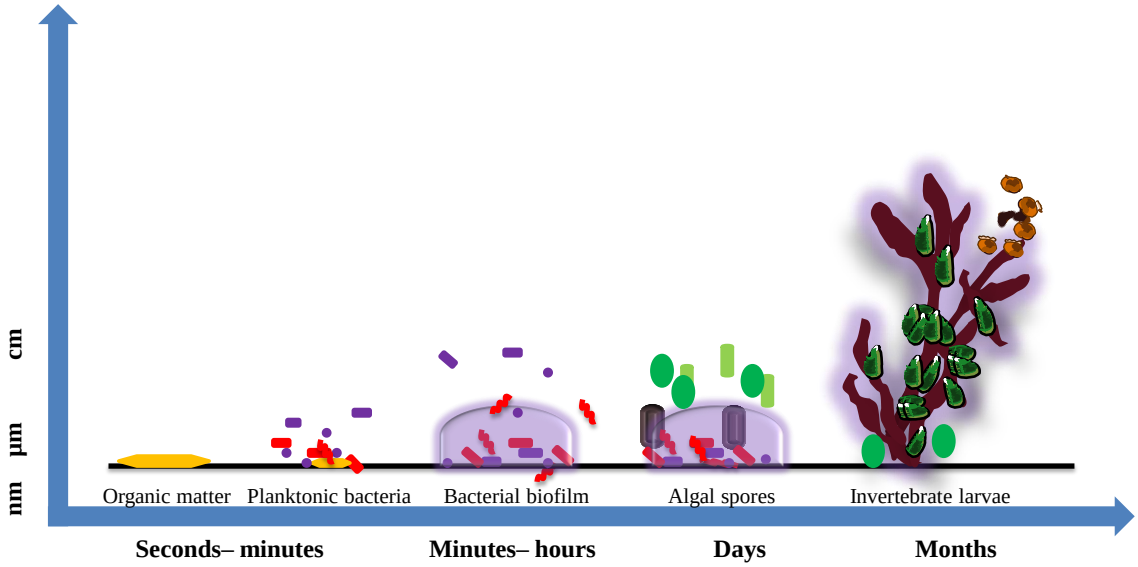

Figure 1.4: Diagrammatic representation of biofouling community formation on a marine substrate. The colonising sequence begins from the spontaneous organic fouling process, which is followed by colonisation by prokaryotes and eukaryotes (left to right). The settlement of invertebrate larvae and macro-algal spores ranges from several weeks to months. The schematic representation is a hypothetical scenario that involves settlement of macro-algal spores in response to a biofilm (modified from Patel et al., 2003). This is followed by development of macro-algae (brown filamentous structure) with associated biofilm (purple hue around macro-algae) that are able to induce settlement of invertebrate larvae (yellow brown veligers) that continue to develop as juveniles (green-mussel spat).

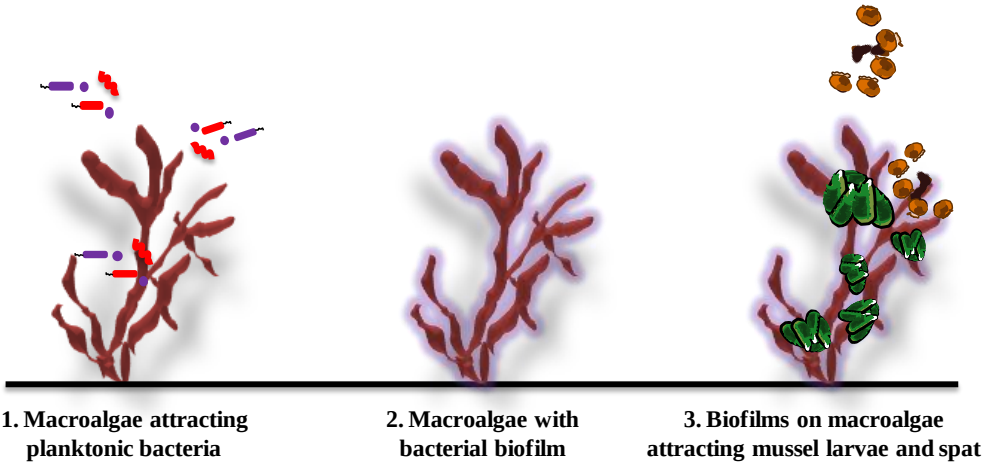

Figure 1.5: Schematic representation of 1. planktonic bacteria attaching on a macro-algal surface and 2. formation of biofilm on macro-algae (purple hue around the surface) and biofilm on macro-algae attracting mussel larvae which continue to develop as juveniles.

1.1.5 Marine bacterial-macro-algal associations

In the marine environment, chemically-mediated interactions and colonisation processes on macro-algal surfaces by a wide diversity of prokaryotes (bacteria) and eukaryotes (e.g., invertebrates) are common phenomena (Goecke et al., 2010; Steinberg & de Nys, 2002). It is likely that macro-algae produce chemical cues that "invite" bacterial cells to settle on them and/or produce nutrients which support bacterial associations (Saha et al., 2011; Sieburth, 1969). These symbiotic/epiphytic (surface colonising) associations may attract more bacterial cells and promote the growth of a biofilm (Amsler et al., 1992). An alternative way to produce this macro-algal-bacterial association is by the induction of an already established biofilm on surfaces that induce macro-algal zoospores to settle onto the biofilm, and thus promote algal growth (Armstrong et al., 2001; Fukami et al., 1997; Patel et al., 2003) (Fig. 1.5). Moreover, symbiotic bacteria are known to facilitate algal growth and nutrition(Croft et al., 2005; Goecke et al., 2010). Interestingly, cross-kingdom signalling has been identified for zoospores of the macro-algae *Ulva reticulata* and other *Ulva* sp., which are able to exploit the quorum sensing signals of associated bacteria (Dobretsov et al., 2007b; Joint et al., 2007). Moreover, Joint and colleagues (2007) were able to show that the number of settled macro-algal spores of *Ulva* sp. on the biofilm was directly proportional to the density of bacteria, highlighting the relevance of density-dependent QS pathways in bacteria. These studies not only demonstrated the complexity of algal-bacterial associations, but they also pointed to the potential for marine invertebrate larvae to respond to algal, bacterial, or algal-bacterial cues during the settlement process.

1.1.6 Larval settlement on marine bacterial-macro-algal associations

Natural chemicals from macro-algae are known to induce settlement and metamorphosis on a wide variety of marine invertebrates (Alfaro et al., 2006; Birrell et al., 2008; Fusetani, 2003). However, many of these studies do not adequately separate bacteria from the macro-algal materials. Thus, it is possible that bacterial cues rather than macro-algal cues are being tested in their assays. For example, larvae of mussel *Perna perna* and *P. canaliculus* have shown increased settlement when exposed to the extracts of red and brown macro-algae (Alfaro et al., 2006; Soares et al., 2008). However, in those experiments, the macro-algal cues were not separated from bacterial cues and hence it is possible that the cue might also be produced from symbiotic or epiphytic bacteria associated with macro-algae. Moreover, Alfaro et al., (2006), highlighted the relevance of bacterial biofilm in macro-algal extracts that induced larval settlement of *P. canaliculus*. Therefore, these results need further investigation to identify the actual source of the settlement cues. This type of experimental bias was convincingly illustrated by Johnson et al. (1991b), who tested the settlement of various invertebrate larvae, including the sea star, *Acanthaster planci*, on natural coralline algal substrates and such substrates treated with antibiotics to remove epiphytic bacteria. Their results indicated that when the bacteria were removed from the algal surfaces, larval settlement was inhibited rather than promoted. Similar results also have been reported for sea urchin larvae (Dworjanyn & Pirozzi, 2008; Huggett et al., 2006; Lamare & Barker, 2001; Pearce & Scheibling, 1991). In addition to positive effects, epiphytic bacteria also may have detrimental effects on larval settlement (Ma et al., 2009; Nasrolahi et al., 2012). Such information has been used extensively to create anti-fouling agents for ship hulls, marinas, and other human-made surfaces (Yebra et al., 2004).

1.1.7 Biofilms as nutrition for invertebrates

Nutrition is indeed an essential parameter for the settlement and recruitment of mussel larvae and juveniles (Carton et al., 2007; Pettersen et al., 2010; Phillips, 2002). The majority of bivalve larvae rely on phytoplankton as their primary food source (Ragg et al., 2010; Whyte, 1987). Nonetheless, bacteria (Brown et al., 1996; Douillet, 1993; Moal et al., 1996) and organic matter (Manahan & Crisp, 1982) have been shown to provide energy. After the initial work by Zobell and Feltham (1938), the possibility that marine invertebrates may utilise bacteria to meet their nutritional demands has been a subject for subsequent investigations. Bacterivory alone has been shown to enable growth and development in many invertebrates, especially gastropods (Adams & Angelovic, 1970), copepods (Rieper, 1978) and bivalves (Brown et al., 1996; Stuart & Klumpp, 1984) by providing a rich source of organic carbon. The presence of essential nutrients, such as proteins and carbohydrates enhances the use of bacteria as an alternate food source in bivalve aquaculture (Brown et al., 1996). Moreover, bacterial products are easily digested and contain crucial trace elements necessary for invertebrate growth (Charles et al., 1999). This is especially true for early stages of invertebrates when they require food particles of small size and easy digestibility, such as bacteria (Robert & Trintignac, 1997). Earlier studies on diets of adult clams (*Venus verrucosa*) showed that clams consumed more bacterial exudates of *Lactobacillus* sp. than algal exudates of *Pavlova lutheri*, highlighting the importance of easily digestible bacterial products as preferential diets for bivalves (Amouroux, 1986).

Thus far, microalgal diets have been the most commonly used diets for bivalve aquaculture (Brown, 2002; Ragg et al., 2010). Yet, culturing microalgae for

feeding larvae and juveniles is a major hurdle in bivalve hatcheries, since an average of 30% of the hatchery cost could be attributed to algal culturing (Borowitzka, 1997; Knauer & Southgate, 1999). Therefore, for marine bivalves, bacterial diets or mixed diets (bacteria with microalgae) should be a good replacement or complement to microalgal diets for sustainable larval and juvenile production (Robert & Trintignac, 1997). Bacteria are known to be an integral part of any hatchery tank environment, and the use of antibacterial agents, such as antibiotics, have been shown to retard the growth of invertebrates (Anguiano-Beltrán & Searcy-Bernal, 2007). Moreover, some bacteria possess growth-promoting nutrients, such as vitamins, which may be absent in microalgae (Croft et al., 2005; Douillet & Langdon, 1993). Conversely, polyunsaturated fatty acids (PUFAs), are essential growth promoting substances present in algae, and were thought to be absent in all bacteria (Brown et al., 1996; Robert & Trintignac, 1997) until Russell and Nichols (1999) identified the presence of PUFAs in certain marine bacteria. Furthermore, oyster larvae (*Crassostrea gigas*) have been shown to grow better (16–21%) and have enhanced survival (21–22%) when fed with the microalga, *Isochrysis galbana* in the presence of bacteria CA2 strain, compared with larvae fed with axenic (bacteria-free) cultures of *I. galbana* (Douillet & Langdon, 1993). This bacterium at a concentration of 1.5×10^7 cells ml^{-1} was able to provide 41% of dietary carbon required by the oyster larvae (Douillet, 1993). Similarly, mixed diets containing bacteria and microalgae promoted the shell length of Sydney rock oyster larvae, *Saccostrea commercialis*, compared with control larvae fed with algae alone (Brown et al., 1996; Nell et al., 1994).

Apart from providing nutrition, bacterial enzymes produced by gut-associated bacteria are essential in the digestion of complex materials for many

invertebrates (Harris, 1993). For example, symbiotic bacteria associated with the gut aid in the degradation of complex polysaccharides in seaweeds and thereby assist in the digestion process of abalone *Haliotis midae* (Erasmus et al., 1997) and *H. discus hannai* (Sawabe et al., 1998). Also, *Vibrio* sp. AS1 present in the gut of brittle stars (*Amphipholis squamata*) assists in the nutrient acquisition in their early embryonic stages (Lesser & Blakemore, 1990). Moreover, some gut-associated bacteria fix nitrogen from detritus, which is provided as a food source for the sea urchin *Strongylocentrotus droebachiensis* (Guerinot & Patriquin, 1981). The gut micro-flora of abalone also have been shown to assist in the digestion of certain macro-algae (Guest et al., 2008). Recently two novel probiotic bacteria (*Alteromonas macleodii* 0444 and *Neptunomonas* sp. 0536) were found to increase mussel, *P. canaliculus*, larval survivability by increasing their resistance against two pathogenic *Vibrio* sp. (Kesarcodi-Watson et al., 2010). Similarly, the *Roseobacter* sp. was found to have an agonistic effect against *Vibrio* sp., thereby improving the overall survivability of scallop, *Pecten maximus* larvae. Thus, bacteria can assist in digestion, improve survivability and can be used as an alternate food source for invertebrates, especially during the early stages of larval development.

1.2 **Purpose for the study**

1.2.1 Commercial perspective

The endemic Greenshell™ mussel, *P. canaliculus* contributes to the New Zealand aquaculture industry, with exports valued at over NZ$220 million in 2011(Ministry of Fisheries, 2012). New Zealand's advantage in the international market is based on the fact that farming is conducted in relatively clean water (surrounded by open ocean with low disease risk), and that this mussel species has a

good taste and an attractive green shell colour. This high-quality product is sold to more than 60 countries around the globe and accounts for 83.4% of the total aquaculture production volume (Food and Agriculture Organization, 2012; Ministry of Fisheries, 2011b). There are a total of 1018 authorised mussel farms in New Zealand, mostly concentrated in the Marlborough Sounds and Coromandel areas (Ministry of Fisheries, 2009). Commercial farming began over 40 years ago and mussels are grown in farms using the Japanese long-line technology (FAO, 2012). The farms almost entirely depend on wild-caught spat, 20% of which are caught on fibrous ropes suspended near mussel farms. The majority of the wild spat (about 80%) are collected from Ninety Mile Beach while still attached to drift macro-algae (Alfaro & Jeffs, 2002; Alfaro et al., 2004; Hickman, 1976). The stranded seaweeds, with associated spat, are collected from the surf zone all along the beach and shipped in refrigerated trucks to the farms. Once at the farm, the spat and seaweed are placed on the farm lines, which are surrounded by a stocking to hold the material in place (Fig. 1.6). Within a few weeks, the stocking degrades, and the spat "jump" onto the farm rope. After a few months, when the juveniles are securely attached to the ropes, the mussels are stripped from the lines and re-seeded at lower density to maximise production (Fig. 1.7).

Figure 1.6: Spat and seaweed held next to farm line with a degradable stocking (photo by A. C. Alfaro).

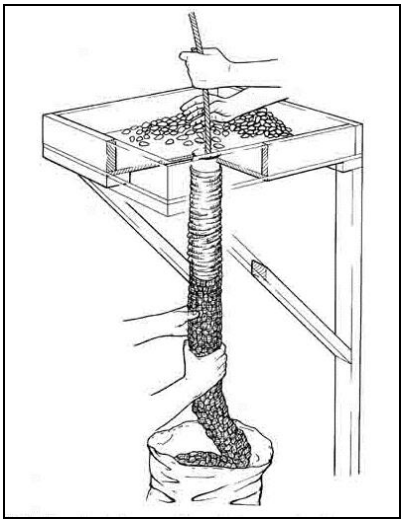

Figure 1.7: Re-seeding mussel juveniles on ropes surrounded by degradable stockings (from Jenkins, 1985)

The period between the first seeding and the re-seeding is a critical phase for the mussel industry, since losses of 50% and occasionally up to 90% can take place. Poor retention is not well understood and is one of the major problems that the industry faces today. It is believed that inadequate nutrition, predation, disease and other environmental stresses may play a part in the exodus of spat from ropes

(Carton et al., 2007; Hayden & Woods, 2011). However, it is likely that rope surface conditions (i.e., structure, chemistry, and biofilms) also have a significant effect on whether the mussels stay attached or "parachute" to find better substrates. Indeed, a well-seasoned rope (i.e., covered with a biofilm) is known to improve retention, at least during the first few days. Thus, studies on how biofilms induce, modulate and retain mussels to surfaces would likely improve our understanding of this enigmatic phenomenon.

While mussel hatcheries have been targeted as potential avenues to increase spat production, alleviate the reliance of the sometimes-unpredictable wild-caught spat, and improve genetic control over stock, this type of production is not without its faults. Hatchery-produced spat can suffer low settlement, high mortality, and high variability from batch to batch. Thus, further research is needed to develop a financially feasible hatchery production operation. The obvious area in which to focus initial investigations is settlement and early nutrition.

1.2.2 Ecological and evolutionary implications

Settlement and recruitment are essential events that affect the life cycle of many marine invertebrates, including mussels. For the green-lipped mussel, *P. canaliculus* larvae, the planktonic larvae are known to delay their settlement process up to several weeks before they find a suitable settlement substrata. These larvae are known to selectively choose filamentous red seaweeds and hydroids to attach themselves (Alfaro & Jeffs, 2002; Alfaro et al., 2004). The preference for certain macro-algae over others is not fully understood, but may be related to the bacterial biofilms that routinely form on their surfaces. At the base of this system is a well-orchestrated process of larval-surface recognition, which ensures that larvae "sense"

suitable substrates, and can quickly attach to primary settlement sites and continue to develop as juveniles. It is likely that this stimulus-response process is mediated by bacterial biofilms routinely present on the settlement sites. Thus, a better understanding of the role that biofilms play in the settlement and early nutrition of mussels is likely to enhance our knowledge of adaptive strategies for pelagic dispersal, and successful transfer to benthic life for mussels and other species with planktonic larvae.

1.3 Aims and objectives of this Study

Macro-algae and their surface biofilms provide a complex micro-environment with the potential to induce or deter settlement of mussel larvae and may provide nutrients for early mussels to feed on. As a first step to identifying and characterising the role that biofilms derived from macro-algal surfaces play on the early life of mussels, this study focussed on the following general aims:

1. To isolate and identify some representative marine bacterial species that induce (or deter) mussel larval settlement.
2. To identify bacterial phases (planktonic bacteria, biofilm) and/or biofilm products (exudates) that induce larval settlement.
3. To characterise chemical cues contained within bacterial biofilms that induce larval settlement.
4. To test the effect on spat retention of substrates inoculated with selected biofilms.
5. To determine whether mussel larvae and juveniles feed on selected bacterial biofilm.

The mussel, *P. canaliculus* was selected for this study, since it is a commercially significant endemic species to New Zealand. Also, there have been a number of studies on the adult stage of this species, and there is limited understanding of the

larval stage. Furthermore, knowledge of larval settlement, larval and juvenile retention and nutrition processes of this mussel could provide information and opportunities to enhance production in a commercial setting.

The foremost objective of this research was to obtain culturable single species of marine bacteria, which had the ability to modulate settlement of mussel larvae. Previous studies on the settlement of other planktonic larval species in response to natural cues (i.e., macro-algae, conspecifics, microalgae) clearly indicate that associated biofilm cues may not necessarily have a positive role in settlement of larvae. Therefore, *in-vitro* static-water larval settlement assays were conducted to test the effect of single-species marine bacteria on the settlement of *P. canaliculus* larvae. In order to conduct an effective settlement assay for this species, the settlement assay had to be optimised. The effect of settlement substrata, settlement medium and duration of settlement were optimised in this study to conduct efficient reproducible settlement assays. Then, fourteen mono-species culturable marine bacteria obtained from various sources (seawater, seaweed and mussels) were tested for settlement. Based on initial results, three marine bacteria (*Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1) were identified using biochemical and phylogenetic analysis, and were incorporated in the subsequent study. The effect of various substrates with biofilm also was tested to observe if these settlement surfaces modulated the activity of biofilms on the settlement of larvae. Moreover, it was essential to note that key mono-species biofilms did not interact with the physical cue of the settlement substrata and consistently induced similar rates of larval settlement across three substrates. Whilst the cue for larval settlement was found to be associated with the biofilm and not the

settlement substrate, the putative nature of the bacterial cue had to be established. Since the origin of settlement cues associated with bacteria could have been physical (bacterial cell structure) or chemical (cell-surface bound or in exudates), bacteria were cultured in different phases (planktonic and biofilm) and types (biofilm cells alone, biofilm cells with exudates and exudates alone). These culturing techniques pinpointed the exact location of the settlement cue. Once the exudates were found to induce/inhibit larval settlement, characterisation of these exudates was conducted to identify the chemical signature of the settlement cue. Hence, physical (heat treatment and molecular weight fractionation) and chemical (crude, sub-sample, sequential and lipid fractionation, and enzymes) treatments were carried out and distinct fractions were tested for their effect on larval settlement. These results clearly identified the biochemical class of the molecules and shed-light on techniques that can be used to characterise other bacterial exudates.

Another topic of interest was to analyse the effect of biofilms on the retention of juvenile mussels, since biofilms have been routinely reported to be present on macro-algal surfaces where spat are retained in the wild. For this purpose, the three marine bacteria (previously known to modulate settlement of mussel larvae) were tested for their ability to retain juvenile mussels over a period of time. The underlying rationale for this study was to introduce targeted species of bacteria to enhance mussel seed production.

Finally, the role of biofilms as potential sources of larval nutrition also was investigated in this study. Once again, the three marine bacteria (*Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1) were chosen for these experiments. The techniques used in this study provide an innovative

approach to investigate the importance of bacteria as an alternate food source for mussel larvae and juveniles.

1.4 Thesis layout and hypotheses

There are a total of ten chapters in this thesis. Chapters 1 and 10 are the general introduction and final discussion, respectively. Briefly, chapters 2–7 detail the investigation of mussel larval settlement in response to marine mono-species bacteria. Chapter 8 describes the nutrition studies of mussel larvae and juveniles. The attachment and retention studies of juvenile mussels have been specified in chapter 9. Each of the chapters 2–9, has a separate abstract, introduction, materials and methods, results, and a discussion section. Chapters 2–4 were conducted as preliminary work to successfully conduct settlement assays. Also, chapters 5 and 6 have been published (Ganesan et al., 2010; Ganesan et al., 2012a; Ganesan et al., 2012b) and chapter 7 is in preparation for publication. Chapters 8 and 9 were conducted as trial experiments and techniques used in the study, and the results are being prepared for publication. The following comprises the chapter titles and hypothesis/hypotheses listed under each of them.

Chapter 2: Optimising mussel larval settlement assays

- Settlement substrate has no effect on the mussel larval settlement
- Settlement medium has no effect on the mussel larval settlement
- Rate of larval settlement is dependent on the incubation time

Chapter 3: Isolation, identification and screening of culturable mono-species marine bacteria for mussel larval settlement

- Single-species marine bacteria induce larval settlement

Chapter 4: The role of substrate physical properties in modulating bacterial cues for the settlement of mussel larvae

- Settlement substrates do not change properties of bacterial cues for larval settlement

Chapter 5: Effects of mono-species bacterial biofilms, biofilm exudates and bacterial cell-suspensions on *P. canaliculus* larval settlement

➢ For *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1

- Bacterial biofilm (with exudates) and exudates (alone) induces larval settlement
- Planktonic bacteria and washed biofilm cells (without exudates) do not induce larval settlement

➢ For *Pseudoalteromonas* sp. AMGP1

- Bacterial biofilm (with exudates) and exudates (alone) kill larvae
- Planktonic bacteria and washed biofilm cells (without exudates) do not kill larvae

Chapter 6: Characterisation of biofilm exudates and their effects on settlement of mussel larvae.

- Polar proteins and non-polar lipoproteins of *Macrococcus* sp. AMGM1 induce larval settlement
- Glycolipids of *Bacillus* sp. AMGB1 induce larval settlement

Chapter 7: The effects of fractionated biofilm exudates of *Pseudoalteromonas* sp. AMGP1 on the settlement of *P. canaliculus* larvae.

- Polar proteins kill larvae
- Non-polar lipoproteins induce larval settlement

Chapter 8: Attachment and retention of mussel juveniles (spat) in response to bacterial biofilms

- Bacterial biofilms on glass slides and ropes increase attachment of spat
- Bacterial biofilms on glass and ropes do not retain spat

Chapter 9: Role of marine bacteria in nutrition of mussel larvae and juveniles

- Marine bacteria provide nutrition for larvae
- Marine bacteria provide nutrition for spat

Chapter 2.

OPTIMISING MUSSEL LARVAL SETTLEMENT ASSAYS

2. Optimising Mussel Larval Settlement Assays

2.1 Abstract

Larval settlement of the commercially renowned green-lipped mussel, *Perna canaliculus* has been a topic of interest for over a few decades now. Yet there are no standardised protocols available, which can be used as exemplars for conducting *in-vitro* settlement assays. This study focuses on optimising three key experimental parameters (i.e., settlement substrate, settlement medium and duration of the settlement assay) in order to achieve controlled, efficient and reproducible settlement assays. The results from the effect of three substrates (agar, glass and Petri plate) on larval settlement indicated ideal control settlement rates of < 20% across all treatments. Furthermore, the study demonstrates that the inherent physical nature of these substrates may not have a significant role in larval settlement process. Also, sterile natural seawater (by filtration, autoclaving, or filtration and autoclaving), synthetic (SSW) and artificial (ArSW) seawater induced < 20% of larvae to settle. However, SSW and ArSW induced greater larval mortalities of over 70% when compared with sterile natural seawater media. These results clearly show the suitability of natural seawater as a potential medium to conduct settlement assays. Finally, the optimum duration of the experiment for larval settlement was found to be 48 hours. Results from this study also indicate that the assay duration can be extended up to 60 hours in case of counting larvae from a large number of treatment plates. Overall, the methodologies optimised in this study can aid in repeatedly testing several cues using still water settlement assays for *P. canaliculus* larvae, and potentially other marine invertebrate species.

2.2 Introduction

Several filamentous macro-algae and hydroids provide the primary substrate for settlement of larvae of the indigenous green-lipped mussel, *P. canaliculus*. This remarkable phenomenon can be observed in the surf zone at the Ninety Mile Beach in northern New Zealand, where juvenile mussels (spat) arrive at the shore, attached to drifting macro-algae. The primary and secondary settlement processes were first described for the blue mussel, *Mytilus edulis*, larvae that were observed to settle initially on filamentous substrates and later transfer to rocky substrates during their juvenile phase (Bayne, 1964). Furthermore, during the late pediveliger stage (> 2.5mm), juvenile mussels can secrete long strands of mucus several times in order to commute in the ocean until they successfully encounter a suitable settlement site (Lane et al., 1985; Sigurdsson et al., 1976). This knowledge has been extrapolated to understand settlement behaviour of green-lipped mussel (*P. canaliculus*), and the dynamic settlement and re-settlement processes of mussel larvae have been extensively investigated (Alfaro, 2005, 2006c; Alfaro & Jeffs, 2002; Alfaro et al., 2004; Buchanan & Babcock, 1997). For example, Buchanan and Babcock (1997) and Alfaro and Jeffs (2002) have documented primary settlement on morphologically distinct filamentous substrates, while Alfaro (2005, 2006a) investigated settlement patterns within artificial and natural substrates under various water dynamics and oxygen concentrations. In addition, chemical cues have been found to induce mussel settlement under field and laboratory conditions (Alfaro et al., 2006; Gribben et al., 2011). Although larval settlement studies conducted in the field may aid the identification of generic characteristics of settlement cue, a more specific identity of these cues may be achieved only through laboratory guided assays. Unlike field assays, studies conducted in the laboratory are more

advantageous in terms of controlling numerous extrinsic factors (i.e., pollution, weather conditions, temperature and salinity) that may influence larval settlement behaviour (Pawlik, 1992). Moreover, unfavourable conditions can often delay settlement of larvae for up to several weeks until they find a suitable substrata and continue to develop as juveniles (Bayne, 1964). Thus, a more controlled *in-situ* assay is preferred to obtain reliable, reproducible and efficient analyses of larval settlement responses (Carl et al., 2011).

Settlement assays conducted in the laboratory have been routinely conducted to understand the effects of natural (Alfaro et al., 2006; Ganesan et al., 2010; Ganesan et al., 2012a), and synthetic (Young, 2009; Young et al., 2011) chemicals on the settlement of *P. canaliculus* larvae. Organisms used in these experiments were obtained from a controlled hatchery environment to minimise the likelihood of drastic genotypic and phenotypic differences, thereby improving the reproducibility of these settlement assays. Another key factor to optimise settlement assays and to obtain favourable results within short experimental periods was to incorporate larvae of a desired age. Within hatcheries, settlement-competent larvae have been randomly observed after about 18 days post-fertilisation to be over 200 µm in size and have morphologically distinct characters (i.e., presence of foot, velum and eyespots). Interestingly, these parameters are similar among mussel species, such as *P. canaliculus* (Jenkins, 1985), *Aulacomya maoriana* (Alfaro et al., 2011b), *Mytilus galloprovincialis* (Satuito et al., 2005) and *Mytilus edulis* (Bayne, 1964). However, conditions (i.e., time, temperature, duration) for the settlement assay vary among mussel species, indicating that there are no established protocols for standardising these settlement assays.

For *P. canaliculus*, the effect of larval age (16–23 days post-fertilization), larval batches (within and between hatcheries), and duration of the assay (24 and 48 hours) have been assessed and optimised (Young, 2009). However, the effects of various substrates on the settlement of mussel larvae were not analysed in that study. Sterile polystyrene Petri plate (60 mm diameter × 14 mm depth) was the most common substrate used in the present study, as these have been previously used as settlement substrates for *P. canaliculus* larvae (Young et al., 2011) and for a variety of invertebrate larvae (Alfaro et al., 2011b; Dobretsov & Qian, 2006; Qian et al., 2003; Young et al., 2011). Other types of substrates, such as glass (Zardus et al., 2008), agar (Stott et al., 2004), poly-vinyl chloride (PVC) plates (Hung et al., 2008) and acrylic sheets (Keough, 1998) have also been used as substrates to conduct settlement assays. Quite often, these settlement substrates have been randomly chosen without any underlying rationale. Yet, many studies have suggested that artificial substrates may contain specific physical signatures (i.e., surface topography, surface charge and wettability), which can affect larval settlement in the absence of a settlement cue (Carl et al., 2012; Gribben et al., 2011; Maki et al., 1989; Petrone et al., 2011; Rittschof & Costlow, 1989). For example, larvae of the barnacle *Balanus improvisus* were shown to settle preferentially on low wettablility (hydrophobic) polystyrene surfaces, whereas highly wettable (hydrophilic) glass surfaces induced attachment of *B. amphitrite* larvae (Dahlström et al., 2004; O'Connor & Richardson, 1994). These results demonstrate to the need for carefully choosing an optimal substrate for *P. canaliculus* larvae to use as controls (no intrinsic physical cue effect) in settlement assays.

A standard settlement assay medium used has been sterile seawater to restrict the effect of resident micro-organisms present (Little et al., 1987). Sterilisation of seawater by ultra-violet (UV) radiation (Gallardo & Buen, 2003) membrane filtration (Unabia & Hadfield, 1999), autoclaving (Lam et al., 2005), and filtration followed by autoclaving (Dobretsov & Qian, 2004) has been commonly reported for preparation of settlement assay media. Although UV irradiated seawater has been routinely used in large-scale hatchery settings (Brown & Russo, 1979; Bullock & Stuckey, 1977), production of toxic residues, prevalence of resistant strains, high cost and the need to pre-filter seawater to remove suspended particles have been some of the known disadvantages of this sterilisation technique (Björnsson, 2004; Liltved & Cripps, 1999; Liltved et al., 2006; Wardell et al., 1986). Unlike UV treatment, seawater subjected to (0.45 µm) filtration may remain free of bacteria and viruses, yet virus like particles, dissolved organic matter and other toxic residues from microbes appear to remain in the retentate (Berry & Noton, 1976; Sano et al., 2004). Therefore, pre-filtering seawater to remove colloidal particles followed by autoclaving would be best suited for settlement assay experiment that require small volumes of seawater (Berry & Noton, 1976). Alternatively, artificial seawater by mixing pre-made salts (e.g., Instant ocean® and nine salt solution) have also been incorporated to achieve axenic larval assay media (Fitt et al., 1989; Holmström et al., 1992; Maki et al., 1989; Mihm & Banta, 1981). The underlying rationale to replace artificial seawater with natural counterparts has been to achieve a solution that is reproducible and chemically well-defined (Kester et al., 1967). Furthermore, natural seawater remains susceptible to several man-made pollutants (e.g., oil, antifouling biocides, sewage effluents and heavy metals), which may lead to developmental defects of some invertebrates from gametes through to larvae (Bellas, 2006; Bellas et

al., 2004; Loya & Rinkevich, 1980; Pastorok & Bilyard, 1985). Nevertheless, a number of commercially available sea salts (i.e., Instant Ocean, Marinemix, Coralife etc.) used in the preparation of artificial seawater contain a hundred–thousand times greater concentration of heavy metals (copper, zinc, lead and titanium) when compared with natural seawater (Atkinson & Bingman, 1997; Marulla & O'Toole, 2005). The increased heavy metals in artificial seawater, especially copper also have shown to affect growth of phytoplankton and development of invertebrate larvae (Davey et al., 1970). For example, Shimek (2003) demonstrated that synthetic seawater containing Instant Ocean® salts had an increased proportion of heavy metals (i.e., cadmium, lead, copper, nickel and zinc) than natural seawater, and induced greater mortality (over 90%) for the sea urchin (*Arbacia punctulata*) larvae. Nonetheless, the toxicity of heavy metals from both natural and synthetic seawater can be eliminated by pre-filtering them using heavy metal chelating column like Chelex-100 prior to their use in the larval assays (Davey et al., 1970; Zaroogian et al., 1969). While, artificial seawater has been constantly manipulated to suit the specific micro- and macro-nutrients needs of larvae, their toxicity on larvae needs to be analysed (Berges et al., 2001; Courtright et al., 1971; Kester et al., 1967).

Apart from settlement substrates and media, the settlement assay period is a critical parameter when evaluating the effects of various treatments on larvae. Rittschof & Costlow (1989) assessed the settlement of bryozoan (*Bugula neritina*) after 30 minutes and barnacles (*Balanus amphitrite*) after 22 hours. These differences in settlement assay duration point to the need to optimise incubation time for every species of invertebrate larvae. The incubation period of the settlement assay may also depend on the time taken for larvae to respond to specific inductive

cues tested in a study. For instance, Dobretsov and Qian (2003) preferred a 24 hour over 48 hour assay period to observe the effect on the blue mussel (*Mytilus edulis* L) larvae as there was no observed increase in larval settlement after the 24 hour time period. Conversely, Thiyagarajan et al. (2002) clearly demonstrated that the settlement rates of barnacle, *Balanus amphitrite* larvae, were more variable during the initial 12 hour assay period when compared with a 48 hour assay. Similarly, Young (2009) observed a greater increase in settlement rates and lower variability among treatment plates during a 48 hour settlement period when compared with the 24 hour assay period for *P. canaliculus* larvae. The observed variability in the number of settled larvae during the initial 24 hour period was attributed to the initial acclimatisation phase of larvae to their new environment. Regardless, an accepted level of variability in larval settlement may allow reliable comparison of results obtained from two or more treatments incorporated in a study. Nonetheless, scoring larval settlement is a time consuming process, time exposure variations may be up to 10–12 hours. Thus, a better understanding of the potential effects of responses over different periods of time (ideally every 12 hours) is essential to undertaking meaningful settlement assays.

Thus the aim of this study was to focus on the effect of three parameters (settlement substrates, settlement media and duration of settlement) to induce settlement of larvae. The rationale for choosing these three parameters was to obtain ideal control plates that presented no hidden extraneous factors while accounting for larval settlement and mortality. Also, it was essential to achieve an exemplar settlement assay in order to conduct future settlement assays with *P. canaliculus* larvae. To this extent, the current study attempts to optimise settlement assays by

standardising the settlement substrates (polystyrene, glass and agar), medium (sterile, synthetic and artificial seawater) and duration (12, 24, 36, 48, 60 and 72 hours) for *P. canaliculus* larvae.

2.3 Materials and Methods

2.3.1 Mussel larvae

Free-swimming *P. canaliculus* veliger larvae were sourced from Cawthron Institute and Shellfish Production and Technology New Zealand Limited (SPATnz), both in Nelson, New Zealand. At the hatchery, mussel larvae were maintained at 18 ± 1°C in filtered (0.1 µm) seawater of 35 ppt salinity. The protocol for larval rearing and maintenance has been described by King et al. (2005) and reported by Young (2009). Microalgal diets were provided at the hatchery for the larvae from 48 hours post-fertilisation (D-stage larvae), and were comprised of *Chaetoceros calcitrans* and *Isochrysis galbana* (T-Iso clone). Between 18–20 days post-fertilisation (depending on the batch), around 10,000–40,000 eyed larvae were screened through a 70–100 µm nylon mesh (about 20 cm^2), to retain larvae on the mesh. These larvae were placed in mesh surrounded by wet paper towels and ice packs and transported overnight to Auckland University of Technology's (AUT) aquaculture facility in a polystyrene foam bin. With some batches, swimming larvae were transported in seawater (1 µm filtered) in a 20 ml screw-capped polystyrene container. However, greater mortality rates (50–90%) were observed when larvae were transported in this manner.

Once the larvae arrived at AUT, they were immediately transferred to a 2 l beaker containing 1 l of seawater that was filtered through a 0.45 µm membrane

filter (mixed cellulose ester, Advantec®, Japan), autoclaved to remove any resident bacteria present in the natural seawater and cooled to 17°C prior to use. Larvae in autoclaved filtered seawater (AFSW) were left undisturbed for 30 minutes in order to acclimatise to their new environment. During this period, about 30–50 larvae were randomly selected and observed under a dissecting stereomicroscope (Leica Zoom™ 2000, U.S.A) at 20 × magnification to assess their age (presence of eye spots, movement of gills, velum and foot) and health (presence of dead/malformed or crushed larvae and presence of protozoans). After the acclimatisation period, if no larvae were observed swimming in the water column and if more than 50% of the larvae were found dead, the batch was discarded. Otherwise, the larvae that were swimming in the water column were decanted to another 1 l beaker that contained a small (20–30 mm) magnetic bar. The beaker was immediately placed on a magnetic stirrer set to 100–200 rpm to avoid larvae being crushed by the magnetic bar and to obtain even dispersion of larvae throughout the beaker. Prior to the settlement assay, about 10 replicate samples of 1 ml larval suspensions were withdrawn from various corners and depths of the beaker to obtain a density of 20–30 larvae ml^{-1}. Larval density counts were initially conducted by observing under a stereomicroscope at 10.5 × magnification, but visual checks with the naked-eye proved sufficient for this purpose. After addition of larvae to ten treatment plates, the larval density was corroborated to ensure the presence of a similar number of larvae across all treatments.

2.3.2 Preparation of settlement substrates

Small polystyrene Petri plates (60 mm diameter × 14 mm depth, Biolab Inc., New Zealand) were mostly used as substrates for larval settlement. However, large

(90 mm diameter × 15 mm depth, Biolab Inc., New Zealand) Petri plates were used as secondary substrates to hold other primary settlement substrates such as agar (5–10 ml), polystyrene sheets (80 mm length ×30 mm width) and glass microscope slides (76 mm length × 26 mm width). Initially, large Petri plates were used to examine the effect of Zobell marine agar 2216 medium (refer chapter 3 for medium composition) as a potential substrate for larval settlement. However, marine agar provided nutrients to epizoic (surface) bacteria present on larvae and these in turn killed the larvae during the settlement period. Polystyrene sheets were buoyant in the assay medium and microscope glass slides created crevices when placed on Petri plates. The gaps allowed the larvae to settle on underside surfaces of the glass and in-turn crushed some larvae during the counting process, which made it difficult to estimate actual larval settlement and mortality. To resolve this issue, 5 ml of 1.5% noble agar (Coast Biologicals Ltd, Auckland, New Zealand) was used as the primary substrate instead of marine agar. When glass slides were placed prior to the solidification of 5 ml of noble agar, the undersides of the glass slides were completely sealed. However, polystyrene sheets were unable to attach on the noble agar, and the substrate was eliminated from the study.

Thus, for this study three substrates namely, large Petri plates containing 10ml of 1.5% noble agar (noble agar), glass slides in large plates with 5 ml of 1.5% noble agar (glass slide on noble agar) and small sterile Petri plates were tested. For the larval settlement assay, the experiments were conducted in replicates of five per substrate (noble agar, glass slide on noble agar and small Petri plate) containing 9 ml of AFSW and 1 ml of larvae in AFSW.

2.3.3 Preparation of settlement media

Seawater was routinely obtained from Mission Bay, Auckland, New Zealand and stored in the aquaculture facility at AUT. Prior to use in the settlement experiments, water pH was confirmed to be 8.0 ± 0.2, and a salinity of about 35 ppt (at 21°C) was checked using a hand-held refractometer to show a specific gravity of 1.025 g cm^{-3}. To remove macro- and micro-organisms, seawater was subjected to five treatments and each of these treatments was checked for their ability to influence larval settlement and mortality. Natural seawater was subjected to filtration using a 0.45 µm membrane filter (FSW), sterilised by autoclaving (ASW), and also filtered and autoclaved (AFSW). In addition, synthetic seawater (SSW) was prepared according to the manufacturer's instruction (Instant Ocean®, 400 g in 12 l of distilled water). Artificial seawater (ArSW) was prepared by mixing the salts present in the Zobell marine agar medium (i.e., without peptone, beef extract and agar). The pH and salinity were adjusted for SSW and ArSW, and all five media were maintained at 17°C prior to their use in the assay. Experiments were conducted in replicates of three per medium (FSW, ASW, AFSW, SSW or ArSW) and each polystyrene Petri plate (60 mm diameter × 14 mm depth) received 9 ml of the respective medium and 1 ml of larvae in AFSW.

2.3.4 Settlement assay duration

The ideal duration of the settlement assay was tested over periods of 12, 24, 36, 48, 60 and 72 hours. Experiments were conducted in replicates of ten for each time interval on polystyrene Petri plates (60 mm diameter × 14 mm depth) containing 9 ml of AFSW and 1 ml of larvae in AFSW.

2.3.5 Settlement assay

All settlement assays were carried out in AUT's aquaculture facility. All assay plates received 20–30 larvae ml^{-1} and were maintained at 17 ± 1°C under ambient light conditions. The number of replicates were usually ten plates for each treatment and controls, but ranged from three to five replicates per treatment for this study. During the incubation period, the plates were left undisturbed, and water was not exchanged. Food was not provided to the larvae during the settlement experiments. At the end of this period (48 hours unless stated otherwise), the number of settled larvae was counted under a stereomicroscope at 12 × magnification. Settled larvae were observed attached to the bottom of the Petri plate with a thin visible transparent mucous thread-like structure. Attachment was corroborated by placing a 200 µm pipette within close proximity (~1mm) to the larvae and by applying gentle suction. This method had been previously tested by Young (2009), who found this to be the most effective way of testing settlement. Larvae were termed settled only when alive (see section below) and not dislodged when gentle suction was applied. In some instances, larvae were found attached to each other and only one of them were counted as settled. During the counting process, all larvae were transferred to a clean Petri plate to ensure that the larvae were not counted twice, and for mortality counts.

2.3.6 Mortality assays

The mortality assays were carried out by noting larval behaviour (e.g., opening of valves, movement of foot, gills and velum) under a stereomicroscope at 20–45 × magnification. Unlike live larvae, dead ones appeared darker and their internal organs were not entirely visible. While observation of larval movement was

essential to establish mortality, it was a time consuming process (5–10 minutes per plate). In addition to observing larval behaviour and colour, vital stains such as Rhodamine-B or Neutral red were used to differentiate live from dead larvae. These stains were prepared in AFSW and the final concentration of the stain in the assay medium was 10 ppm for Rhodamine-B and 20 ppm for Neutral red (Simon, 1974). Larvae containing stains were incubated for 10–20 minutes and observed under the stereomicroscope at 20 × magnification. Unstained larvae showing no signs of movement were recorded as dead. In rare instances, live protozoans present in dead larvae were able to absorb these vital stains resulting in false positive colouration of the larvae. Therefore, both visual and staining techniques were adopted in conjunction to minimise errors and to determine true larval mortality counts.

2.3.7 Statistical analyses

All percent larval settlement and mortality data were arcsine-transformed and analysed using the Predictive Analysis Software (PASW®) Statistics 18. The data were tested for normality using the Shapiro-Wilk test (Shapiro & Wilk, 1965) and homogeneity of variance using the Levene's test (Levene, 1960). Data that met these parametric assumptions ($p \geq 0.05$) were subjected to one-way Analysis of Variance (ANOVA) followed by *post-hoc* Tukey tests for pair-wise comparisons.

2.4 **Results**

2.4.1 Settlement substrates

Larval settlement was uniformly low (< 20%) on substrates containing noble agar, a glass slide on noble agar and small Petri plates (Fig. 2.1 A; ANOVA $F_{2,12} = 0.3$, $p = 0.758$). In addition, there were no significant differences in larval mortality

across all three settlement substrates (Fig. 2.1 B; ANOVA $F_{2,12} = 0.1$, $p = 0.879$). Individual *post-hoc* Tukey tests revealed non-significant differences ($p > 0.001$) among the three substrates for both larval settlement and mortality, indicating that any one of the three substrates could potentially be incorporated in the settlement assay.

2.4.2 Settlement media

Results from the larval settlement assay clearly indicated low settlement of < 20% across all five assay media (Fig. 2.1 C; ANOVA $F_{4,10} = 0.3$, $p = 0.886$). Tukey tests showed non-significant ($p > 0.001$) pairwise comparisons differences between filtered seawater (FSW), autoclaved seawater (ASW), autoclaved filtered seawater (AFSW), synthetic seawater (SSW) and artificial seawater (ArSW). However, significantly greater mortality (mean% ± SE) was observed in treatments containing SSW (83.1 ± 2.6) and ArSW (71.3 ± 5.0) when compared with lower mortality between 8–12% in FSW, ASW and AFSW (Fig. 2.1 D; ANOVA $F_{4,10} = 107.58$, $p = 0.001$). Moreover, individual Tukey tests revealed non-significant differences ($p < 0.001$) between FSW, ASW and AFSW clearly indicating that either one of three media could be used for larval settlement assay.

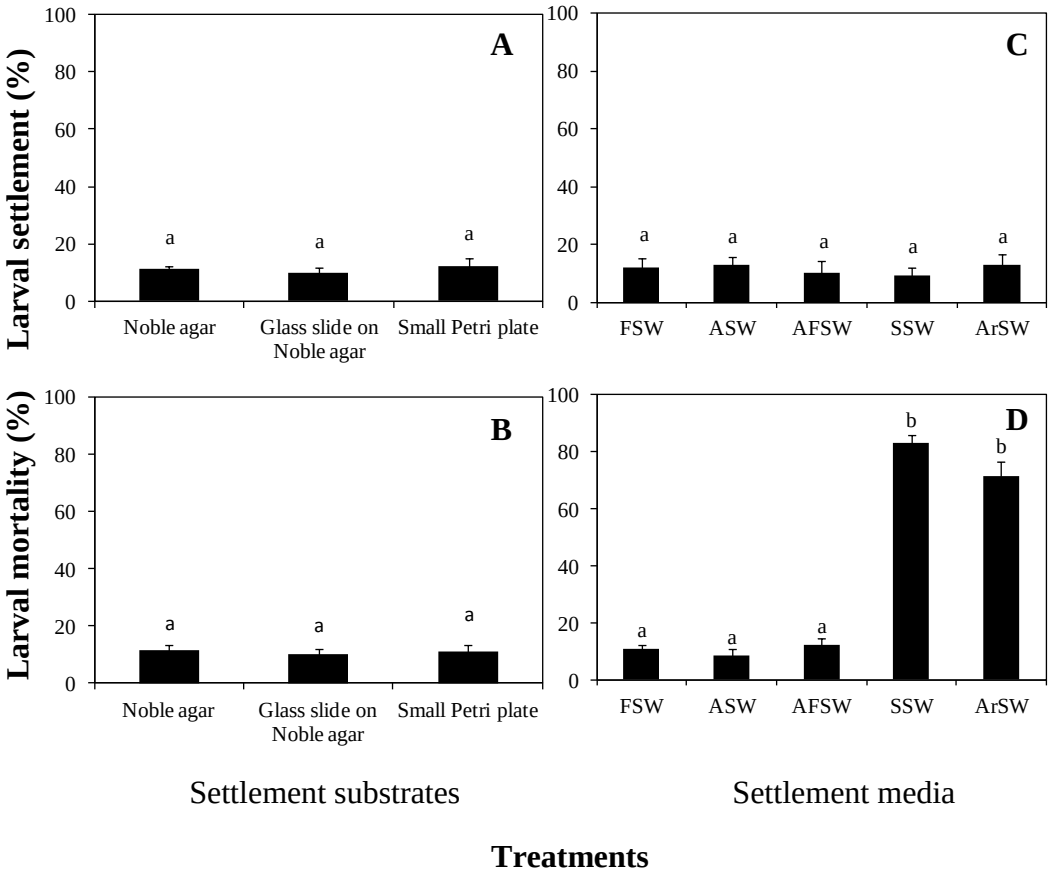

Figure 2.1: Effect of settlement substrates and settlement media for mussel larvae. Data represent mean (±SE) larval settlement (top row) and mortality (bottom row) of *P. canaliculus* after 48 hours on three settlement substrates (A&B, n = 5) namely noble agar, glass slides on noble agar and small Petri plate and five settlement media (C&D, n = 3) namely, filtered seawater (FSW), autoclaved seawater (ASW), autoclaved filtered seawater (AFSW), synthetic seawater (SSW) and artificial seawater (ArSW). Non-significant Tukey tests are denoted by the same letter over the bars.

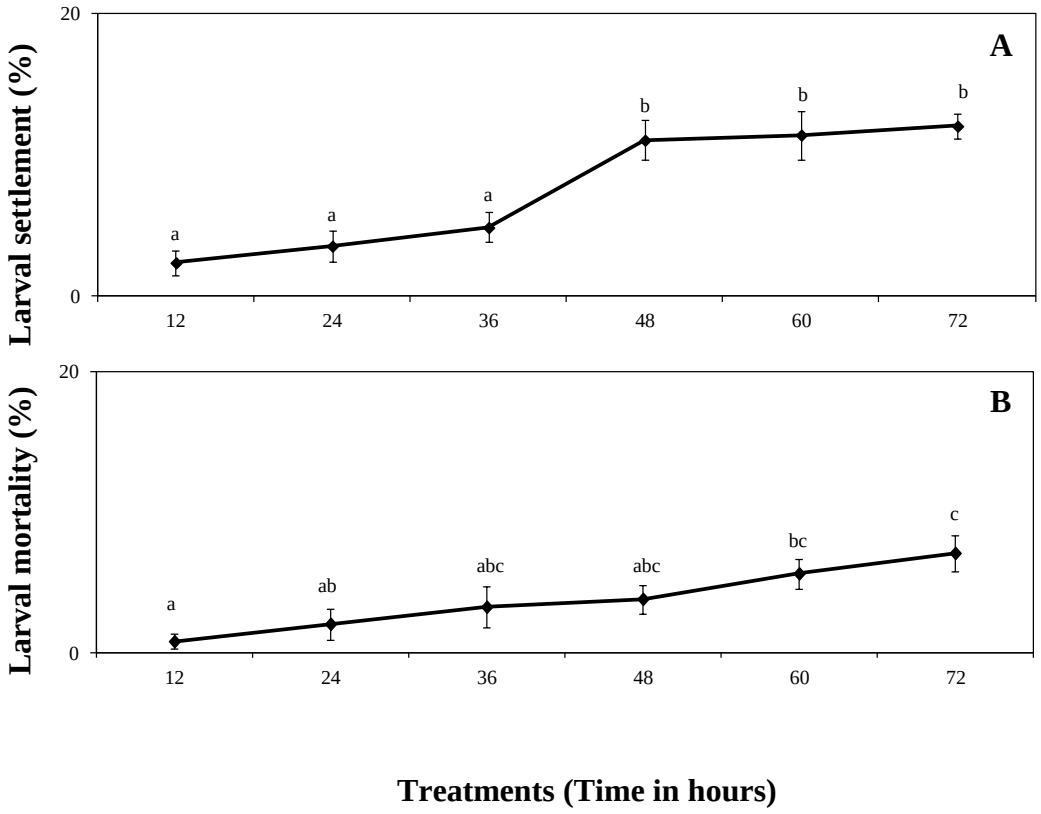

Figure 2.2: Effect of settlement assay duration for mussel larvae. Data represent mean (±SE, n = 10) larval settlement (A) and mortality (B) of *P. canaliculus* after 12–72 hours on polystyrene Petri plates containing autoclaved filtered seawater. Non-significant Tukey tests within (biofilm exudates) are denoted by the same letter over the bars.

2.4.3 Settlement assay duration

The optimal duration of the settlement assay was assessed from experiments conducted at 12, 24, 36, 48, 60 and 72 hours (Fig. 2.2 A). Significantly (ANOVA $F_{5,54} = 12.3$, $p = 0.001$) greater larval settlement (10–12%) was observed after the 48 hours, while < 5% was recorded in the first 12–36 hour period. Tukey tests showed non-significant differences ($p < 0.001$) between 48, 60 and 72 hours assay period. In addition, non-significant differences ($p > 0.001$) were observed during 12, 24 and 36 hour periods, but all three were significantly different ($p < 0.001$) to the 48 hour assay, indicating that mussel larvae needed 48 hours to exhibit a settlement response. Results from mortality assays (Fig. 2.2 B; ANOVA $F_{5,54} = 5.1$, $p = 0.001$) clearly indicate greater larval death counts after the 60 (5.6 ± 1.0) and 70 (7.1 ± 1.3) hour

assay periods. Tukey tests revealed significant differences ($p < 0.001$) between 12 hours when compared with 60 and 72 hours. Moreover, Tukey tests showed non-significant ($p > 0.001$) between 12, 24, 36 and 48 hour assay period.

2.5 Discussion

This study provides results that contribute to the optimisation of still-water laboratory settlement assays for hatchery reared *P. canaliculus* larvae, and potentially other mussel species. For an effective laboratory assay, parameters such as testing settlement substrates, settlement media and duration can be easily achieved and similar assays can be conducted to test the effect of a variety of settlement cues for *P. canaliculus* larvae.

The results from the present study clearly indicate that mussel larvae do not show differences in percentage settlement on three different substrates (polystyrene, glass and agar). This may also indicate that *P. canaliculus* larvae do respond to the inherent physical cue (i.e., surface charge, surface topography and wettability) of these substrates. Since none of these physical parameters was measured in this study; further studies will be needed to elucidate the effects of such factors. Previous studies indicated that larvae respond to surface topography (Gribben et al., 2011), and especially to the morphology of macro-algae and hydroids (Alfaro & Jeffs, 2002; Alfaro et al., 2004; Buchanan & Babcock, 1997). This indicates that surface contour has a potential effect on larval settlement. Since the three substrates used in this study were macroscopically smooth, the effect of surface contour of these substrates may have no relevance to the settlement of larvae. Nonetheless, the effects of wettability and surface charge on settlement of *P. canaliculus* larvae need to be addressed in future studies. An additional observation from the present study was

that the settlement and mortality of larvae were similar, despite the differences in settlement surface area. Large Petri plates (90 × 15 mm) were used to accommodate noble agar and glass slides on noble agar, both of which had a greater surface area for larval settlement when compared with small Petri plates (60 × 14 mm). Similar settlement patterns were observed for the barnacle larvae (*Semibalanus balanoides* Linnaeus), which showed no differences in settlement on substrates with dissimilar surface area (Pineda & Caswell, 1997). Another reason for *P. canaliculus* larvae to settle evenly on all three substrates (noble agar, glass on noble agar and Petri plate) in this study could be the absence of settlement inducing cues (i.e., bacterial cues). However, the suitability of the settlement substrates needs to be reassessed when testing the effects of bacteria on larval settlement. This is because the physical cues of substrates have been shown to influence the inductive capacity of bacterial cues, which may have an overall effect on the settlement of larvae (Huggett et al., 2009). Nevertheless, the use of small Petri plates is practical and appropriate for scoring settlement and mortality of larvae within general settlement assays. Unlike Petri plates, the agar surface was translucent when observed under the stereo-microscope, which increased the time required to count larvae.

Sterilisation of natural seawater by filtration, autoclaving, and/or both was found to be effective when compared with the use of synthetic or artificial seawater. Both filtration and autoclaving can alter the organic composition, which may have a drastic effect on the seawater chemistry (Manahan & Stephens, 1983). Moreover, this inherent chemistry of seawater has been shown to influence larval behaviour and development (Jaeckle & Manahan, 1992). Therefore, to ensure the isolation of the treatment effect, and to negate the intrinsic effects of seawater chemistry on larval settlement, the protocols for sterilising seawater used in the study are recommended

for future larval settlement studies. Unlike natural seawater, the effect of artificial and synthetic seawater on larval mortalities was quite surprising. This may have been due to the lack of specific ions and/or enrichments in the artificial seawater which may have affected larval health. Courtright et al. (1971) explained that commercially available alternatives for natural seawater may not suit all invertebrate larvae and each species requires specific nutritional parameters, which need to be individually examined. The authors further demonstrated that the larvae of *Mytilus edulis* required specific organic components in seawater, and its absence led to malformed or dead larvae. Similarly, the larvae of red king crab, *Paralithodes camtschaticus* were unable to survive when exposed to Instant ocean®, synthetic seawater, prepared as instructed by the manufacturer (Persselin & Daly, 2010). This may have been due to a high concentration of heavy metals, especially copper in commercially available sea salts that may have affected larval health (Marulla & O'Toole, 2005; Shimek, 2003). Nevertheless, artificial seawater can be modified by removing toxic metal ions and adding specific nutrients to suit larval requirements (Courtright et al., 1971; Jaeckle & Manahan, 1992; Mihm & Banta, 1981; Zaroogian et al., 1969). Such modified and chemically well-defined seawaters have been incorporated in a number of larval settlement assays (Fitt et al., 1990; Fitt et al., 1989; Mihm & Banta, 1981; Webster et al., 2004). Without prior knowledge of the effect of each chemical in an artificial seawater medium on larval settlement and survivability, it may be most economic and advisable to use natural seawater for laboratory-guided settlement assays.

Regarding the duration of the settlement assay, Young (2009) suggested a 48-hour assay over 24 hours to observe clearly the effect of treatments on the settlement of larvae. The results from the present study demonstrated that larvae

need to be counted only after a 48-hour assay period. Since observing each settlement plate under the microscope and accounting for larval settlement and mortality involves between 10–12 minutes, the total duration of the assay period can last up to 60 hours. Any further delay after the 60 hours incubation may lead to false counting of larval mortality. Data on larval mortality can be especially crucial in assays in which the larvae are not fed. Quite often, when the settlement assay extends over a week, water is exchanged and larvae are fed to increase their survivability (Pechenik & Gee, 1993), but this practice may have other additional effects on settlement, which cannot be eliminated from the study. Therefore, for the static-water settlement assay, it is best to avoid feeding larvae, and to restrict settlement duration.

In summary, some key larval settlement parameters for settlement assays were examined in this study. However, the effect of several other parameters (i.e., effect of light, incubation temperature, density of larvae, pH and salinity of assay medium) on larval settlement could be investigated. The results from this study identified that the three substrates (agar, glass and polystyrene) can be effectively used as controls owing to the low levels of settlement and mortality observed across these plates. In addition, it was determined that sterilisation of natural seawater by filtration, autoclaving and/or both do not affect settlement or mortality and either can be effectively used as the assay medium. However, artificial and synthetic seawater need to be modified to suit *P. canaliculus* larvae, and to increase larval survival. Finally, the optimal duration for scoring larval settlement was found to be after a 48-hour period, but counting larval settlement may be carried up to 60 hours if required. Overall, the present study provided information to significantly improve *in-situ* settlement assays for *P. canaliculus* larvae. The protocols described here can be

easily achieved and reproduced to analyse the effect of different natural and/artificial cues on the settlement of larvae when using static-water settlement assays.

Chapter 3.

ISOLATION, IDENTIFICATION AND SCREENING OF CULTURABLE MONO-SPECIES MARINE BACTERIA FOR MUSSEL LARVAL SETTLEMENT

3. Isolation, Identification and Screening of Culturable Mono-Species Marine Bacteria for Mussel Larval Settlement

3.1 Abstract

The present study involved the isolation of fourteen marine bacteria from marine surfaces, and their screening for their ability to induce settlement of mussel larvae. Bacteria were isolated on marine agar and biochemically characterised using routine macroscopic (i.e., colony morphology), microscopic (Gram staining and motility) and biochemical (oxidase) tests. Biofilm cell suspensions of the bacterial groups were prepared for larval settlement assays. Of fourteen isolated bacteria, six were chosen for subsequent settlement tests. Results from the final screening demonstrated the ability of five mono-species marine bacteria to induce greater settlement over 40–60% settlement rates when compared with controls and one other bacterial species. The bacterial species with low settlement results also showed significantly greater mortality (over 70%) when compared with all other bacterial species and controls. Three out of the five best performing bacteria were chosen for more detailed settlement experiments. These bacterial species were identified as *Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1, respectively using 16 S rRNA sequence. These results indicated that the mussel, *P. canaliculus* larvae have the ability to distinguish their settlement substrata and settle differentially to cues from biofilms of mono-species marine bacteria.

3.2 Introduction

Bacteria are extremely successful prokaryotic organisms, which can survive and succeed in just about every environmental condition, including extreme geographical conditions (e.g., hydrothermal vents and deep ice). Owing to their abundance and unique survival strategies, these prokaryotic microbes are able to produce a range of biologically active substances and hence they often are commercially exploited. Examples of such resource utilisation include the production of antibiotics (Rahman et al., 2010), anti-cancer (El-Sersy et al., 2012) and anti-tumour (Chang et al., 2011) drugs for human therapeutics. From an aquaculture perspective, marine bacteria have been investigated for their probiotic (Kesarcodi-Watson et al., 2010), antifouling (Qian et al., 2007) and settlement inducing properties (Hadfield, 2011). Thus far, numerous studies have demonstrated the ability of single species of marine bacteria as potential inducers for settlement of larvae, such as spirobids (Kirchman et al., 1982a), bryozoans (Maki et al., 1989), hydroids (Leitz & Wagner, 1993), oysters (Fitt et al., 1990), polychaete (Harder et al., 2002; Lau et al., 2002; Lau & Qian, 2001), barnacles (Faimali et al., 2004; Khandeparker et al., 2006), sea urchins (Huggett et al., 2006), mussels (Alfaro et al., 2011b; Bao et al., 2007b; Ganesan et al., 2010) and corals (Negri et al., 2001; Tebben et al., 2011). These studies have a common theme of isolating mono-species marine bacteria from the wild to conduct laboratory-guided settlement assays. Therefore, to test the efficiency of mono-species marine bacteria, appropriate isolation and production of pure cultures is a prerequisite.

Ever since Zobell (1941) described a culturing medium for marine bacteria, the knowledge of culturable heterotrophic bacteria has been increasing

exponentially. Traditionally, isolation of pure cultures of bacteria have been conducted by physically separating mixed populations on agar plates, followed by sub-culturing until a single colony of mono-species bacteria can be obtained. A major drawback of this procedure is the inability of slow growing oligotrophs to compete with the fast growing individuals (Joint et al., 2010). Oligotrophic bacteria require nutrient deficient media to survive and multiply. For this reason, many studies have concentrated on developing suitable media to encourage growth of obligate oligotrophic and fastidious marine bacteria (Connon & Giovannoni, 2002; Joint et al., 2010; Schmidt & Konopka, 2009). Despite the advancement in technology, over 99.99% of marine bacteria have been estimated to be un-culturable to date (Ferguson et al., 1984; Schmidt & Konopka, 2009). Such discrepancies in the number of marine bacteria that can be cultured when compared with those occurring naturally were described as "the great plate count anomaly" by Staley & Konopka (1985). This anomaly has been resolved with the incorporation of high-throughput DNA sequencing to account for the un-culturable microbes in a habitat. Nonetheless, DNA sequencing may only aid in determining bacterial identity, and to conduct experiments (e.g., larval settlement assays) bacterial cultures are essential. Hence, to date conventional plating of marine bacteria has been regarded as the best isolation technique (Joint et al., 2010).

The isolation of mono-species marine bacteria is usually followed by their identification. Biochemical characterisation (i.e., indole, methyl red, Voges-Proskeur and citrate utilisation test) has been used in some studies to phenotypically categorise marine bacteria (Johnson et al., 1991a; Leitz & Wagner, 1993; Unabia & Hadfield, 1999). In addition, commercially available Analytical Profile Index (API) test kits

have been routinely incorporated for such identification purposes (Breschel & Singleton, 1992; Ivanova et al., 1999; Logan & Berkeley, 1984). The former test is usually prepared in the laboratory in volumes of over 5 ml, and the latter is pre-prepared in micro-quantities, which consumes less time. Regardless, neither of these techniques encourage growth of many marine bacteria and this impacts their identification (Joint et al., 2010). Moreover, API test kits were primarily designed to analyse terrestrial pathogens and hence not recommended for the analysis of most marine bacteria (Høvik Hansen & Sørheim, 1991). So far, 16 S rRNA sequencing of marine bacteria has been found reliable for their identification. For example, several marine bacteria belonging to the genus *Vibrio* (Kim & Jeong, 2001), *Pseudoalteromonas* (Holmström et al., 1998) and *Bacillus* (Ash et al., 1991) have been identified through this method but the ability to identify marine bacteria depends on existing databases containing either the phenotypic or genotypic characteristics of the exemplar microbe.

For the present study, mono-species marine bacteria were isolated from the wild and cultured on Zobell's marine agar 2216 (Zobell, 1941). No other cultural media were prepared or tested to encourage growth of new bacterial groups. This was mainly due to the priority of the study, which was to isolate bacteria for larval settlement and not to catalogue the diversity of marine bacteria. Once isolated, groups of bacteria were screened for their ability to induce larval settlement. Key bacterial species that modulated larval settlement alone were characterised further to obtain their identity. Initially, biochemical tests were conducted in an attempt to characterise them. Due to the inability of the biochemical analysis to reveal the identity of marine bacteria, was determined by sequencing the 16 S rRNA gene.

Therefore, the present study aimed to isolate, identify and screen mono-species marine bacteria for their ability to induce settlement of mussel larvae.

3.3 Materials and Methods

3.3.1 Sample collection

Seaweed and mussel samples were obtained on 8th May, 2008 from Muriwai Beach (36° 49' 0" South, 174° 27' 0" East), located on the west coast of North Island, New Zealand since this location has both seaweeds (macro-algae) and mussel populations (Hayward & Morley, 2004). Seaweeds that were present on the day of sampling and previously shown to induce larval settlement (Alfaro et al., 2006; Alfaro et al., 2011a) were chosen for this study. The rationale for choosing these samples was to obtain bacteria that could have emanated from the seaweed and retained in the seawater at the time of sampling. In-order to capture bacteria that could have entered mussel gut from seawater during bivalve-filtration process, mussels adjacent to seaweeds was also sampled. Thus, these samples cannot be linked to their source as the sampling was conducted only once during this study. Therefore, information present in Table, 3.2 may only provide some indication of the sample source and repeated sampling need to be conducted to confirm these results.

Seaweeds belonging to four different genera, including *Dictyota* sp., *Gigartina* sp., *Melanthalia* sp., and *Ulva* sp., green-lipped mussels [spat (3–6 mm), juvenile (15–20 mm) and adult (50–60 mm)], and seawater were collected for this study. All samples were placed in individual sterilised, 20 ml polystyrene container, placed in an ice box and maintained at 15–17°C during transport to the AUT's

Applied Sciences Laboratory. All samples were processed immediately to isolate culturable bacteria present on these surfaces.

3.3.2 Isolation of marine bacteria

Individual seaweed samples were placed in beakers containing 50 ml of autoclaved 0.9% saline (0.9 g of NaCl in 100ml of distilled water). The seaweed samples were washed several times to remove debris and particulate matter present on them. Once the seaweeds appeared visually clean, individual seaweed pieces were randomly dissected using a sterilised scalpel to achieve 7–10 mm sections. These sections were washed thrice with a total of 15 ml of sterile saline and transferred using sterile forceps onto a sterile Whatman® filter paper (Grade 1) to dry. Approximately 4–5 seaweed sections per seaweed species were placed on a single Zobell marine agar 2216 media (hereafter referred to as "marine agar"; refer Table 3.1 for composition and media preparation). The seaweed sections were gently pressed into the agar using sterile forceps to ensure that the sections did not move during the incubation period.

The byssal threads of mussels were removed, processed as described for seaweeds and cultured on marine agar. Using a sterile cotton swab saturated in autoclaved saline solution, the bacteria on the surface of all mussel samples were cultured onto marine agar. Individual mussels also were dissected to obtain gut contents, which were transferred onto marine agar using a swab. Since dissection of spat was not achievable due to their small size, the entire spat was crushed using a sterilised rod and mixed with 10 ml of saline. From this, 100 µl of the spat solution was also inoculated on marine agar using L-shaped glass rods by the spread-plate

method. Similarly, two 100 µl of seawater was also inoculated on two marine agar plates.

From each sample source, two replicate plates were cultured and subjected to 15 and 34°C for 24–48 hours to observe the differences in bacterial growth. Numerous isolated bacterial colonies were identified on each plate and differentiated based on the colony morphology (size, surface, texture, colour, and elevation). Bacteria subjected to two different temperatures (15 and 34°C) showed no difference in growth. Moreover, no new colonies were found after the first 24 hours of incubation. Therefore, the isolated colonies were sub-cultured on marine agar plates and incubated at 34°C for 24 hours. The pure culture plates were sealed using Parafilm M® and stored at 5°C for subsequent biochemical tests.

Bacterial cultures necessary for on-going settlement experiments were sub-cultured from stock cultures maintained at -20°C. To prepare stock cultures, pure colonies were cultured in 50 ml of Zobell marine broth medium containing 15% glycerol and incubated at 34°C for 24 hours. One ml of bacterial were aseptically transferred to 50 sterile Eppendorf vials and maintained at -80°C for prolonged storage.

Table 3.1: Chemical composition of Zobell marine agar 2216 to prepare 1l of the culture medium

Chemicals	**Amount required (g l^{-1})**	**Manufacturer**
Peptone	5.0	Bacto™ Peptone, BD, France
Yeast extract	1.0	Bacto™ Yeast extract, BD, France
Ferric citrate	0.1	BDH Ltd, Poole, England
Sodium chloride	19.45	Unilab, Australia
Magnesium chloride	8.8	BDH Ltd, Poole, England
Sodium sulphate	3.24	Scharlau, Spain
Calcium chloride	1.8	BDH Ltd, Poole, England
Potassium chloride	0.55	BDH Ltd, Poole, England
Sodium bicarbonate	0.16	BDH Ltd, Poole, England
Potassium bromide	0.08	BDH Ltd, Poole, England
Strontium chloride	0.034	BDH Ltd, Poole, England
Boric acid	0.0220	BDH Ltd, Poole, England
Sodium silicate	0.0040	Scharlau, Spain
Sodium fluoride	0.0024	BDH Ltd, Poole, England
Ammonium nitrate	0.0016	BDH Ltd, Poole, England
Disodium phosphate	0.0080	BDH Ltd, Poole, England
Agar	15.0	Lab chem., Ajax finechem. Pty. Ltd.

3.3.3 Identification of key mono-species marine bacteria

The fourteen pure culture isolates obtained from various sources (seaweed, seawater and mussels) were characterised based on their distinct colony morphology, such as colour, shape, and size. For identification purpose, these groups of bacteria were labelled as 'Bacterium' 1, 2, 3, up to Bacterium 14. Table 3.2 provides information on preliminary tests performed on the isolated colonies, including Gram staining, motility and oxidase tests that were performed to segregate these isolates into distinct groups. Key bacterial species that were chosen for the settlement experiments were subjected to biochemical and genetic identification tests. Initial biochemical analyses, such as Voges-Proskauer, citrate, triple sugar iron, starch hydrolysis and catalase tests were conducted on the three species of bacteria. Pre-

made media (Difco™, BD, U.S.A.) and reagents (Sigma-Aldrich®, U.S.A) were used to conduct these biochemical tests and the media were prepared in AFSW instead of distilled water. About 1–3 isolated colonies were inoculated in each of the biochemical media and incubated for 24 hours. After this period, the tubes were observed for biochemical changes against control tubes. For more in-depth biochemical tests, the analytical profile index (API) system was used to identify the bacteria to the lowest possible taxonomic level. For this purpose, bacteria were cultured on nutrient agar instead of marine agar. Of the three bacteria, only two species were able to grow on nutrient agar and hence subjected to biochemical analysis. The API® staph and 20E strips contained arrays of micro tubes with pre-made chemicals that were the substrates for the biochemical analyses. The analyses were conducted as per the instructions provided by the manufacturer (bioMérieux®, France).

In order to obtain more definitive taxonomic identification of the three selected bacterial strains, samples were submitted to Ecogene™ (Landcare Research, Auckland, New Zealand) for 16 S rRNA gene sequencing. The pure culture isolates on marine agar plates were submitted along with unique bacterial codes for each of the three bacterial isolates. At Ecogene™, the colonies were drawn from the culture plates and the bacterial genomic DNA was extracted. Complete (approximately 1.5 kb) sequencing of the gene were carried out and the sequence was amplified using the PCR technique (Forward primer: 27F 5′-AGAGTTTGATCMTGGCTCAG-3′, Reverse primer: 1525R 5′-AAGGAGGTGATCCAGCC-3′) and the resultant nucleotide sequences (1.5 kb) for the three bacteria were provided by Ecogene™. The homologous nucleotide sequence for each bacterium was identified in Genbank

(at the National Center for Biotechnology Information at www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search tool, BLASTn 2.2.22+ (Altschul et al., 1990; Zhang et al., 2000).

3.3.4 Preparation and enumeration of biofilm cell suspensions

Pure culture isolates from various sources (seaweed, seawater and mussels) were amplified by sub-culturing the bacteria. Log-phase cultures were chosen for the preparation of biofilm cell suspensions. The colonies from five replicate culture plates containing the single species of bacteria were transferred to 10 ml autoclaved seawater, which had been sterilised previously through a 0.45 µm membrane (AFSW). The suspensions in AFSW were briefly vortexed and centrifuged at 3000 × g for 10 minutes. The supernatants were discarded and the cells alone were transferred to 10 ml of AFSW. This procedure was followed thrice to remove any nutrients present in the suspensions. The final suspensions were mixed with 10 ml of AFSW resulting in a concentration of 10^6 to 10^7 cells ml^{-1}. The concentration of biofilm cell suspensions (BCx) were enumerated by serial dilution and 100 µl of the sample were cultured on marine agar. The concentration of cells was re-confirmed by checking for an optical density of 0.8 to 1.0 at a wavelength of 610 nm (Pharmacia biotech®, Ultra spec, model 21000). Only BCx with appropriate cell concentrations were used for the screening experiments.

3.3.5 Settlement assay

The settlement assays were conducted as described in chapter 2. All polystyrene Petri plates (16 × 14 mm) received 1 ml of seawater containing about 20–30 swimming larvae. The control plates contained larvae in 10 ml of AFSW and the treatment plates contained larvae in 9 ml of AFSW and 1 ml of biofilm cell

suspensions (BCx). The initial screening experiments (14 groups of bacteria) were conducted in duplicates, but subsequent screening (6 and 3 groups) of bacteria was conducted with replicates of 10 for each treatment and controls. After the 48 hour incubation period, the larvae were observed under a dissection microscope with 12 × magnification. Live larvae observed as attached to the bottom of the Petri plates were termed settled. However, settlement was always corroborated by placing a 200 µl pipette close to (~1 mm) larvae and gentle suction was applied. Larvae that resisted suction were termed settled and the larvae that were dislodged during the process were termed unsettled.

Mortality was recorded as described in chapter 2 by observing larval behaviour (movement of the velum, foot, or gut) and confirmed using vital stains. After 20 minutes of incubation, the larvae were observed under the dissection microscope at 20 × magnification. Stained larvae were recorded alive and unstained with no signs of movement were recorded dead.

3.3.6 Statistical analyses

All settlement and mortality data obtained were arcsine-transformed prior to statistical examination using the Predictive Analysis Software (PASW®) Statistics 18. Data that were tested for and met parametric assumptions were subjected to one-way Analysis of Variance (ANOVA) followed by *post-hoc* Tukey tests for pair-wise comparisons.

3.4 **Results**

3.4.1 Isolation and initial identification of mono-species marine bacteria

A total of 25 bacterial isolates were obtained from seaweed, seawater and mussels. With the help of preliminary, macroscopic, microscopic and biochemical examination, the 25 groups of bacteria were segregated into 14 distinct bacterial groups (Table 3.2). These groups were characterised based on the microscopic shape (i.e., cocci or bacilli) and their reaction to Gram's staining.

3.4.2 Biochemical and genetic identification of mono-species marine bacteria

Initial biochemical tests on the three bacteria used in the settlement assay were characterised as: a) Bacterium 3: Gram-positive, catalase positive, oxidase negative, non-motile coccus (collected from seawater); b) Bacterium 12: Gram-positive, catalase negative, oxidase positive, non-motile bacillus (collected from mussel guts); and c) Bacterium 7: Gram-negative, catalase negative, oxidase positive, halophilic, motile bacillus (collected from the green alga, *Ulva lactuca*). Furthermore, the Voges-Proskauer test showed positive for bacterium 3 and 12 and not for bacterium 7. Similarly, results from the TSI test indicated that bacterium 3 and 12 were able to ferment glucose and not lactose or sucrose, whereas bacterium 7 was unable to utilise the three sugars (glucose, sucrose and lactose) to obtain energy. Sodium citrate was not utilised as the sole carbon source by any of the bacteria as demonstrated by the citrate test. Unlike the other two bacteria, bacterium 12 was able to hydrolyse starch.

Further biochemical tests were conducted on API® staph and 20E strips for the three bacteria. Unlike bacterium 7, bacteria 3 and 12 were able to grow on nutrient agar without the addition of salt. Therefore, these tests were conducted only

on the two species of bacteria. Due to lack of an appropriate database for marine bacteria, the biochemical test results could not be compared for these species of bacteria, but have been catalogued for future reference (Tables 3.3 & 3.4).

The results for the BLASTn analyses for the three bacterial strains revealed that the sequence of the Gram-positive cocci (Bacterium 3) was 99% similar with an uncultured bacterium (FM872860) and *Macrococcus caseolyticus* JCSC5402 (AP009484), and was reported as *Macrococcus* sp. AMGM1. The Gram-positive rod (Bacterium 12) had a sequence which was 100% similar to *Bacillus thuringiensis* serovar berliner (EU429665) and *Bacillus cereus* G9842 (CP001186). As a result of this ambiguity in species identification for this bacterium, it is reported as *Bacillus* sp. AMGB1. The Gram-negative motile rods (Bacterium 7) had a 99% similarity with *Pseudoalteromonas* sp. BSw20001 (GenBank Accession no. EU365590), and *Pseudoalteromonas* sp. ArcB8453012 (GU120031) and reported as *Pseudoalteromonas* sp. AMGP1. Finally, the 16 S rRNA sequences (Table 3.5) were submitted to GenBank (BankIt) to receive an accession number for future references. The unique accession numbers provided by the Genbank were, GU322006 (*Macrococcus* sp. AMGM1) GU322007 (*Bacillus* sp. AMGB1) and GU322008 (*Pseudoalteromonas* sp. AMGP1).

Table 3.2: Preliminary isolation and characterisation of marine bacteria

Bacteria	Sample source	Macroscopic examination (Colony morphology)					Microscopic examination			Biochemical examination
		Shape	Size (ø in mm)	Surface and texture	Colour and optical characteristics	Elevation	Margin	Shape	Reaction to Gram staining	Motility test	Oxidase test
Bacterium 1	*Melanthalia* sp., *Gigartina* sp., *Dictyota* sp., byssal thread, and adult mussel (surface)	Circular	Punctiform, (0.5–1)	Smooth and moist	Yellow and translucent	Convex	Entire	Cocci	+	NM	-
Bacterium 2	Juvenile mussel (gut)	Irregular	Large (4–5)	Glistening and mucoid	Dirty-white and opaque	Raised	Undulate	Cocci	+	NM	-
Bacterium 3	Seawater	Circular	Punctiform (0.5–1)	Smooth and moist	White and translucent	Raised	Entire	Cocci	+	NM	-
Bacterium 4	*Gigartina* sp., seawater, juvenile and adult mussel (gut)	Circular	Medium (~ 3)	Smooth and moist	White and translucent	Umbonate	Entire	Bacilli	-	M	+
Bacterium 5	Juvenile mussel (gut)	Circular	Small (1.5–2)	Smooth and moist	Peach and opaque	Raised	Entire	Bacilli	-	M	+
Bacterium 6	Mussel spat	Circular	Medium (2–2.5)	Glistening and butyrous	Dull-white and translucent	Raised	Entire	Bacilli	-	M	+
Bacterium 7	*Ulva* sp.	Circular	Small (1.5–2)	Glistening and moist	Yellowish-white and translucent	Raised	Entire	Bacilli	-	M	+
Bacterium 8	Byssal thread	Circular	Small (1.5–2)	Smooth and moist	Orange and opaque	Raised	Entire	Bacilli	-	M	-
Bacterium 9	Adult mussel (gut)	Circular	Medium (2–2.5)	Smooth and moist	Yellowish-white and opaque	Raised	Entire	Bacilli	-	M	-
Bacterium 10	Adult mussel (gut)	Circular	Medium (2–2.5)	Smooth and moist	Yellow and opaque	Convex	Entire	Bacilli	-	M	-
Bacterium 11	Byssal thread	Circular	Small (1.5–2)	Glistening and moist	White and opaque	Raised	Entire	Bacilli	+	NM	+
Bacterium 12	Juvenile mussel (gut)	Irregular	Medium (2–2.5)	Dull and moist	White and opaque	Flat	Undulate	Bacilli	+	NM	-
Bacterium 13	Mussel spat	Irregular	Large (3–5)	Wrinkled and dry	Off-white and opaque	Flat	Undulate	Bacilli	+	NM	-
Bacterium 14	Adult mussel (surface and gut)	Circular	Medium (2–2.5)	Glistening and mucoid	White with red centre and translucent	Umbonate	Entire	Bacilli	+	NM	-

Table 3.3: Catalogue of biochemical test results obtained from API® staph for two species of marine bacteria.

Biochemical tests (API® staph)	**Bacterium 3** (***Macrococcus* sp. AMGM1**)	**Bacterium 12** (***Bacillus* sp. AMGB1**)
Fermentation/oxidation of D-glucose	+	+
Fermentation/oxidation of D-fructose	+	+
Fermentation/oxidation of D-mannose	+	-
Fermentation/oxidation of maltose	+	+
Fermentation/oxidation of lactose	-	-
Fermentation/oxidation of D-trehalose	+	+
Fermentation/oxidation of D-mannitol	±	-
Fermentation/oxidation of xylitol	-	-
Fermentation/oxidation of D-melibiose	-	-
Nitrate reduction	+	+
Alkaline phosphatase test	+	-
Voges Proskauer	+	+
Fermentation/oxidation of raffinose	-	-
Fermentation/oxidation of xylose	-	-
Fermentation/oxidation of sucrose	±	-
Carbohydrate utilisation	-	-
N-acetyl-glucosamine test	-	+
Arginine dihydrolase reaction	-	+
Urease	-	±

Table 3.4: Catalogue of biochemical test results obtained from API® 20 E for two species of marine bacteria.

Biochemical tests API ® 20 E	**Bacterium 3** (***Macrococcus* sp. AMGM1**)	**Bacterium 12** (***Bacillus* sp. AMGB1**)
Fermentation/oxidation of D-glucose	+	+
Fermentation/oxidation of D-fructose	+	+
Fermentation/oxidation of D-mannose	+	-
Fermentation/oxidation of maltose	+	+
Fermentation/oxidation of lactose	-	-
Fermentation/oxidation of D-trehalose	+	+
Fermentation/oxidation of D-mannitol	±	-
Fermentation/oxidation of xylitol	-	-
Fermentation/oxidation of D-melibiose	-	-
Nitrate reduction	+	+
Alkaline phosphatase test	+	-
Voges Proskauer	+	+
Fermentation/oxidation of raffinose	-	-
Fermentation/oxidation of xylose	-	-
Fermentation/oxidation of sucrose	±	-
Carbohydrate utilisation	-	-
N-acetyl-glucosamine test	-	+
Arginine dihydrolase reaction	-	+
Urease	-	±

Table 3.5: The 1.5 kilo base pair (kb) data from partial sequence of 16SrRNA for three species of marine bacteria.

Bacteria	**Partial sequencing of 16S ribosomal RNA (1.5 kb)**
Bacterium 3 **(*Macrococcus* sp. AMGM1)**	CTAATACATGCAAGTCGAGCGAATTGACAGAGGTGCTTGCACCTCTCGATTTTAGCGGCGGACGGGTGAGTAAC ACGTGGGTAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAATATTTAGCTTCG CATGAAGCAATAGTGAAAGACGGTTCTGCTGTCACTTATAGATGGACCTGCGGTGTATTAGCTAGTTGGTGAGGT AACGGCTCACCAAGGCAACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCC CAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGA GTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTAAGGGAAGAACAAGTACGTTAGTAACTGAACGTACCTTG ACGGTACCTTACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATC CGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTCTCTTAAGTCTGATGTGAAAGCCCCCGGCTCACCCGGGGA GGGTCATTGGAAACTGGGAGACTTGAGTACAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGCA GAGATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGTGCGAAAGCGTGGG GATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGGGGGTTTCCGCCC CTCAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTG ACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACA TCCTTTGACAACTCTAGAGATAGAGCTTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGC TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCTTTAGTTGCCATCATTAAGTTGGGC ACTCTAGAGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTT GGGCTACACACGTGCTACAATGGATGGTACAAAGGGCAGCAAAACCGCGAGGTCAAGCAAATCCCATAAAACC ATTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCT ACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGCCGGTG GAGTAACCTTTTAGGAGCTAGCCGTCGAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTAT CGGAA
Bacterium 12 **(*Bacillus* sp. AMGB1)**	CCTAATACATGCAAGTCGAGCGAATGGATTGAGAGCTTGCTCTCAAGAAGTTAGCGGCGGACGGGTGAGTAACA CGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACTGC ATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGT AACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCC CAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGA GTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTT GACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTAT CCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGG AGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGT AGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGG GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCC CTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATT GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGAC ATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGC TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGC ACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCT GGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACC GTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCC GCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTG GGGTAACCTTTTTGGAGCCAGCCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGTAT CGGAA
Bacterium 7 **(*Pseudoalteromonas* sp. AMGP1)**	GCCTAACACATGCAAGTCGAGCGGTAACAGAAAGTAGCTTGCTACTTTGCTGACGAGCGGCGGACGGGTGAGTA ATGCTTGGGAACATGCCTTGAGGTGGGGGACAACAGTTGGAAACGACTGCTAATACCGCATAACGTCTACGGAC CAAAGGGGGCTTCGGCTCTCGCCTTTAGATTGGCCCAAGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCA AGGCAACGATCCCTAGCTGGTTTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG GAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCT TCGGGTTGTAAAGCACTTTCAGTCAGGAGGAAAGGTTAGTAGTTAATACCTGCTAGCTGTGACGTTACTGACAG AAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGG GCGTAAAGCGTACGCAGGCGGTTTGTTAAGCGAGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATTTCGA ACTGGCAAACTAGAGTGTGATAGAGGGTGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGG AATACCGATGGCGAAGGCAGCCACCTGGGTCAACACTGACGCTCATGTACGAAAGCGTGGGGAGCAAACAGGA TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTAGAAGCTCGGAGCCTCGGCTCTGTTTTTCAAAGCT AACGCATTAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCAC AAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACACTTGACATACAGAGAACTTAC CAGAGATGGTTTGGTGCCTTCGGGAACTCTGATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAGATGT TGGGTTAAGTCCCGCAACGAGCGCAACCCCTATCCTTAGTTGCTAGCAGGTAATGCTGAGAAACTCTAAGGAGAC TGCCGGTGATAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGTGTAGGGCTACACACGT GCTACAATGGCGCATACAGAGTGCTGCGAACTCGCGAGAGTAAGCGAATCACTTAAAGTGCGTCGTAGTCCGGA TTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGTATCAGAATGACGCGGTGAATACGT TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAGTAGATAGTCTAACCCTCGG GAGGACGTTTACCACGGAGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCCTAGGGGA

3.4.3 Initial screening of 14 marine bacteria for larval settlement

Results from the initial screening experiments suggested that bacterium 1, 2, 3, 4 and 12 induced greater larval settlement levels when compared with other bacterial groups and sterile controls (Fig. 3.1 A; ANOVA; $F_{14,15} = 285.8$, $p < 0.001$). Individual Tukey tests revealed non-significant ($p > 0.001$) differences between bacterium 1 when compared with bacterium 2, 3, 4 and 12. Conversely, bacterium 7 induced settlement at a much lower level than the other bacterial groups and almost equal to the control group. In addition, *post-hoc* Tukey tests showed significant ($p < 0.001$) differences between bacterium 7 and the remaining thirteen groups of bacteria, except controls. Moreover, bacterium 7 induced significantly greater larval mortality (> 70%) when compared with other bacteria and controls (Fig. 3.1 B; ANOVA; $F_{14,15} = 225.3$, $p < 0.001$). Tukey tests revealed significant ($p < 0.001$) differences between bacterium 7 and all treatments and controls. While a one-way ANOVA suggested that there were significant differences between treatments, these results may only provide some indication of the settlement inducing properties of the fourteen bacteria tested in this study. Due to the low number of replicates per treatment group, the normality of these samples cannot be reliably established. This was resolved by increasing the number of replicates per sample ($n = 10$) in the final screening assay. Thus, based on their properties, bacterium 1, 2, 3, 4, 7 and 12, were chosen to understand their ability to induce or inhibit larval settlement.

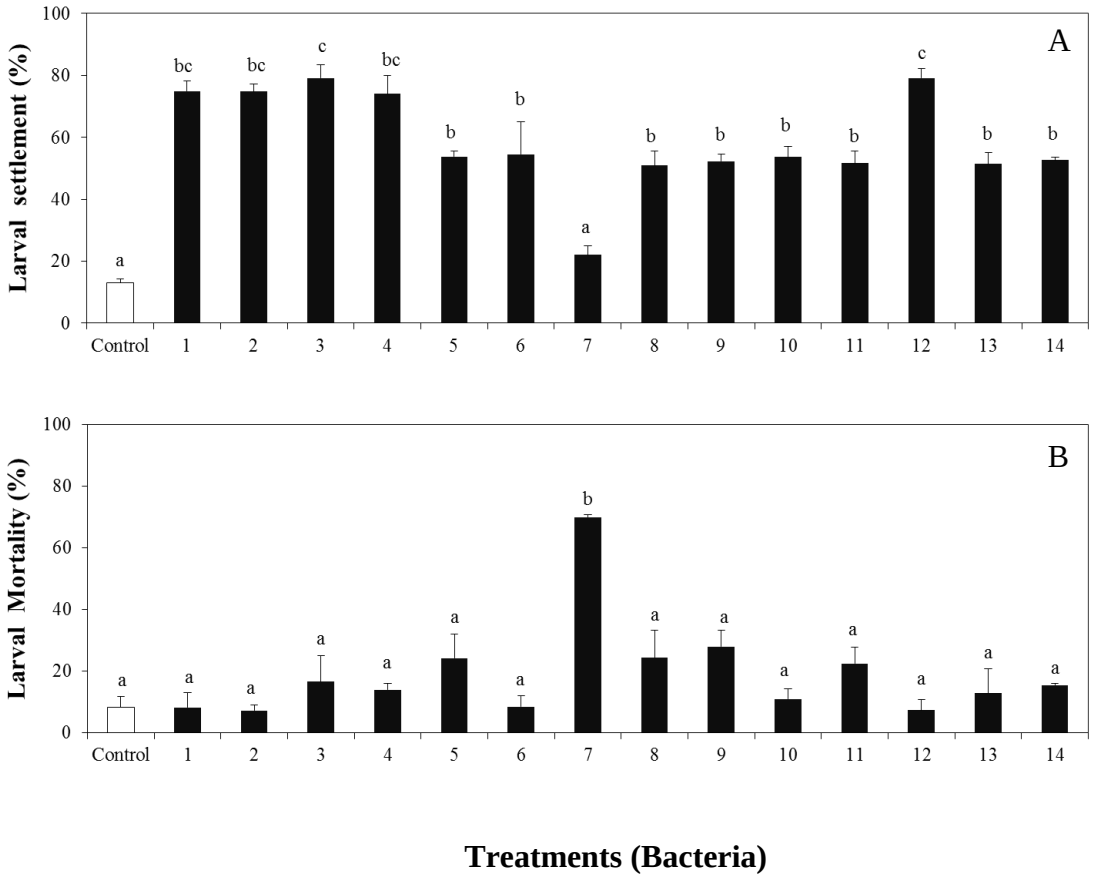

Figure 3.1: Effect of biofilm cell suspensions (BCx) of fourteen bacteria on mussel larvae. Data represent mean (±SE, n = 2) larval settlement (A) and mortality (B) of *P. canaliculus* after 48 hours on polystyrene Petri plates containing BCx of Bacterium 1–14 represented as numerals (1, 2, 3 etc.). The open bars represent sterile control plates and shaded bars represent the treatment plates. Non-significant Tukey tests within are denoted by the same letter over the bars.

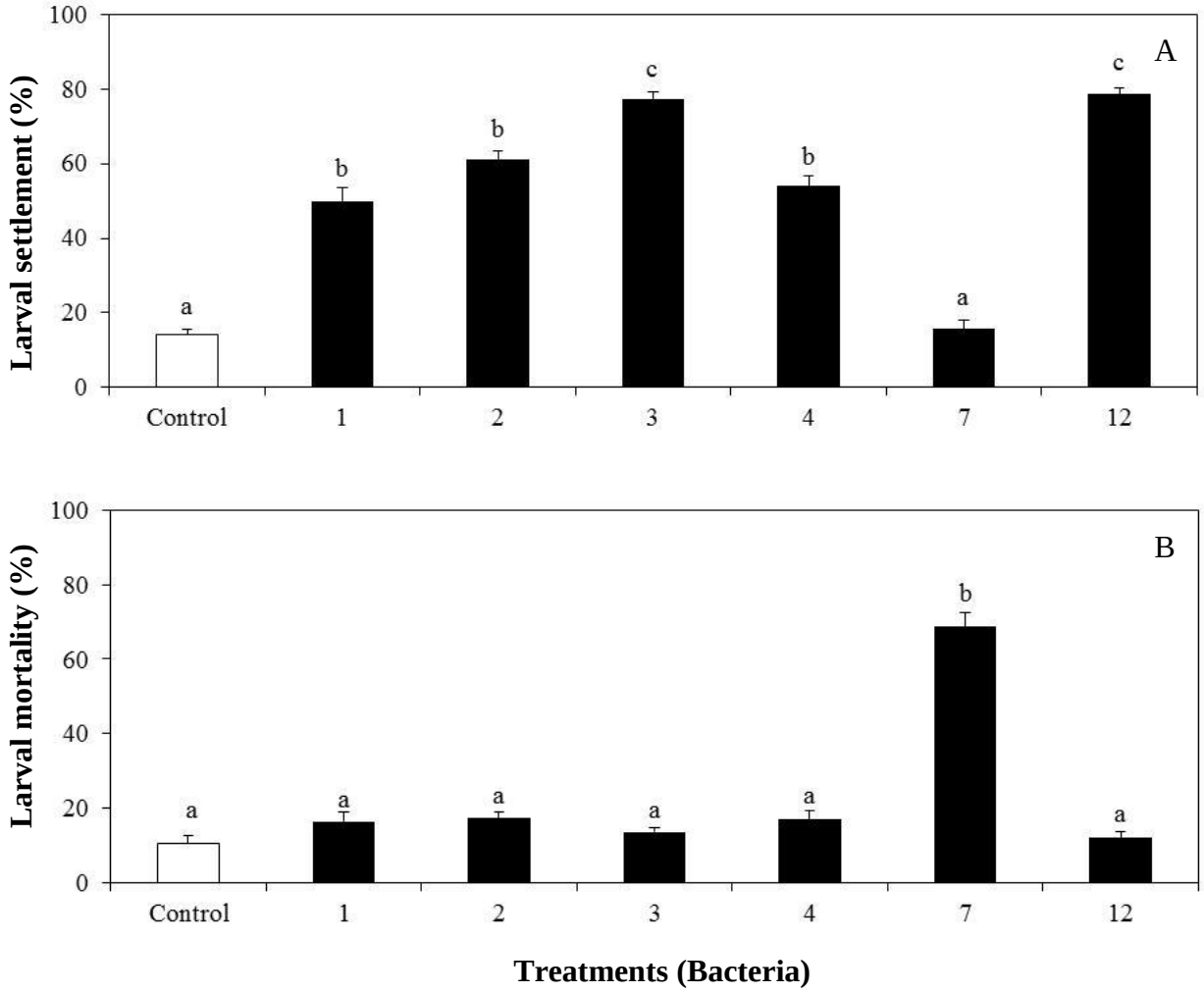

Figure 3.2: Effect of biofilm cell suspensions (BCx) of six bacteria on mussel larvae. Data represent Mean (±SE, n = 10) larval settlement (A) and mortality (B) of *P. canaliculus* after 48 hours on polystyrene Petri plates containing BCx of Bacterium 1, 2, 3, 4, 7 and 12 represented as numbers. The open bars represent sterile control plates and shaded bars represent the treatment plates. Non-significant Tukey tests within are denoted by the same letter over the bars.

3.4.4 Final screening of 6 marine bacteria for larval settlement

The results from the final screening of six bacteria clearly indicated that bacterium 3 and 12 significantly induced greater settlement compared with other bacteria (1, 2, 4 and 7) and sterile controls (Fig. 3.2 A; ANOVA; $F_{6,63} = 106.2$, $p < 0.001$). Also, bacterium 1, 2 and 4 significantly ($p < 0.001$) induced greater

settlement (40–60%) compared with the lower settlement (< 20%) observed in bacterium 7 and controls. Individual Tukey *post-hoc* tests revealed non-significant ($p > 0.001$) differences between bacterium 3 and 12 and also between bacterium 7 and controls. These results indicate that bacterium 3 and 12 were the best settlement inducers, and bacterium 7 induced the lower settlement values.

Conversely, bacterium 7 induced greater mortality (mean% ± S.E; 68.8 ± 3.6) when compared with the significantly lower mortality of between 10–20% in all other treatment and control plates (Fig. 3.2 B; $F_{6,63} = 54.4$, $p < 0.001$). In addition, Tukey tests revealed non-significant difference ($p > 0.001$) between all treatment and control pairs indicating that this bacterium had the unique ability to produce toxins for mussel larvae.

3.5 **Discussion**

In this study, mono-species bacterial biofilms were shown to differentially induce settlement of mussel *P. canaliculus* larvae. Of the fourteen groups of bacteria tested, two species of bacteria (Bacterium 3 and 12) induced significantly greater larval settlement (over 60%) when compared with the controls. Conversely, bacterium 7 induced only up to 20% larval settlement, quite similar to controls. Yet this bacterium induced greater rates of larval mortality (over 60%) when compared with other bacteria and sterile controls. These results indicated that *P. canaliculus* were able to differentiate cues from mono-species biofilms of bacteria. Similar results have been reported by Lau et al. (2002) for the settlement of polychaetes, *Hydroides elegans* larvae in response to mono-species marine bacteria isolated from Hong Kong waters. Those results indicated that some bacteria (e.g., *Vibrio, Staphylococcus, Micrococcus, Alteromonas*) isolated from the same source were able

to induce greater (> 50%) settlement compared with other bacteria (e.g., *Vibrio, Bacillus, Micrococcus, Cytophaga*), which induced lower (< 20%) settlement. The differences in settlement patterns could be due to the ability of bacteria on a substrata to actively induce on specific settlement substrates to perhaps avoid competition for nutrition and space (Hadfield, 2011). Thus, most single species of bacterial biofilm do not universally induce settlement of all invertebrate larvae, and specific bacteria need to be isolated and screened for larval settlement induction purposes.

Of the fourteen culturable bacteria described in this study, 57% belonged to the Gram-negative group and the remaining (43%) belonged to the Gram-positive group (Table 2). The proportion of Gram-positive to Gram-negative marine bacteria obtained in this study was almost equal. However, Zobell (1946) stated that 95% of marine bacteria are Gram-negative and conversely, Jensen & Fenical (1995) pointed the relative abundance of Gram-positive bacteria in their marine samples. These facts may be true for only culturable bacteria isolated by traditional culturing techniques and not for the overall population of bacteria present in the sea, as over 99.99% of marine bacteria are still considered un-culturable (Joint et al., 2010). Moreover, only a few thousand bacterial species have been verified and appropriately described to date (Achtman & Wagner, 2008; Schmidt & Konopka, 2009). The lack of information in the existing databases on marine microbes may also explain why the three key bacterial species isolated in this study (*Macrococcus*, *Bacillus* and *Pseudoalteromonas*) were classified only up to their genus level.

Thus far, the genus *Macrococcus* has been tested only for the settlement of mussel species (Alfaro et al., 2011b; Ganesan et al., 2010). Species of *Macrococcus* have not been tested with any other invertebrate larvae despite their occurrence

among marine microbial populations (Lauzon et al., 2010; Tanaka et al., 2010). A plausible reason for *Macrococcus* not having been studies for larval settlement could be due to this bacterium being classified into its own genus relatively recently (Kloos et al., 1998), which would limit the availability of information for this bacteria on larval settlement. Unlike *Macrococcus*, several species of *Bacillus* have been routinely used in a settlement assays, possibly due to their abundance in marine microbiota (Gontang et al., 2000; Jensen & Fenical, 1995; Siefert et al., 2000). Species of *Bacillus* also have been shown to display an array of contrasting properties for the settlement of invertebrate larvae. For example, the biofilms of *Bacillus* sp. AMGB1 were able to induce greater settlement for *P. canaliculus* larvae (Ganesan et al., 2010) but achieved less than 20% settlement for the ribbed mussel (*Aulacomya maoriana*) larvae (Alfaro et al., 2011b). Moreover a strain of *B. amyloliquefaciens* has been shown to display anti-larval properties against the bryozoan *Bugula neritina* (Gao et al., 2010). These studies clearly indicate that culturable heterotrophic bacteria may belong to a variety of genera, and their settlement inducing properties may not relate to their phylogeny (Lau et al., 2002; Tran & Hadfield, 2011). Similarly, the biofilm of *Pseudoalteromonas* sp. AMGP1 that induced greater larval mortality in this study, did not induce mortality of ribbed mussel, *A. maoriana* larvae (Alfaro et al., 2011b; Ganesan et al., 2010). Due to the differences in chemo-sensory pathways across different species of invertebrate larvae, responses to bacterial cues may not be the same (Hadfield, 2011). Hence, it is essential to isolate, screen and identify specific bacteria preferably from the microbiota of the larvae to achieve optimum results.

In summary, the present study focused on culturing marine bacteria to enhance settlement of *P. canaliculus* larvae through laboratory assays. The study indicated that these larvae were able to choose substrates for settlement and actively responded to bacterial cues. Moreover, the techniques used in this study would be a prototype for isolating, identifying and characterising bacterial settlement cues for other invertebrate larvae. The next step would be to analyse the activity of these selected microbes on different settlement substrata to ensure that the cue for larval settlement originated from bacteria and not the substrata.

Chapter 4.

THE ROLE OF SUBSTRATE PHYSICAL PROPERTIES IN MODULATING BACTERIAL CUES FOR THE SETTLEMENT OF MUSSEL LARVAE

4. The Role of Substrate Physical Properties in Modulating Bacterial Cues for the Settlement of Mussel Larvae

4.1 Abstract

The physical properties of substrates in regulating bacterial cues for the settlement of mussel, *Perna canaliculus* larvae were analysed in this study. Biofilm cell-suspensions (BCx) of fourteen mono-species bacteria were subjected to a total of four treatment methods, including BCx on Petri plates, noble agar, glass slides and noble agar wells. From the fourteen mono-species bacteria, three species (*Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1) were selected and tested with two additional methods, which included BCx on noble agar with a top layer of sterile agar and noble agar wells with a top layer of sterile agar. The effects of chemicals exuded by BCx for each bacterial species were analysed across all substrate treatments. Results obtained from this study showed that BCx of nine out of fourteen mono-species bacteria induced uniform larval settlement rates on all substrates. Conversely, BCx from three bacterial species resulted in significantly greater larval settlement on Petri plates and glass slides when compared with noble agar and noble agar well, indicating that these bacteria are unable to release cues contained in an inert noble agar matrix. In addition, BCx of another bacterial species significantly enhanced (up to 60% induction) larval settlement on Petri plates compared with glass and noble agar plates, which achieved < 30% larval settlement. BCx of a bacteria was able to induce up to 70% settlement on noble agar and lowest (< 50%) on noble agar wells. These results highlighted that some species of marine bacteria may interact with physical cues of the substrata and alter the ability of chemicals to induce mussel larval settlement. Furthermore, three

key mono-species bacteria (*Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1) induced similar settlement percentages across all substrates, asserting the role of bacterial cues for mussel larval settlement. Finally, results from the present study suggested that the physical properties of the substrata, as well as the chemical properties of the bacterial biofilms on these substrates are important variables in the larval settlement process.

4.2 Introduction

The most important process in the life cycle of planktonic invertebrates is the identification of optimal settlement substrates in order to successfully undergo settlement. As settlement is a precursor to metamorphosis and adulthood, larvae need to precisely respond to appropriate settlement cues to undergo this crucial transformation. Certainly, physical cues (e.g., light, temperature, flow, salinity and depth) have been shown to mediate settlement for a number of invertebrate larvae, such as oysters (Turner et al., 1994), sponges (Maldonado & Young, 1996), corals (Mundy & Babcock, 1998), barnacles (Olivier et al., 2000) and mussels (Dobretsov & Miron, 2001). In addition, physicochemical properties of the settlement substrates, such as surface colour (Su et al., 2007), texture (Holmes et al., 1997), orientation (Glasby, 2000), wettability (Rittschof & Costlow, 1989) and charge (Petrone et al., 2011) have been shown to influence larval settlement. For instance, cyprids of the barnacle, *Balanus improvisus* preferentially settle on to low wettable polystyrene plates, whereas, *B. amphitrite* prefers high wettable glass surfaces (Dahlström et al., 2004; O'Connor & Richardson, 1994). Conversely, both high and low wettable surfaces have been found to equally attract polychaetes, (*Hydroides elegans*) larvae

(Huggett et al., 2009). However, the mechanism by which these larvae explore the wettabilities of their settling substrata remains largely unknown.

Interestingly, surface physical properties (e.g., topography and wettability) can also affect biofilm composition (Dexter et al., 1975; Pedersen et al., 1986), which may subsequently influence larval settlement (Faimali et al., 2004; Huggett et al., 2009; Maki et al., 1989). Moreover, the structures of certain macroscopically-rough substrates (i.e. rocks) contain cavities large enough to accommodate both bacteria as well as larvae (Holmes et al., 1997; Hutchinson et al., 2006; Su et al., 2007). Preferences to hide in voids of rough substrates may shelter these organisms from hydrodynamic forces and prevent them from shearing (Carl et al., 2012; Donlan, 2002). While this rugophilic behaviour has been well-documented (Holmes et al., 1997; Su et al., 2007; Wethey, 1986), it still remains unclear if the physical cues of the substrate discretely or in combination with their associated biofilms influence larval settlement. This question remains unanswered, especially for field-based studies, when settlement inducing cues (i.e., physical and bacterial) are inseparable. For instance, in a field experiment by Faimali et al., (2004), cyprids of barnacle *B. amphitrite* were shown to preferentially settle on topographically uneven quartz plates containing greater diversity of diatom and bacterial biofilms than even-surfaced glass substrates which contained only bacterial biofilms. Since the research was conducted in the field; biofilms may have interacted with other extraneous factors (e.g., light, temperature, and habitat) that could have potentially affected those results. The effects of such extrinsic factors on biofilm make-up and their combined consequences on larval settlement have been extensively studied (Dobretsov & Qian, 2006; Huang & Hadfield, 2003; Lau et al., 2005; Qian &

Dahms, 2009). To eliminate such confounding results from field studies, some researchers have focused on conducting *in-vitro* larval settlement assays on a variety of substrates with known physical properties and exposing them to laboratory grown mono-species bacteria (Maki et al., 2000; Maki et al., 1989; Neal & Yule, 1994). For example, O'Connor & Richardson (1996) clearly showed differences in attachment patterns for barnacle (*Balanus improvisus*) larvae across two routinely used settlement substrates. Polystyrene substrates containing biofilms of *Deleya marina* induced up to 40% larval settlement compared with biofilms on glass vials that achieved < 20% settlement. Results from such experiments clearly indicate that settlement substrates may have a potential role in modulating the effect of mono-species bacteria cues on larval settlement. Thus, the effects of physical and biological cues need to be investigated individually and in combination to identify their roles in larval settlement of marine invertebrates.

For the New Zealand green-lipped mussel (*P. canaliculus*), physical substrate properties such as surface charge and topography have been shown to influence settlement of larvae and juveniles (Alfaro & Jeffs, 2002, 2003; Alfaro et al., 2004; Buchanan & Babcock, 1997; Gribben et al., 2011; Young, 2009). Moreover, the effect of less-textured substrates (i.e., glass, noble agar and polystyrene) with varying wettabilities revealed no differences in larval settlement (chapter 2). However, the effect of mono-species bacteria on these substrates and their combined effect on larval settlement were not investigated until now. For this purpose, three settlement substrates, namely polystyrene Petri plates, microscope glass slides and noble agar on plates were chosen to test for their ability to modulate bacterial cues for mussel larval settlement. Results from this study may indicate the nature of the

inductive cue, whether cell surface-bound and water soluble (cells on Petri plates and glass slides) or only water soluble (cells encompassed in noble agar). Finally, it was essential to identify if three key mono-species marine bacteria (*Macrococcus* sp., *Bacillus* sp. and *Pseudoalteromonas* sp.) induced similar larval settlement percentages, regardless of the substrate physical properties. This information was used as a key criterion for choosing bacterial biofilms for subsequent studies. Moreover, if a biofilm cue displayed the capacity to induce effectively despite changes to substrate physical properties; it could potentially be used on any commercial substrate (e.g., ropes) to increase larval settlement and to improve mussel seed production.

4.3 Materials and Methods

Three settlement substrates (polystyrene Petri plates, microscope glass slides and noble agar) that were previously examined without biofilms (chapter 2) were chosen for this study. All substrates accommodated 1 ml of biofilm cell suspensions (BCx) containing 10^6 to 10^7 cells ml^{-1}. Preparation of BCx from mono-species bacteria was as described in chapter 3. Briefly, biofilm cells were withdrawn from five replicate culture plates containing mono-species bacterial colonies grown on marine agar plates. The bacterial cultures were pooled in 10 ml of autoclaved filtered seawater (AFSW), washed thrice with a total of 30 ml AFSW and finally re-suspended in 10 ml of AFSW, resulting in a concentration of 10^6 to 10^7 cells ml^{-1}. Once the required BCx concentration was achieved, they were added to the respective settlement substrates.

The settlement experiments were conducted in large polystyrene Petri plates (90 mm diameter × 15 mm depth) that accommodated BCx and the settlement

substrates (i.e., noble agar and glass slides). The methodology for the preparation of the settlement substrates containing mono-species BCx is explained below (Fig. 4.1). AFSW was used instead of BCx as controls in this study.

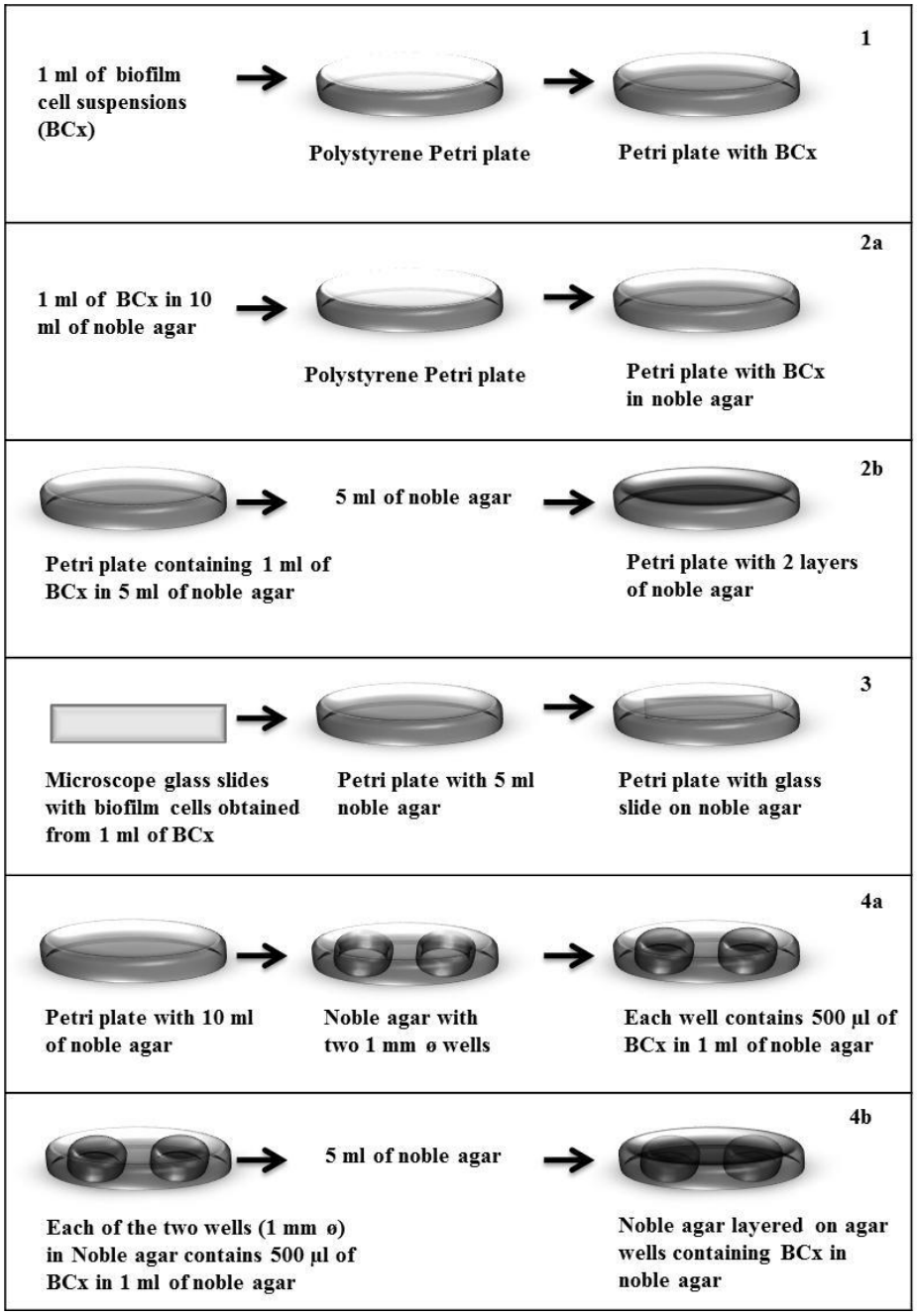

Figure 4.1: Details of the procedure for the preparation of settlement substrates with mono-species biofilm cell suspensions (BCx) on polystyrene Petri plate (1), noble agar (2a & b), microscope glass slides (3) and noble agar wells (4a & b).

4.3.1 Substrate 2a. BCx in noble agar

To mimic conditions in the wild (e.g., bacteria on seaweed surfaces) and to avoid direct interaction of bacterial cells with larvae, BCx in noble agar was prepared. For this, 1 ml of BCx (10^6 to 10^7 cells ml^{-1}) was added to the Petri plate along with 10 ml of 1.5% noble agar maintained at 45–50°C. BCx in noble agar were mixed gently by swirling the plate clockwise and counter-clockwise three times to achieve an even suspension of the cells. The agar was cooled down in order to solidify the material prior to use in the settlement assays.

4.3.2 Substrate 2b. BCx in noble agar (sandwiched)

Preparation of substrate 2b was a continuation of the substrate 2a to completely ensure that larvae did not interact with the cells of BCx during the settlement assay. Instead of 10 ml of noble agar, 1ml of BCx (10^6 to 10^7 cells ml^{-1}) was added to 5 ml of 1.5% noble agar. Once the agar cooled, another layer of noble agar (5 ml of 1.5%) was added to this layer. The two layers of noble agar containing BCx were left undisturbed until the agar firmed up.

4.3.3 Substrate 3. BCx on glass slides

Biofilms were grown on microscope glass slides (76 mm length × 26 mm width) by inoculating with respective mono-species BCx. Hence, 1 ml of mono-species BCx (10^6 to 10^7 cells ml^{-1}) was added to sterile polypropylene Coplin jars containing 50 ml of 0.5% peptone in AFSW. Each Coplin jar accommodated a maximum of five glass slides when vertically placed in the jar. The jars containing glass slides were incubated on a rotary incubator set to 100 rpm at 34°C for 24 hours. After the incubation period, all slides were removed and washed thrice with a total of 30 ml of AFSW. Biofilms from one side of the slide were removed by gently wiping

the slide with absolute ethanol (99%). The side that contained mono-species biofilm was placed facing up and mounted on the Petri plate containing 5 ml of pre-solidified noble agar. The slide was gently depressed on the agar to ensure that the agar completely sealed the bottom surface. The noble agar containing the glass slide was allowed to set prior to their use in the settlement assay.

4.3.4 Substrate 4a. BCx in noble agar wells

Petri plates containing 10 ml of solidified noble agar were used and 2 wells (1 mm in diameter) were created using a sterile stainless-steel cork borer. Each well received 1 ml of noble agar (1.5% at 50°C) containing 500 µl of BCx (10^6 to 10^7 cells ml^{-1}) and the wells were left to solidify. This substrate was used to test if larvae settled on the wells containing BCx or surrounding areas without BCx.

4.3.5 Substrate 4b. BCx in noble agar wells (sandwiched)

These substrates were prepared as in 4a, but instead plates containing 5 ml of noble agar containing wells with BCx were prepared. Once the two wells containing BCx solidified, another layer of noble agar (5 ml of 1.5%) was added to the plates and left undisturbed to cool down. This technique ensured that larvae were not in direct contact with the BCx, but could respond to chemicals from BCx diffusing from the wells across the agar top layer.

4.3.6 Settlement assays

Initial screening tested the ability of biofilm cell suspensions (BCx) of fourteen bacteria subjected to substrates 1, 2a, 3 and 4a. For the settlement assay, three replicates per treatment were tested. In addition, BCx from three key mono-species bacteria (*Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and

Pseudoalteromonas sp. AMGP1) were exposed to all six substrate treatments (1, 2a, 2b, 3, 4a and 4b). Settlement assays included 10 replicates per treatment for each of the three mono-species bacteria.

Larval settlement assays were carried-out as detailed in chapters 2 & 3. All plates contained 20–30 swimming pediveliger larvae obtained from SPATnz, Nelson, New Zealand. Individual treatment plates exposed to substrate 1 received 8 ml of AFSW and 1 ml of larval solution. Other treatment plates exposed to substrates 2a, 2b, 3, 4a and 4b received 9 ml of AFSW and 1 ml of larvae. The control plates contained 9 ml of AFSW and 1 ml of larval solution. All treatment and control plates containing larvae were incubated at 17°C for 48 hours. After this period, live settled larvae were scored under a stereomicroscope at 20 × magnification. Vital stains and visual techniques were used to determine live and dead larvae (refer chapter 2 for detailed methods).

4.3.7 Statistical analyses

All data were statistically analysed using Predictive Analysis Software (PASW®) Statistics 18. Data for the percentages of live settled, live unsettled, and dead larvae were arcsine transformed prior to statistical analyses. All data that satisfied parametric assumptions were subjected to separate two-way ANOVAs (methods and BCx as fixed factors) for each bacterium. *Post-hoc* Tukey tests were conducted for pair-wise comparisons.

4.4 Results

4.4.1 BCx of fourteen bacterial groups on four settlement substrates

Results from the biofilm cell suspensions (BCx) of fourteen marine bacteria clearly indicated differences in settlement inducing properties of five bacterial groups, subjected to substrates 1, 2a, 3 and 4a to respectively achieve BCx on Petri plate, noble agar, glass slide and noble agar wells (Figs. 4.2 A–N; Table 4.1).

The BCx of bacterium1 demonstrated significantly reduced larval settlement (< 40%) on noble agar and noble agar wells compared with BCx on Petri plates and glass slides, which achieved over 70% larval settlement (Fig. 4.2 A; ANOVA; $\alpha = 0.05$; $p < 0.001$). Tukey tests detected non-significant ($p > 0.05$) differences between BCx on Petri plate and glass slide. These results may indicate that the settlement cue for this species of bacteria may be surface-bound and not water-soluble. Similarly, bacterium 14 had significantly lower settlement between 18–25% on noble agar and noble agar wells compared with the significantly greater settlement of 66.7 ± 3.2 and 62.3 ± 7.6 on Petri plates and glass slides, respectively (Fig. 4.2 N; Table 4.1). Non-significant differences ($p > 0.05$) were observed between BCx of bacterium 14 on Petri plate and glass slides. BCx of bacterium 9 on glass slide induced greater settlement (44%) compared with BCx in noble agar and noble agar wells that induced between 17–23% (Fig. 4.2 I; Table 4.1). Tukey tests detected non-significant ($p > 0.05$) differences between BCx of bacterium 9 on Petri plate compared with BCx on noble agar plates and glass slides. These results indicate that larval settlement cues from this bacterium were mostly surface-bound and may be released into the medium.

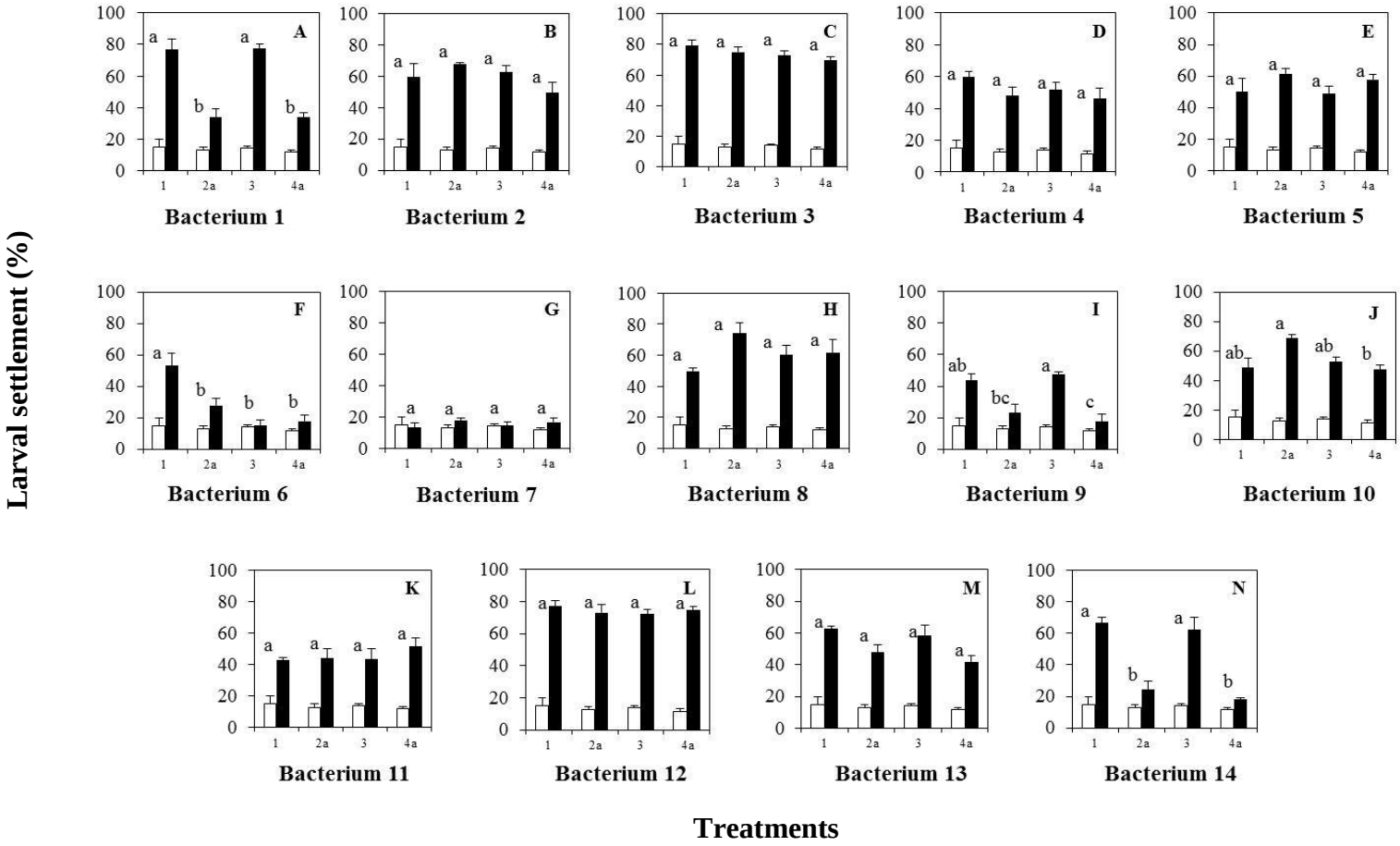

Figure 4.2: Effect of settlement substrates and biofilm cell suspensions (BCx) of fourteen bacteria on mussel larvae. Data represent mean (±SE, n = 3) larval settlement of *P. canaliculus* after 48 hours containing fourteen (A–N) mono-species BCx exposed to four treatment substrates (1, 2a, 3 and 4a) to achieve BCx of individual bacteria on Petri plate, noble agar, glass slide and noble agar wells. The open bars represent sterile control plates and shaded bars represent the treatment plates. Non-significant Tukey tests are denoted by the same letter over the bars.

Table 4.1: Statistical analyses (two-way ANOVA with Tukey test; $\alpha = 0.05$) for *P. canaliculus* larval settlement and mortality on four diverse settlement substrates containing biofilm cell suspensions (BCx) of 14 bacteria. Significant tests ($p < 0.05$) are in bold.

Treatments	*df*	MS	*F*	*p*	Treatments	*df*	MS	*F*	*p*
Bacterium 1					***Bacterium 8***				
Methods (M)	3	388.7	17.1	**0.001**	Methods (M)	3	46.9	1.6	0.236
Biofilm cell suspensions (BCx)	1	4470.9	196.1	**0.001**	Biofilm cell suspensions (BCx)	1	5641.1	188.4	**0.001**
BCx×M	3	294.8	12.9	**0.001**	BCx×M	3	70.4	2.4	0.111
Error	16	22.8			Error	16	29.9		
Bacterium 2					***Bacterium 9***				
M	3	39.0	1.5	0.247	M	3	169.5	7.4	**0.002**
BCx	1	5257.3	205.4	**0.001**	BCx	1	1034.6	45.4	**0.001**
BCx×M	3	24.6	1.0	0.434	BCx×M	3	108.1	4.7	**0.015**
Error	16				Error	16	22.8		
Bacterium 3					***Bacterium 10***				
M	3	22.9	1.8	0.181	M	3	50.5	3.1	**0.055**
BCx	1	8562.8	687.3	**0.001**	BCx	1	4187.8	259.7	**0.001**
BCx×M	3	6.4	0.5	0.681	BCx×M	3	55.4	3.4	**0.042**
Error	16	12.5			Error	16	16.1		
Bacterium 4					***Bacterium 11***				
M	3	30.3	1.4	0.269	M	3	3.4	0.1	0.929
BCx	1	3614.5	171.3	**0.001**	BCx	1	2644.3	116.1	**0.001**
BCx×M	3	9.8	0.5	0.712	BCx×M	3	17.5	0.8	0.529
Error	16	21.1			Error	16	22.8		
Bacterium 5					***Bacterium 12***				
M	3	10.6	0.5	0.703	M	3	5.8	0.4	0.784
BCx	1	4185.2	188.2	**0.001**	BCx	1	8857.0	544.3	**0.001**
BCx×M	3	29.2	1.3	0.305	BCx×M	3	4.2	0.3	0.856
Error	16	22.2			Error	16	16.3		
Bacterium 6					***Bacterium 13***				
M	3	199.0	7.9	**0.002**	M	3	68.0	3.4	**0.043**
BCx	1	630.5	25.0	**0.001**	BCx	1	3837.0	192.3	**0.001**
BCx×M	3	160.0	6.3	**0.005**	BCx×M	3	30.6	1.5	0.244
Error	16	25.3			Error	16	20.0		
Bacterium 7					***Bacterium 14***				
M	3	1.5	0.1	0.957	M	3	403.2	18.0	**0.001**
BCx	1	17.0	1.2	**0.291**	BCx	1	2208.6	98.3	**0.001**
BCx×M	3	11.4	0.8	0.510	BCx×M	3	304.0	13.5	**0.001**
Error	16	14.2			Error	16	22.5		

Interestingly, settlement percentages (mean% ± S.E) of BCx from bacterium 6 were significantly higher on Petri plates (53.0 ± 7.7) compared with three other settlement substrates, which had lower larval settlement between 15–28% (Fig. 4.2 F; Table 4.1). *Post-hoc* Tukey tests showed significant ($p < 0.05$) differences between BCx on Petri plate compared with BCx on glass slide, noble agar and noble agar wells, indicating that BCx of bacterium 6 may induce greater larval settlement on hydrophobic polystyrene surfaces and not on hydrophilic glass and agar surfaces.

For BCx of bacterium 10, the highest settlement (69.0 ± 2.1) was observed on noble agar plates and the lowest (47.4 ± 3.3) on noble agar wells (Fig. 4.2 J; Table 4.1). Tukey tests showed significant ($p < 0.05$) differences between BCx on noble agar compared with BCx on noble agar wells. Non-significant ($p > 0.05$) differences were noted for BCx of this bacterium on Petri plate compared with BCx on three other settlement substrates, indicating that the settlement inducing cues for this bacterium may be surface-bound as well as water borne (present in the medium). These contrasting results on noble agar plates and noble agar wells also may indicate the need to increase the number of replicates per treatment which may aid in clarifying these results.

Unlike other bacteria, BCx of bacterium 2–5, 7, 8 and 11–13 clearly had similar levels of larval settlement across all four substrates tested in this study (Figs. 4.2 B–E, G, H and K–M; Table 4.1). Tukey tests resulted in non-significant ($p > 0.05$) differences between bacterial cell suspensions (BCx) on Petri plate, noble agar plates, glass slide on noble agar and noble agar wells for each of these bacteria.

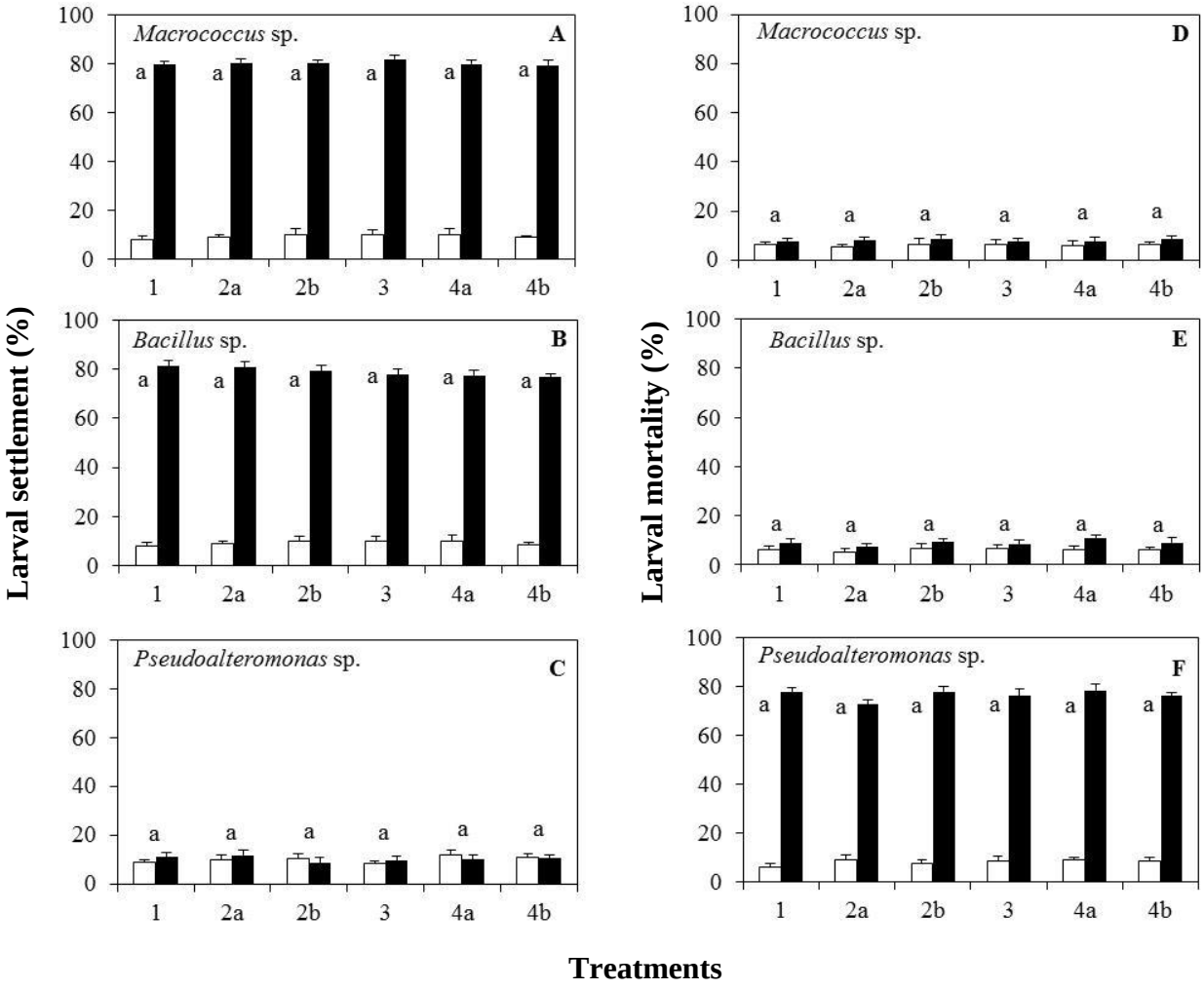

Figure 4.3: Effect of settlement substrates and biofilm cell suspensions (BCx) of three bacteria on mussel larvae. Data represent mean (±SE, n = 10) larval settlement (A, B & C) and mortality (D, E & F) of *P. canaliculus* after 48 hours on polystyrene Petri plates containing BCx subjected to five substrate treatments to obtain settlement plates containing BCx on Petri plate (1), noble agar (2a), noble agar sandwiched (2b), glass slide (3), noble agar wells (4a) and noble agar wells sandwiched (4b). The open bars represent sterile control plates and shaded bars represent the treatment plates. Non-significant Tukey tests are denoted by the same letter over the bars.

Table 4.2: Statistical analyses (two-way ANOVA with Tukey test; α = 0.05) for *P. canaliculus* larval settlement and mortality on six diverse settlement substrates containing biofilm cell suspensions of *Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1. Significant tests ($p < 0.05$) are in bold.

Treatments (% Larval settlement)	*df*	MS	*F*	*p*
Macrococcus sp. AMGM1				
Methods (M)	5	6.5	0.2	0.949
Biofilm cell suspensions (BCx)	1	66078.2	2352.0	**0.001**
BCx×M	5	3.0	0.1	0.991
Error	108	28.1		
Bacillus sp. AMGB1				
M	5	7.7	0.3	0.935
BCx	1	64106.8	2151.6	**0.001**
BCx×M	5	17.7	0.6	0.705
Error	108	29.8		
Pseudoalteromonas sp. AMGP1				
M	5	18.7	0.4	0.816
BCx	1	0.4	0.01	0.924
BCx×M	5	16.9	0.4	0.846
Error	108	41.9		
Treatments (% Larval mortality)	*df*	MS	*F*	*p*
Macrococcus sp. AMGM1				
Methods (M)	5	10.6	0.2	0.952
Biofilm cell suspensions (BCx)	1	362.5	7.6	0.007
BCx×M	5	8.6	0.2	0.968
Error	108	47.8		
Bacillus sp. AMGB1				
M	5	13.5	0.2	0.941
BCx	1	466.3	8.5	0.004
BCx×M	5	33.0	0.6	0.702
Error	108	55.2		
Pseudoalteromonas sp. AMGP1				
M	5	16.1	0.4	0.870
BCx	1	65441.2	1497.6	**0.001**
BCx×M	5	26.7	0.6	0.691
Error	108	43.7		

4.4.2 BCx of three bacterial groups on five settlement substrates

Results from the examination of biofilm cell suspensions (BCx) on five settlement substrates clearly indicated that all three bacteria, *Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1 induced similar settlement levels across all substrates. Uniform settlement levels of over 70% were observed in treatment plates containing BCx of *Macrococcus* sp. on all five settlement substrates (Fig. 4.3 A; Table 4.2). Similar settlement rates (mean% ± S.E) between 76.8 ± 1.2 and 81.5 ± 2.0 were observed on settlement substrates containing BCx of *Bacillus* sp. (Fig. 4.3 B; Table 4.2). In comparison, low larval settlement (between 8.9 ± 2.0 and 11.9 ± 2.1) was observed on treatment plates containing BCx of *Pseudoalteromonas* sp. for all settlement substrates (Fig. 4.3 C; Table 4.2). Individual Tukey tests resulted in non-significant ($p > 0.05$) differences for all substrate treatments for each of three species of bacteria.

Low larval mortality percentages (< 20%) were observed on all settlement substrates containing BCx obtained from both *Macrococcus* sp. and *Bacillus* sp. (Fig. 4.3 D & E; Table 4.2). Tukey tests revealed non-significant differences ($p > 0.05$) across all treatments for both of these bacteria. Conversely, greater mortality percentages (between 73–79%) were noted on all treatments containing BCx of *Pseudoalteromonas* sp. (Fig. 4.3 F; Table 4.2). Non-significant differences ($p > 0.05$) were observed for all treatments containing BCx of this bacterium.

4.5 **Discussion**

Results from previous studies clearly indicated that *Perna canaliculus* larvae do not respond to intrinsic physical cues of the settlement substrata. Consistently low larval settlement percentages (< 20%) were observed on un-filmed glass, polystyrene

and noble agar plates (chapter 2). However, mono-species bacterial biofilms from *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1, grown on to polystyrene plates induced over 60% settlement compared with the low settlement rates (< 20%) observed on sterile un-filmed plates (chapter 3). These results highlight the importance of bacterial cues for mussel larval settlement. This study is the first to investigate the interplay of substrate physical properties and bacterial cues for the settlement of *P. canaliculus* larvae. Screening fourteen mono-species marine bacteria presented contrasting results which explicitly demonstrated the need for this study. Although wettability analyses were not conducted on these substrates, clear differences in larval settlement indicated that there were physicochemical interactions between bacterial cues and their corresponding substrates.

Based on previous studies, it was recognised that bacteria settle largely on hydrophobic (e.g., polystyrene) surfaces rather than hydrophilic (e.g., glass) substrates (Fletcher & Loeb, 1979). This may explain why Bacterium 6 induced greater (up to 60%) larval settlement on polystyrene plates compared with the < 30% observed on the highly wettable (hydrophilic) glass and noble agar plates. Conversely, biofilms of *Halomonas marina* induced greater (30–45%) attachment rates on polystyrene plates compared with lower rates (< 20%) on glass plates for the barnacle (*Balanus amphitrite*) larvae (Maki et al., 2000). Likewise, biofilms of three marine bacteria (*Deleya marina*, *Alteromonas macleodii* and *Pseudomonas fluorescens*) induced greater settlement rates on glass compared with polystyrene surfaces (O'Connor & Richardson, 1996). The differences in attachment of bacteria to abiotic substrates (e.g., glass and polystyrene) and the overall chemistry of the substrata may vary depending on the bacterial species (Loosdrecht et al., 1989;

McEldowney & Fletcher, 1986). This is due to the mode of bacterial attachment on different substrates. These differences are dependent on one or more physicochemical interactions (i.e., electrostatic attraction, Van der Waals forces and steric hindrance), which can influence the overall biofilm chemistry of the settlement substrata (Costerton et al., 1995; Donlan, 2002; Flemming & Wingender, 2010). Thus, it is essential to analyse both individual (without biofilm) and combined (with biofilm) effects of substrates while examining the effect of mono-species bacterial cues for larval settlement.

As well as substrate nature, the noble agar tested in this study provided information on the ability of extracellular products of bacteria as modulators of mussel larval settlement. Noble agar was one of the recommended biopolymers to test the effect of bacterial cues for larval settlement (Salamanca et al., 2010). However, *P. canaliculus* larvae did not settle directly on top of the noble agar wells containing BCx. This could have been due to the effect of water-soluble cues released by BCx of mono-species bacteria that could have emanated through the agar matrix and saturated the assay medium during the incubation period. Similarly, coral (*Acropora millepora*) larvae metamorphosed in greater amounts (over 80%) in water-soluble exudates of *Pseudoalteromonas* strain A3 released from agar drops rather than surface-bound biofilm cues that induced only < 60% larval metamorphosis (Negri et al., 2001). A number of similar studies reported the importance of biofilm exudates over biofilms for the settlement of invertebrate larvae (Bao et al., 2007a; Fitt et al., 1990; O'Connor & Richardson, 1998). Conversely, surface-bound bacterial cues from marine *Roseobacter* and *Proteobacteria* were demonstrated to induce settlement of polychaetes, *Hydroides*

elegans larvae (Lau & Qian, 2001). Likewise from this study, water-borne cues of BCx from bacterium 1, 9 and 14 on agar plates and wells did not induce similar levels of larval settlement, unlike BCx on polystyrene and glass surfaces. Thus, it is essential to understand that settling larvae may respond to one or more cues present in biofilms of mono-species bacteria (Hadfield, 2011) and techniques explained in this study may aid in the identification of such bacterial cues for mussel and other invertebrate larvae.

In conclusion, laboratory assays containing bacterial cell suspensions (BCx) of five mono-species bacteria (bacterium 1, 6, 8, 9 and 14) interacted differently with substrates with different physical properties, and they resulted in variations in inductiveness of mussel (*P. canaliculus*) larval settlement. The properties of nine mono-species bacteria (bacterium 2–5, 7, 8 and 11–13) were consistent on all settlement substrates, indicating similarities of physicochemical responses across all substrata and the ability of biofilm exudates to induce larval settlement. Finally, the three key bacteria (*Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1) were consistent in their ability to induce *P. canaliculus* larval settlement. Nonetheless, the present study did not specifically identify if the bacterial cues were cell-surface bound or present in the medium. Hence, the next research step would be to elucidate the putative nature of bacterial cues for settlement with careful separation of cells and chemicals within bacterial biofilms and testing individual fractions on mussel larvae.

Chapter 5.

EFFECTS OF MONO-SPECIES BACTERIAL BIOFILMS, BIOFILM EXUDATES AND BACTERIAL CELL-SUSPENSIONS ON *PERNA CANALICULUS* LARVAL SETTLEMENT

5. Effects of Mono-Species Bacterial Biofilms, Biofilm Exudates and Bacterial Cell-Suspensions on *Perna canaliculus* Larval Settlement

5.1 Abstract

The ability of bacterial biofilms and biofilm exudates to induce mussel (*Perna canaliculus*) larvae to settle was investigated. Bacterial biofilms and biofilm exudates from *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1 significantly increased larval settlement (over 60%) compared with controls. Conversely, *Pseudoalteromonas* sp. AMGP1 did not induce larval settlement in treatments, whereas biofilms and their exudates resulted in extremely high larval mortality. Thus, *Pseudoalteromonas* sp. AMGP1 biofilms may produce toxins deadly for *P. canaliculus*. These results suggest that settlement cues for *P. canaliculus* may be produced by the biofilm cells and are present in the biofilm exudates. A technique was developed to separate and test bacterial cell surface-bound compounds and their exudates (biofilm matrix) for marine invertebrate larval settlement experiments. A broth culture (preventing biofilm formation) was used to obtain planktonic cell suspensions without exudates (PCo). Biofilm cell suspensions with their exudates (BCx) were obtained by growing the cells on marine agar (allowing biofilm formation) before suspending in seawater. A biofilm suspension without exudates (BCo) was obtained by washing BCx cells thoroughly before suspending in seawater. Three separate marine bacteria (*Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1) were used to test PCo, BCx, and BCo suspensions for their ability to induce settlement of mussel larvae (*P. canaliculus*). BCx suspensions of *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1 significantly induced settlement (over 70%) compared with their corresponding PCo

and BCo suspensions and the sterile control plates. Conversely, BCx suspensions of *Pseudoalteromonas* sp. AMGP1 did not induce settlement, and were highly toxic to the larvae. In addition, PCo and BCo treatments with *Pseudoalteromonas* sp. AMGP1 had no effect on larval settlement or mortality, compared with control plates. Results show that the tested bacterial cells alone (devoid of their biofilm exudates) do not induce settlement or mortality in this mussel species, and that their exudates contain the chemical cues that affect larval development and survivability. Thus, this work highlights the importance of appropriately separating exudates from bacterial cells to test surface-bound compounds in settlement cue experiments.

5.2 Introduction

Marine invertebrate larvae undergo settlement in response to a wide variety of external physical (e.g., light, temperature, salinity, hydrostatic pressure), natural chemical (e.g., macro-algal bi-products, conspecific compounds), synthetic chemical (e.g., neurotransmittors, ions) and biological (e.g., larval age, behaviour) stimuli or cues (Hadfield, 2011; Hadfield & Paul, 2001; Metaxas & Saunders, 2009; Pawlik, 1992; Rodriguez et al., 1993). Numerous studies have investigated the effects of such cues on larval settlement for a multitude of species, such as polychaetes (Harder et al., 2002; Hung et al., 2009; Qian, 1999), barnacles (Faimali et al., 2004; Khandeparker et al., 2002; Qian et al., 2003) and mussels (Alfaro et al., 2006; Alfaro et al., 2011b; Bao et al., 2007a; Young et al., 2011). However, settlement cues stemming from marine biofilms are difficult to elucidate because of the high complexity and diversity of their composition (Qian et al., 2007). Indeed, marine biofilms may contain a range of bacterial strains, microalgal species, fungi and protozoa with their own cue constituents (Schachter, 2003). Field experiments to

investigate settlement induction processes have included natural biofilms of unknown specific composition (Bao et al., 2007a; Dobretsov & Qian, 2006; Keough & Raimondi, 1995). For example, Zhao and Qian (2002) investigated the settlement of slipper limpets (*Crepidula onyx*) as a response to surface cues of natural multi-species biofilms. Although the authors identified that proximity to a marine biofilm induced up to 90% larval settlement, they were unable to determine the bacterial species and/or the specific products involved in the settlement cue. In addition, laboratory experiments have aimed at using mono-specific bacterial biofilms in attempts to identify potential larval settlement inductors for a variety of marine species, including mussels (Satuito et al., 1997), sea urchins (Huggett et al., 2006), barnacles (Khandeparker et al., 2006), hydroids (Leitz & Wagner, 1993), spirobids (Kirchman & Graham, 1982), and polychaete worms (Harder et al., 2002). Furthermore, some of these controlled experiments also have shown inhibitory responses to larval settlement (Dobretsov & Qian, 2006; Rao et al., 2007), and their use as antifouling agents has been explored extensively (Bowman, 2007; Dobretsov & Qian, 2002; Qian et al., 2007). Khandeparker et al. (2003) used single-species biofilms to test settlement of barnacle larvae (*Balanus amphritite*), and found that biofilms containing *Pseudomonas aeruginosa* induced greater settlement (up to 44%) compared with biofilms with *Bacillus pumilis* and *Citrobacter freundii*, which achieved only 10% settlement in 24-hour assays. However, the authors were not able to identify which specific component of the biofilm (i.e., bacterial cells, exudates) actually induced settlement.

Since bacterial biofilms encompass aggregates of cells and exudates (biofilm matrix) (Costerton et al., 1995), some studies have focused on separating the

different components of a biofilm to understand the specific origin of the cue (Harder et al., 2002; Khandeparker et al., 2006; O'Connor & Richardson, 1998). For example, bacterial culture supernatants have been shown to elicit settlement and metamorphosis of oyster larvae, *Crassostrea gigas* (Fitt et al., 1990). On the other hand, some studies have highlighted the possibility that surface-bound bacterial cues also may induce settlement (Huang & Hadfield, 2003; Kirchman et al., 1982a; Lau & Qian, 2001). However, one of the major problems of such studies is that the bacteria used in the experiments were not clearly devoid of biofilm exudates, which likely contained the settlement cue (Harder et al., 2002; O'Connor & Richardson, 1996). Thus, a method to produce bacterial cells without their exudates is essential to specifically test and determine which component of the biofilm (bacterial cells or exudates) induces the settlement process.

For the New Zealand green-lipped mussel, *P. canaliculus*, extensive research on the spat-macro-algal association has identified the role of substrate morphology (Alfaro, 2006c; Alfaro & Jeffs, 2002, 2003; Alfaro et al., 2004) and chemical cues (Alfaro et al., 2006; Young, 2009) in the settlement process. However, the mechanisms by which bacterial biofilms induce settlement of mussel larvae have not been studied in depth. Information about the inductive or repellent properties of specific bacterial biofilms may have a direct application for hatchery production, which often is hindered by low settlement rates. As a first step to address this problem, the effects of mono-specific bacterial biofilms, and their extracellular polymeric substances (exudates) on the settlement of hatchery-reared mussel larval were investigated. Furthermore, planktonic (free-floating) bacterial cells without

exudates, bacterial cells with exudates, and bacterial cells devoid of exudates were produced and tested for their ability to induce the settlement of *P. canaliculus* larvae.

5.3 Materials and Methods

5.3.1 Preparation of bacterial biofilms and exudates

In order to prepare bacterial biofilms and biofilm exudates, 1 ml of biofilm cell suspensions containing 10^6 to 10^7 cells ml^{-1} (refer chapter 3 for preparation and enumeration) of each bacterial isolate (*Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1) was added to polystyrene Petri plates. Then, 4 ml of FSW containing 0.5% peptone were added to each plate to grow the cells. Plates were incubated for 24 hours at 37°C on a rotary incubator at 100 rpm for even biofilm formation. After incubation, the plates were washed thrice with a total of 30 ml FSW, leaving behind the attached cells on the Petri plates. These plates were used as larval settlement substrates.

Production of biofilm exudates was achieved by culturing another set of biofilm plates (as described above), and scraping off the attached cells with a clean glass cover slip into 1 ml of FSW. Ten of these 1 ml samples were pooled and centrifuged at 3500 rpm for 10 minutes. The pellets containing the bacterial cells were discarded. The supernatant was filtered through a 0.22 µm acetate filter to obtain a cell-free extract. This pure biofilm exudate was used for the settlement assays.

5.3.2 Preparation of bacterial cell suspensions

Colonies of each of three marine bacteria (*Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1) were grown in two

different culture media (broth and agar). Broth cultures were used to produce planktonic cell suspensions (PCo), which did not contain exudates. Agar cultures were used to produce biofilm cell suspensions (BCx). Unlike the PCo, BCx contained exudates which are generated when the cells form a biofilm (Costerton et al., 1995) . These bacterial phases were tested for their ability to induce settlement of *P. canaliculus* larvae (see below).

Broth cultures of each bacterial species were grown in 100 ml of 0.5% peptone in autoclaved filtered seawater (AFSW), and were maintained on a rotary incubator at 37ºC. After 24 hours, the cells were separated from the broth culture by centrifuging at 3500 × g for 10 minutes. The pellets were washed thrice with a total of 30 ml of AFSW. At the end of each washing step, the cells in AFSW were vortexed briefly, centrifuged and re-suspended. The cells finally were suspended in 10 ml of AFSW with a concentration of 10^6 to 10^7 cells ml^{-1}. These suspensions were termed planktonic cells without exudates (PCo) for each species.

Suspensions with BCx (containing exudates) were obtained by pooling cells from five replicate marine agar plates, placing in 50 ml of AFSW, and incubated at 37ºC for 24 hours. Then, the cells were transferred to 10 ml of AFSW, washed and suspended as described above.

In order to test BCx suspensions without the effect of their exudates, another set of BCx suspensions was produced and washed to remove the exudates. This was achieved by adding 40 ml of AFSW to 10 ml of BCx suspension, and incubating at room temperature (17–20ºC) for 48 hours. The incubated suspensions were washed

and suspended as described above. These suspensions were termed biofilm cells without exudates (BCo).

5.3.3 Larval settlement assays

Veliger larvae of *P. canaliculus* (19–21 days old) were sourced from Shellfish Production and Technology New Zealand Limited (SPATnz), Nelson, New Zealand for these settlement experiments. Information on larval transportation and maintenance in the laboratory has been explained in chapter 2. Settlement experiments were conducted to test the effect of each bacterial species on mussel larval settlement. Experiments tested bacterial biofilms, biofilm exudates and bacterial cell-suspensions together with a set of controls. In addition, each experiment tested all three bacterial species simultaneously. Sterile polystyrene Petri plates (60 mm diameter x 14 mm depth, Biolab Inc., New Zealand) were used for all mussel larval settlement experiments. Ten replicate plates were used for each treatment and control.

For each bacterial species, 1 ml of biofilm exudate was placed onto a polystyrene Petri dish and topped with 8 ml FSW. For testing biofilm cells, a biofilm was allowed to form on a Petri dish, which was then topped with 9 ml of FSW. For the biofilm cell-suspensions assay, each treatment plate received 8 ml of AFSW, 1 ml of larvae, and 1 ml of cell suspension (10^6 to 10^7 cells) from the corresponding bacterial phase and species. Control plates contained only 9 ml of FSW. About 20 to 25 healthy swimming larvae in 1 ml FSW were placed in each of the treatment and control plates.

The larvae were left to settle for a period of 48 hours. At the end of this period, larvae from individual plates were observed for settlement under a dissection microscope with 20 × magnification. Larvae were termed settled only if they were alive and attached to the bottom of the Petri plate. Larval attachment was checked by applying gentle suction with a 200 µl pipette placed about 1.0 mm from each larva. In addition, attached larvae often had a visible thin mucous thread structure connecting them to the substrata. Unattached larvae were either observed swimming in the Petri plate or were removed from the Petri plate without any resistance. Mortality was determined by noting larval behaviour (e.g., opening of valves, movement of foot, gills), and confirmed by staining with vital stain. Unstained larvae were recorded as dead.

5.3.4 Statistical analyses

The settlement data were quantified as percent settled (live) larvae from the total number of larvae added to the assay. Similarly, the mortality data were quantified as the percentage of dead (settled or unsettled) larvae from the total number of larvae added to the assay. All percentage data for both settlement and mortality were statistically analysed following arcsine transformations. Data were tested for and met all parametric assumptions, and were evaluated using one-way ANOVAs and *post-hoc* Tukey tests. All statistical analyses were performed using SPSS 16 software for Windows®.

5.4 Results

5.4.1 Bacterial biofilms and biofilm exudates

Results from the settlement experiments (Fig. 5.1 A & B) clearly show that bacterial biofilms and biofilm exudates of *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1 provided a settlement cue for *P. canaliculus* larvae. The larval settlement was significantly greater by 50–60% for bacterial biofilms and biofilm exudates compared with the control (*Macrococcus* sp. AMGM1: ANOVA; $F_{2,27}$ = 365.28, p = 0.001, *Bacillus* sp. AMGB1: ANOVA; $F_{2,27}$ = 380.89, p = 0.001). Individual Tukey *post-hoc* tests revealed non-significant differences between biofilms and biofilm exudates (p > 0.001) for both of these bacteria. On the contrary, bacterial biofilms and biofilm exudates of *Pseudoalteromonas* sp. AMGP1 did not induce settlement (Fig. 5.1 C). There was no significant difference in larval settlement among bacterial biofilms, biofilm exudates and the control (ANOVA; $F_{2,27}$ = 1.56, p = 0.229).

Mortality was relatively low (< 15%) in all treatments and control for exposures to *Macrococcus* sp. AMGM1 (Fig. 5.1 D) and *Bacillus* sp. AMGB1 (Fig. 5.1 E), and control for *Pseudoalteromonas* sp. AMGP1. One-way ANOVAs for larval mortality data were non-significant among bacterial biofilms and biofilm exudates and the control for *Macrococcus* sp. AMGM1 ($F_{2,27}$ = 1.73, p = 0.197), and *Bacillus* sp. AMGB1 ($F_{2,27}$ = 0.14, p = 0.868). However, significantly high larval mortality (over 70%) was observed in the *Pseudoalteromonas* sp. AMGP1 biofilm and biofilm exudate treatments, compared with the control (Fig. 5.1 F; ANOVA; $F_{2,27}$ = 266.70, p = 0.001). A Tukey test indicated non-significant differences between the two treatments only (p > 0.001).

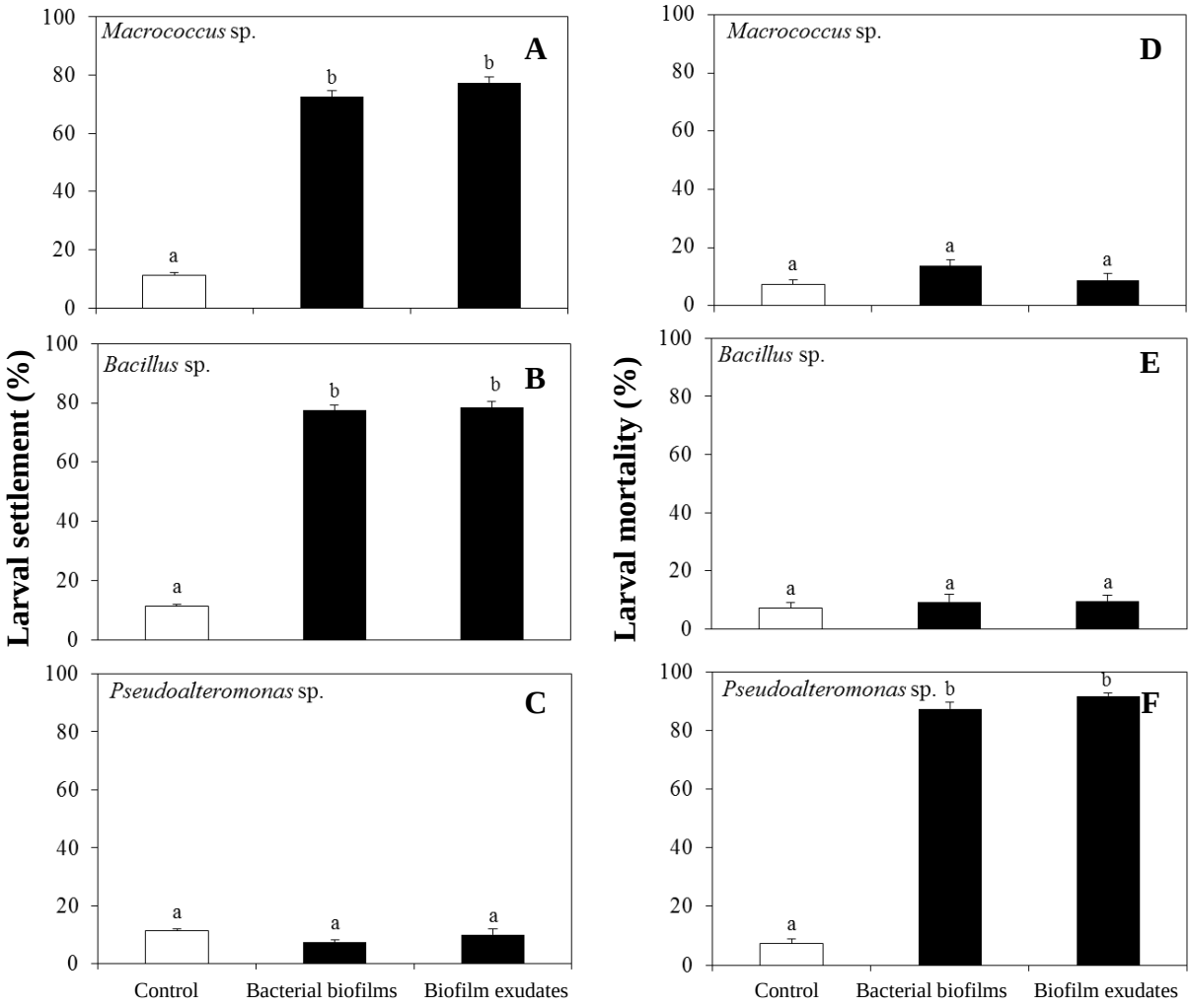

Figure 5.1: Effect of bacterial biofilms and biofilm exudates on larval settlement. Data represent mean (±SE, n = 10) percent larval settlement (A, B & C) and mortality (D, E & F) of *P. canaliculus* after 48 hours on polystyrene Petri plates containing bacterial biofilms or biofilm exudates of *Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1. The open bars represent sterile control plates and shaded bars represent the treatment plates. Non-significant Tukey tests are denoted by the same letter over the bars.

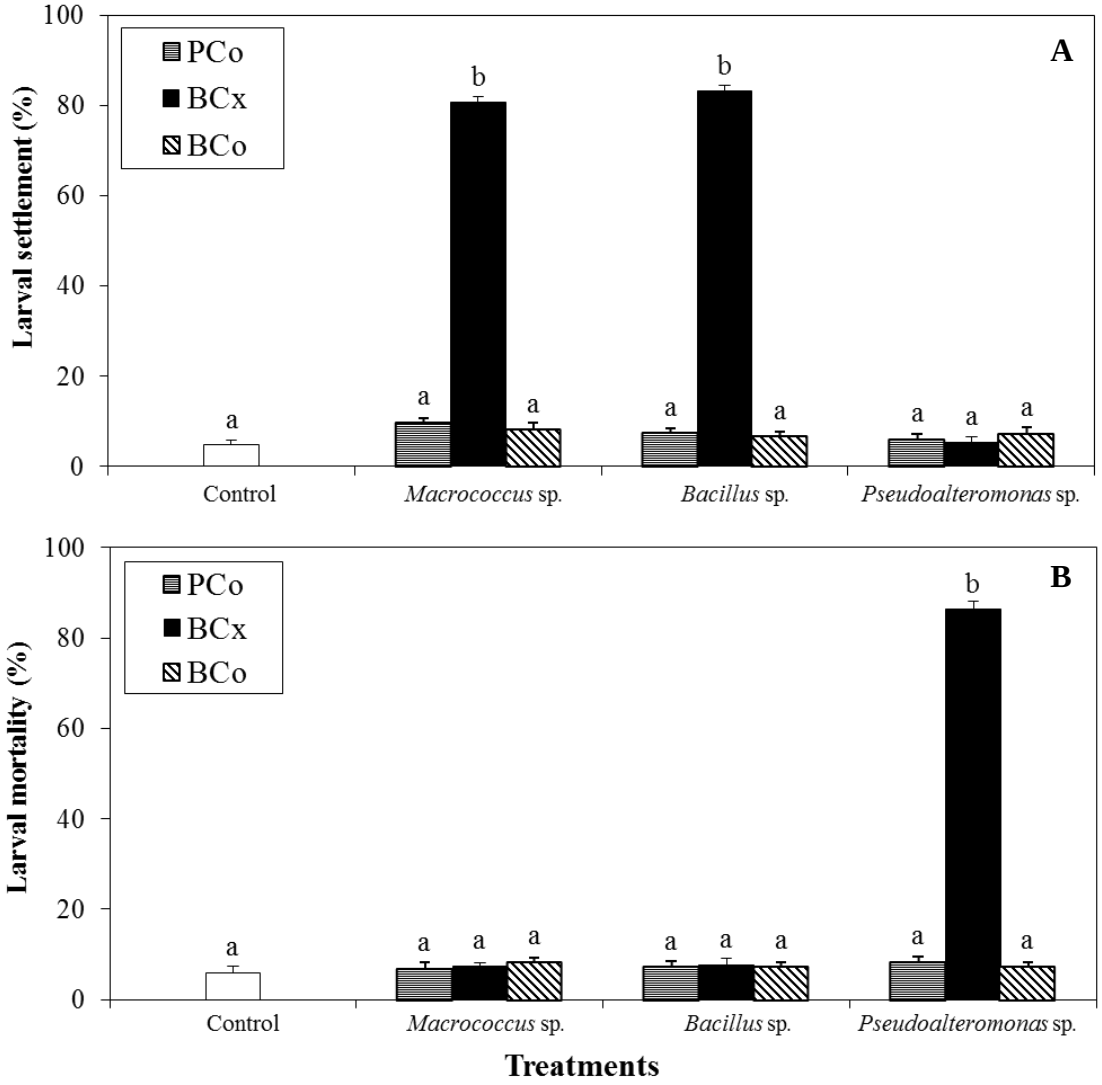

Figure 5.2: Effect of various types of bacterial cell suspensions on larval settlement. Mean (±SE, n = 10) percent larval settlement (A) and mortality (B) of *P. canaliculus* after 48 hours on polystyrene Petri plates containing suspensions of planktonic bacterial cells without exudates (PCo), biofilm cells with exudates (BCx) and biofilm cells without exudates (BCo) of *Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1. The open bars represent sterile control plates and shaded bars represent the treatment plates. Non-significant Tukey tests are denoted by the same letter over the bars.

5.4.2 Bacterial cell suspensions

Results for the larval settlement assay (Fig. 5.2 A) clearly indicate that the biofilm cell suspensions (BCx) of *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1 caused significantly greater settlement (over 70%) compared with

planktonic cell suspension (PCo) treatments, biofilm suspensions without the exudates (BCo), and sterile control plates (ANOVA; $F_{9,90}$ = 173.79, p = 0.001). Tukey tests revealed non-significant differences (p > 0.001) between PCo and BCo treatments for both *Macrococcus* sp. and *Bacillus* sp. In the case of *Pseudoalteromonas* sp. AMGP1, all three bacterial suspensions (PCo, BCo and BCx) did not enhance settlement (all Tukey tests: p > 0.001).

Conversely, significantly greater mortality (Fig. 5.2 B) was observed for BCx treatments of *Pseudoalteromonas* sp. compared with PCo and BCo treatments with the bacterium and controls (ANOVA; $F_{9,90}$ = 120.08, p = 0.001). In addition, there were no significant differences (ANOVA; $F_{9,90}$ = 120.08, p > 0.001) in the mortality for all three treatments of both *Macrococcus* sp. and *Bacillus* sp., when compared with controls.

5.5 **Discussion**

Previous studies on *P. canaliculus*, have demonstrated the importance of substrate morphology (Alfaro, 2006a; Alfaro & Jeffs, 2002, 2003; Alfaro et al., 2004) and chemical cues (Alfaro et al., 2006; Young, 2009) on the larval settlement process. This contribution provides the first evidence that marine bacterial biofilms also induce settlement in this species, and that the induction stimulus is bacterium-specific. In addition, the separation of bacterial biofilms, cells and their exudates was used to determine the origin of the settlement cue.

Results settlement experiments indicated that *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1 produced similar settlement induction responses on mussel larvae, and these responses were significantly greater for bacterial biofilms and biofilm exudates, compared with the control. Species of *Macrococcus* have not been

reported previously to induce larval settlement, but *Bacillus pumilis* was found to induce settlement of barnacle larvae, *Balanus amphitrite* (Khandeparker et al., 2006). Other Gram-positive bacteria have been found to induce settlement in larvae of the polychaete, *Hydroides elegans* (Lau et al., 2002), suggesting that the shared quorum sensing signal pathways (production of peptide molecules) found in this type of bacterium (Fuqua et al., 2001) readily induce larval settlement. The fact that high settlement induction was recorded with *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1 biofilms and their exudates indicates that a settlement cue is released by the bacteria once the biofilm is formed, and it remains within the exudate even after the bacterial cells are removed. Khandeparker et al. (2006) also found that exudates removed from *Pseudomonas aeruginosa* bacteria induced greater settlement in barnacle larvae (*Balanus amphritite*) compared with other bacterial species, while Szewzyk et al. (1991) showed that *Pseudomonas* sp. strain S9 exudates induced settlement of ascidian (*Ciona intestinalis*) larvae. These studies concluded that settlement cues may have originated from bacterial metabolites, but did not characterise the identity of the settlement cue. The next step in this research avenue will be investigated which chemical molecules stimulate settlement of mussel larvae.

The findings of the biofilm and exudates study are in agreement with the bacterial cell-suspensions study, and indicate that when exudates are removed from bacterial cell suspensions (BCo) or when cells are allowed to grow without forming a biofilm (PCo), the previously observed chemical cue effects for all three bacterial strains are no longer present. Similar results were obtained by Tebben et al. (2011), who demonstrated that while the biofilm of bacteria *Pseudoalteromonas* sp. A3 and J010 induced larval metamorphosis in coral (*Acropora millepora*), planktonic cells

(grown in broth cultures) of these bacteria did not have this inductive effect. However, the authors did not analyse the effect of biofilm cells without exudates (BCo), which are likely to have a distinct chemical character and effect on larvae. Some studies have indirectly analysed the effect of BCo by treating biofilms with bactericidal substances (i.e., formalin, glutaraldehyde, heat, ethanol, ultraviolet irradiation, antibiotics), and tested for larval settlement cues (Bao et al., 2007b; Kirchman et al., 1982b; Lau & Qian, 2001). While these treatments (biofilms with dead cells) did not enhance settlement of corresponding invertebrate larvae, such toxic chemicals are likely to denature and/or alter the chemical structure of the cue. Conversely, the present study clearly shows that the production of planktonic bacterial cells in broth media and the thorough washing of bacterial cells grown on agar media are suitable approaches to test the effect of bacterial cells on larval settlement without their exudates and without chemical treatments.

In contrast, larval settlement results with *Pseudoalteromonas* sp. AMGP1 clearly revealed that this bacterial biofilm and biofilm exudate do not induce larval settlement. This bacterium was isolated from the macro-alga, *Ulva lactuca*, where it is frequently present, and has been extensively studied for its antifouling properties (Bowman, 2007; Holmström et al., 1998). Previous studies on different strains of *Pseudoalteromonas* sp. have reported both inductive (Dobretsov & Qian, 2002) and inhibitory (Rao et al., 2007) larval settlement responses. The bacterial strain used in this study appears not only to lack inductive signals for settlement of *P. canaliculus* larvae, but it also produces high larval mortality when a well-formed biofilm or its exudates are present. Another significant finding from the present study is the fact that biofilm cells with exudates (BCx) of *Pseudoalteromonas* sp. AMGP1 caused

significantly higher (over 70%) larval mortality compared with planktonic cells without exudates (PCo) and biofilms cells without exudates (BCo). These results are in agreement with the biofilms and exudates study, and suggest that toxic molecules are associated with the biofilm exudates, are not produced by planktonic cells, and are not biofilm cell surface-bound. Similarly, the biofilm exudates of *Pseudoalteromonas tunicata* $D2^T$ were shown to contain compounds that were toxic to both barnacle (*B. amphitrite*) and ascidian (*C. intestinalis*) larvae (Holmström et al., 1998; Holmström et al., 1992). Furthermore, bacterial biofilms and biofilm exudates of several *Pseudoalteromonas* species have been known to produce antifouling compounds that either kill larvae or deter larval settlement (Dobretsov, 2005; Holmström & Kjelleberg, 1999).

In summary, the inductive properties of two bacterial biofilms (*Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1) and their exudates indicate that the settlement cue is produced by bacteria in the biofilm mode of growth. The settlement experiments also revealed the inhibitory activity of *Pseudoalteromonas* sp. AMGP1, which also produced high larval mortality, suggesting that this bacterium may interact negatively with *P. canaliculus* larvae. Therefore, identification and use of appropriate bacteria should enhance mussel larval settlement and thereby improve the production of hatchery-reared mussel seed. The inductive and toxic properties of the respective mono-species biofilm were shown to be contained within their exudates, as washed biofilm cells and planktonic bacteria, (without exudates) did not exhibit the above mentioned properties for mussel larvae. Investigations on the origin and characterisation of these chemical cues necessitate the clear separation of bacterial phases (planktonic, biofilm) and components (cells and exudates). Also,

this contribution presents a novel technique for extracting cells from biofilms, and it provides a comprehensive approach to test the effects of each bacterial phase and component in larval settlement assays.

Chapter 6.

CHARACTERISATION OF BIOFILM EXUDATES AND THEIR EFFECT ON MUSSEL LARVAL SETTLEMENT

6. Characterisation of Biofilm Exudates and their Effects on Mussel Larval Settlement

6.1 Abstract

Settlement-inducing cues for larvae of the green-lipped mussel, *Perna canaliculus*, were characterised from biofilm exudates of mono-species bacteria. Biofilm exudates of *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1, previously shown to induce settlement of *P. canaliculus* larvae, were chosen for this study. An innovative step-wise multi-technique approach, including physical methods (molecular weight fractionation and heat treatment) and chemical methods (crude fractionation, sub-sample fractionation, sequential fractionation, lipid fractionation and protease treatment), was used to characterise the chemical cues from these bacterial biofilm exudates. The resulting fractions obtained from these methods were individually tested for their ability to induce larval settlement. Results from the settlement assays indicate that settlement cues from *Macrococcus* sp. AMGM1 were between 1–3, 3–10 and 10–30 kDa. These molecules were thermo-labile over 70°C, and contained polar proteins and non-polar lipoprotein molecules. Protease-treated biofilm exudates did not elicit a settlement response, indicating the involvement of proteins from *Macrococcus* sp. AMGM1 in the larval settlement process. In addition, settlement cues from *Bacillus* sp. AMGB1 were found to be between 10–30 and 30–50 kDa, were stable up to 100°C, and contained glycolipid molecules. These results provide evidence for the presence of multiple settlement cues in the biofilm exudates from both *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1. This study provides a comprehensive and inexpensive set of biochemical techniques, which can be used in a step-wise manner, to deduce partial chemical identity of both inductive

and non-inductive settlement cues produced by biofilm exudates. The results can be used to decipher complex bacterially mediated larval settlement processes for *P. canaliculus* and other marine invertebrates.

6.2 Introduction

There is a crucial phase in the life cycle of many marine invertebrates wherein competent planktonic larvae are able to settle and metamorphose to a sedentary juvenile stage (Thorson, 1950). Settlement can be described as a behavioural change that occurs as a result of the larvae interacting with external environmental cues or stimuli (Hadfield, 2011; Hadfield & Paul, 2001; Metaxas & Saunders, 2009; Pawlik, 1992; Rodriguez et al., 1993). Indeed, environmental cues of biological origin, especially those from naturally formed biofilms (multi-species bacteria), are known to induce settlement responses in many species of marine invertebrate larvae (Hung et al., 2007; Keough & Raimondi, 1995; Rahim et al., 2004; Tamburri et al., 2008; Zhao et al., 2003). Since naturally formed biofilms consist of a complex micro-biota (e.g., bacteria, fungi, microalgae, protozoans) contained within extracellular polymeric substances (Decho, 1990), it is difficult to identify the specific microorganism(s) and/or the chemical molecules that mediate larval settlement (Qian et al., 2007). Consequently, no published studies have reported thus far on the characterisation of inductive cues from bacterial biofilms to the specific molecules, except for tetrabromopyrrole, which was isolated from *Pseudoalteromonas* bacteria, and induces metamorphosis in coral larvae (Tebben et al., 2011). Furthermore, Hung et al. (2009) was able to narrow down the settlement induction cues from a multi-species biofilm to a hydrocarbon and a fatty acid compound for the polychaete larvae, *Hydroides elegans*. However, a more specific

identification of the molecules or the key microorganisms responsible for the cues was not achieved in that study.

Identification of inductive cues also is hindered by the fact that naturally produced biofilms with multi-species bacteria are susceptible to changes in substrate type, temperature, pH, salinity, zonation and toxic metals, to name a few (Faimali et al., 2004; Lau et al., 2005; Thiyagarajan et al., 2006; Witt et al., 2011a; Witt et al., 2011b). Such external factors affect the bacterial communities and their corresponding larval settlement cue/s (Qian & Dahms, 2009). In order to reduce this complexity, researchers have chosen to investigate mono-species bacterial biofilms cultured in the laboratory that have been previously isolated from natural biofilms (Alfaro et al., 2011b; Ganesan et al., 2010; Harder et al., 2002; Khandeparker et al., 2003). Previous studies have suggested that settlement cues obtained from mono-species bacterial biofilms can be associated with the biofilm surface (Lau & Qian, 2001), released into the medium (Fitt et al., 1990) or a combination (Bao et al., 2007b). Based on this mono-species biofilm approach, some studies have investigated bacterial cells without exudates and *vice versa* as potential sources of inductive cues (Ganesan et al., 2010; Ganesan et al., 2012a) as presented in chapter 5.

Settlement cues from bacterial biofilms have been characterised based on physical properties, such as molecular weight and susceptibility to heat. Molecular weight separation has shown that supernatant molecules within 1000 to 10,000 Daltons (Da), isolated from cell suspensions of the bacterium *Vibrio* sp., were capable of inducing larval settlement of the scyphozoan, *Cassiopea andromeda* (Neumann, 1979). Furthermore, larval settlement inductive cues for the oyster,

Crassostrea gigas, were obtained from culture supernatants of *Alteromonas colwelliana* and *Vibrio* sp., and the inductive fraction was determined to be ≤ 300 Da. Unlike molecular weight analysis, heat treatment (40–100°C) can affect the chemistry of macromolecules present in biofilm exudates. Yet many studies have used this technique to distinguish the heat-labile (mostly proteins) and heat-stable (i.e., carbohydrates and lipids) macromolecules from biofilm exudates that induce larval settlement (Bao et al., 2007a; Fitt et al., 1990; Satuito et al., 1997; Unabia & Hadfield, 1999). For instance, the larval settlement-inhibitory compounds for ascidian (*Ciona intestinalis*) and barnacle (*Balanus amphitrite*) larvae, which was obtained from biofilm exudates of *Pseudoalteromonas tunicata*, were shown to be heat-stable and found to contain carbohydrate molecules (Holmström et al., 1998; Holmström et al., 1992).

A different approach for the characterisation of bacterial cues is based on chemical properties of the biofilm. Separation and identification of chemical compounds can be achieved through a range of fractionation techniques (e.g., crude, sub-sample, sequential, and lipid fractionations) and chemical treatments (e.g., enzyme treatment). Such methods generally allow for characterisation of broad molecular types such as carbohydrates, proteins, and lipids responsible for the inductive cue. Carbohydrates and related molecules from bacterial exudates have been reported to induce settlement of invertebrate larvae (Steinberg et al., 2002). Bacterial carbohydrates are known to attract and non-covalently bind to larval lectins (sugar binding proteins). This binding activity initiates a settlement response for a number of marine invertebrate larvae (Bao et al., 2007b; Khandeparker et al., 2003; Kirchman et al., 1982b; Maki & Mitchell, 1985). Although specific bacterial proteins

have not been successfully characterised as any settlement/metamorphosis cue, several studies have suggested the role of protein and/or associated molecules in the larval settlement of polychaete (Lau & Qian, 2001; Unabia & Hadfield, 1999), ascidians (Szewzyk et al., 1991), oysters (Fitt et al., 1990) and barnacles (Khandeparker et al., 2002). Furthermore, lipids (fatty acids and glycolipids) from bacteria also have been reported to induce larval settlement (Schmahl, 1985). For example, Leitz and Wagner (1993) reported that lipids extracted from the bacteria, *Alteromonas espejiana*, induced larval metamorphosis of the hydroid, *Hydractina echinata*.

Previously, *P. canaliculus* larvae were shown to settle in response to natural and artificial substrates under field and laboratory settings (Alfaro, 2005, 2006a, 2006c; Alfaro & Jeffs, 2002, 2003; Alfaro et al., 2004; Buchanan & Babcock, 1997). In addition, synthetic chemical cues have been shown to induce larval settlement in this mussel species (Alfaro et al., 2006; Gribben et al., 2011; Young et al., 2011). Mono-species bacterial biofilms and biofilm exudates of *Macrococcus* sp., AMGM1 and *Bacillus* sp. AMGB1 also have been reported to induce settlement of *P. canaliculus* larvae (Ganesan et al., 2010). The inductive cue was found to be associated with biofilm exudates (chemicals alone) and not the cells in the bacterial biofilm (Ganesan et al., 2012a). However, characterisation of biofilm exudates and the effects of the fractionated exudates on settlement of this mussel species have not been examined until now. As an initial step to address this issue, the physical and chemical properties of the inductive cue were elucidated in this study. Specifically, physical characterisation included identification of molecular weight and thermal lability of the inductive fractions. Identification of the settlement inducing

macromolecules (e.g., proteins, lipids, carbohydrates) was achieved by chemical fractionation procedures, including crude, sub-sample, sequential, and lipid fractionations and protease treatment (Figs. 6.1–6.4). These fractionation procedures were adopted to confirm the chemical nature of the inductive cues, and to also negate the possibility of other undetected settlement-inducing macromolecules. This novel approach allowed for systematic elimination of molecules that clearly did not induce larval settlement, and identification of classes of molecules that conclusively induced settlement. This approach incorporated routine and inexpensive techniques, contrasting the use of high-end analytical instrumentation previously used to generate databases of potential cues. Moreover, the objective of the study was not to characterise and catalogue all molecules within the biofilm exudates, but to understand the differences in larval settlement responses to specific chemicals within single-species bacterial biofilms. From an aquaculture perspective, such economic procedures can be adopted to obtain specific molecules from biofilm exudates, which may enhance larval settlement under hatchery conditions. For this purpose, mono-species biofilm exudates were characterised and the fractionated exudates were tested for their ability to induce *P. canaliculus* larval settlement.

6.3 Materials and Methods

Marine bacteria (*Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1) were isolated from the guts of mussels and from seawater, as described previously (chapter 3). Biofilm exudates of these two bacteria were subjected to physical (molecular weight fractionation and heat treatment) and chemical (crude fractionation, sub-sample fractionation, sequential fractionation, lipid fractionation and protease treatment) methods of characterisation (Fig. 6.1). The treated exudates,

along with untreated exudates and sterile control plates, were tested for their ability to induce settlement of mussel larvae (*P. canaliculus*).

6.3.1 Preparation of biofilm exudates

Small scale (≤ 50 ml) biofilm exudates were prepared, as described in chapter 5. For large scale (≥ 50 ml) biofilm exudates preparation, mono-species bacteria were grown on marine agar 2216 (Zobell), and isolated colonies were amplified by transferring the colonies to 100 ml of marine broth. Bacterial cultures were maintained on a rotary platform set to 100 rpm at 37°C. After 24 hours, 1ml of the culture containing 10^6 to 10^7 cells ml^{-1} was transferred to a polystyrene Petri plate (90 × 15 mm), and 9 ml of peptone (0.5%) made up in 0.45 µm filtered, autoclaved seawater (AFSW) was added. Fifty replicate plates were established for each bacterial species, and were maintained at 37°C for 24 hours to allow biofilm formation. The unattached cells were removed from each plate by washing thrice with a total of 30 ml of AFSW. The attached biofilm cells were removed by adding 1 ml of AFSW and gently scraping using glass cover slips. A total volume of 50 ml containing cells from 50 plates were pooled and filtered through a 0.45 µm membrane filter. The retentate containing cells was discarded and the filtrate containing biofilm exudates was again filtered through a 0.22 µm syringe filter to obtain a cell-free extract.

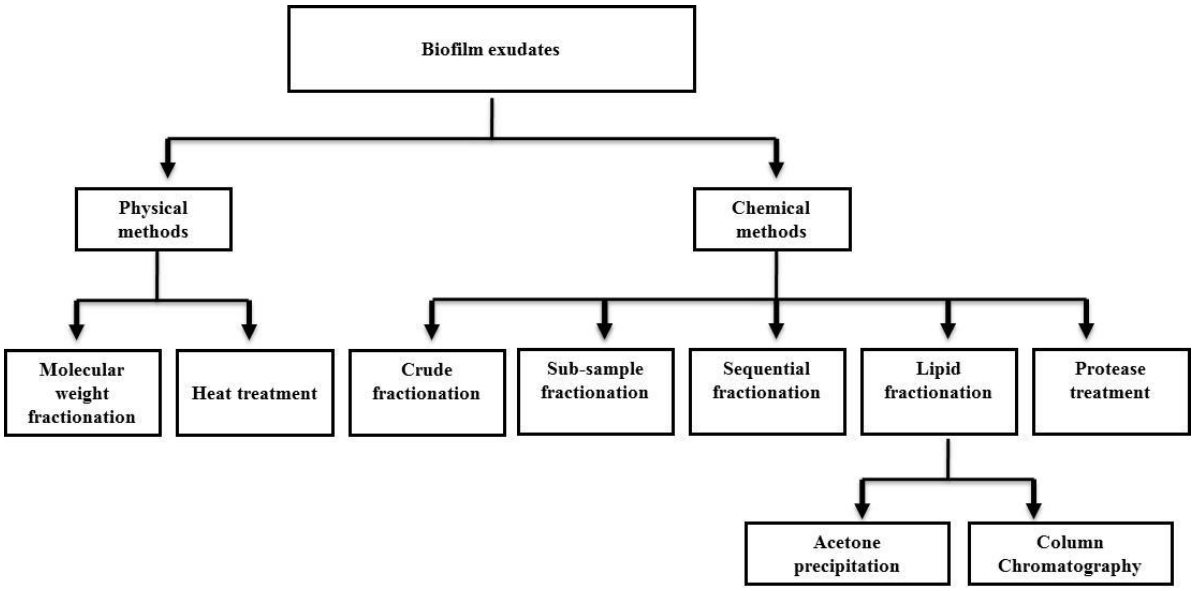

Figure 6.1: Experimental design for the characterisation of biofilm exudates of mono-species marine bacteria, showing the physical and chemical methods used.

6.3.2 Molecular weight fractionation of biofilm exudates

Biofilm exudates were size-fractionated based on molecular weight by centrifugal filtration. Amicon® Ultra-15 (Millipore Corporation, U.S.A) centrifugal filters with a molecular weight cut-off of 3, 10, 30, and 50 kDa were used to fractionate biofilm exudates of *Bacillus* sp. AMGB1 and *Macrococcus* sp. AMGM1. Also, Macrosep® centrifugal filters (Omega^{TM} membrane; Pall Corporation, U.S.A) with a molecular cut-off of 1 and 3 kDa were used to fractionate biofilm exudates of *Macrococcus* sp. AMGM1 alone. A sequential centrifugation procedure was carried out with a total of 80 ml of biofilm exudates using Amicon® filters and 40 ml of biofilm exudates using Macrosep® filters. The filtrates obtained from each high molecular weight filter were loaded to the subsequent low molecular weight filter. Each centrifugal filter received 10 ml of biofilm exudates and was centrifuged according to the manufacturer's instructions. After centrifugation, the lower

reservoir contained 9.95–9.98 ml of the filtrate and the upper reservoir contained 200–500 µl of the retentate. For the settlement assay, the filtrate and retentate were made up to 10 ml with AFSW and tested for their ability to induce settlement.

6.3.3 Heat treatment of biofilm exudates

A total of 40 ml of exudates were distributed equally to four 10 ml test tubes. The tubes were placed in four different water baths maintained at temperatures of 30, 50, 70 and 100°C, respectively. After 60 minutes, the tubes were cooled down to 17°C, and used in the settlement assay.

6.3.4 Chemical treatments of biofilm exudates

Biofilm exudates were exposed to various chemical treatments and characterised by three different experiments, namely: crude fractionation, sub-sample fractionation and sequential fractionation (Figs. 6.2–6.4). Samples with only AFSW also underwent the same chemical treatment and these fractions were used as controls.

6.3.5 Crude fractionation

A total of 50 ml of biofilm exudates and AFSW were used in this experiment. Of the total volume, 20 ml were used in the precipitation of proteins, 20 ml were used for extraction of lipids and the remaining 10 ml were used to degrade carbohydrates from biofilm exudates and AFSW (Fig. 6.2).

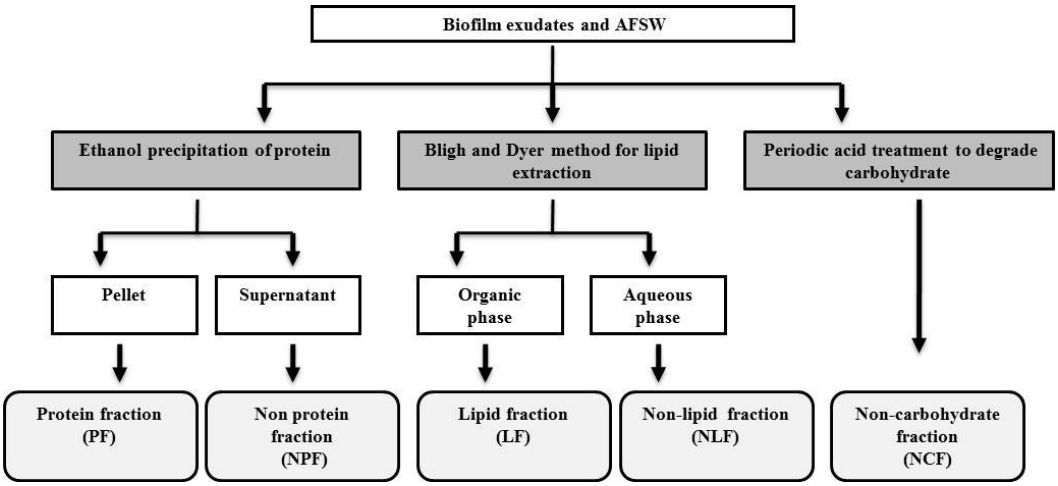

Figure 6.2: Protocol for crude fractionation of biofilm exudates. Five fractions (protein, non-protein, lipid, non-lipid, non-carbohydrate) were tested for their ability to induce settlement.

6.3.5.1 Ethanol precipitation of protein

A modified protocol from Bollag and Edelstein (1991) was used in the ethanol precipitation of proteins from biofilm exudates. Briefly, beakers containing 20 ml of biofilm exudates and AFSW were placed in an ice bath with a magnetic stirrer. Equal volumes of chilled (-20°C) absolute ethanol (99%) were added to each beaker and mixed thoroughly by gentle stirring for 10−15 minutes. Treated exudates were transferred to 50 ml centrifuge tubes and centrifuged at 10,000 × g for 10 minutes at 5°C (Sorvall® RC5C centrifuge, Dupont, U.S.A.). The residual ethanol present in the pellets and supernatant was evaporated under nitrogen gas. The pellets with the protein fraction (PF) and the supernatant containing the non-protein fraction (NPF) were finally re-suspended in 10 ml of AFSW and tested for their ability to induce larval settlement.

6.3.5.2 Bligh and Dyer method for lipid extraction

A micro-scale version of the Bligh and Dyer method (Bligh & Dyer, 1959) was used to extract lipids from biofilm exudates. For every 1 ml of exudates, 3.75 ml of chloroform-methanol (1:2, v/v) was added and vortexed well. Then, 1.25 ml of chloroform was added and vortexed. Finally, 1.25 ml of AFSW was added, vortexed and the whole sample was transferred to a 50 ml centrifuge tube. The treated exudates were centrifuged at 3000 × g for 5 minutes at room temperature. Approximately 90% of the aqueous (top and middle) phase was recovered with the help of a Pasteur pipette and transferred into a test tube. In addition, about 90% of the organic (bottom) phase was recovered by inserting a Pasteur pipette through the top phase by applying gentle positive-pressure (bubbling) so that the top phase did not enter the pipette. The contents of the organic phase were transferred into another centrifuge tube and the solvents were completely evaporated under nitrogen gas. The dried organic phase was dissolved with 2 ml of dimethyl sulfoxide (DMSO) to increase miscibility in seawater. To this mixture, 8 ml of AFSW were added to achieve a final volume of 10 ml. For the settlement assay, 10 ml of aqueous phase (i.e., lipid fraction [LF]) and 10 ml of the organic phase (i.e., non-lipid fraction [NLF]) were tested for larval settlement induction.

6.3.5.3 Periodic acid treatment to degrade carbohydrates

A solution of 2 ml of 0.5 M periodic acid was added to a 50 ml conical flask containing 10 ml of exudates. The solutions were mixed thoroughly and covered with aluminium foil and incubated in the dark at room temperature (24°C). After 1 hour, pH was adjusted to 8.2 ± 0.1 with 2 M sodium hydroxide. The solution was

filtered through a 0.22 µm filter and tested for settlement induction. The fraction was termed as non-carbohydrate fraction (NCF).

6.3.6 Sub-sample fractionation

Another set with 50 ml of untreated biofilm exudates and AFSW was used in the fractionation protocol (Fig. 6.3). The untreated exudates and AFSW were subjected to ammonium sulphate precipitation, as described below. In order to remove ammonium sulphate, molecules ≤ 3 kDa also were removed and hence a sub-sample of the exudates (molecules ≥ 3 kDa) was available for characterisation. At the end of each extraction procedure, 10 ml of the fraction were retrieved and a total of five unique fractions were tested for induction of larval settlement.

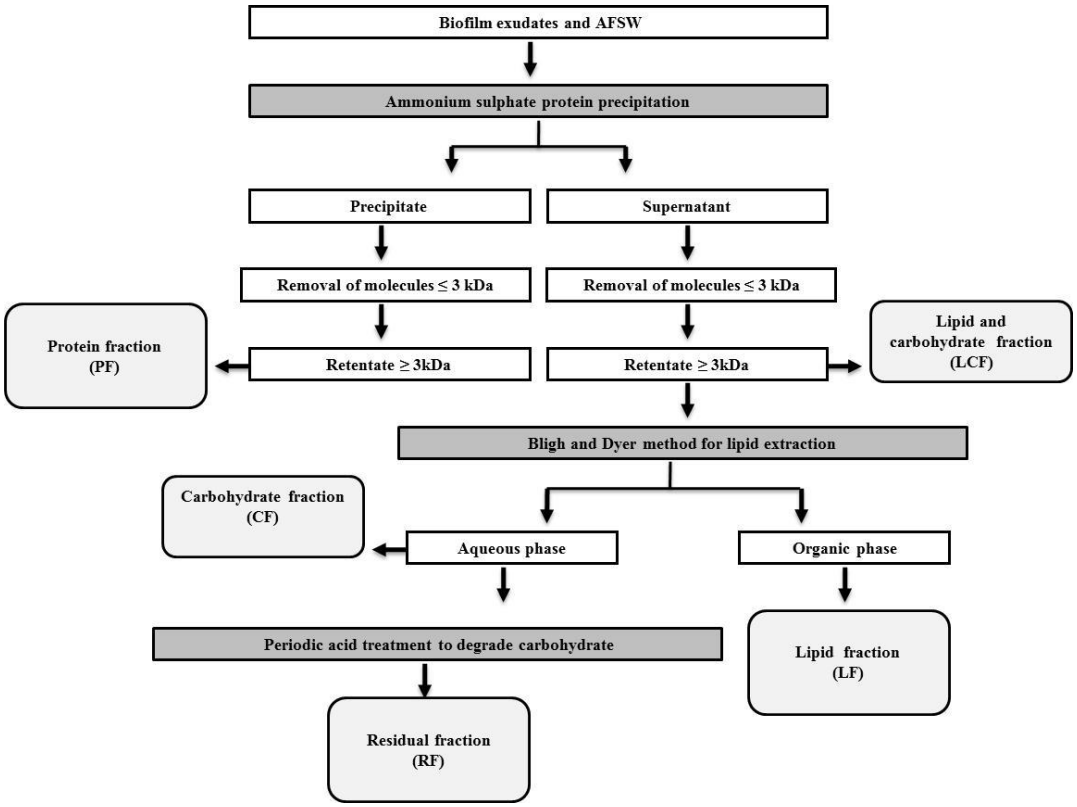

Figure 6.3: Protocol for sub-sample fractionation of biofilm exudates. Five fractions (protein, lipid-carbohydrate, lipid, carbohydrate, lipid and residual) were tested for their ability to induce settlement.

6.3.6.1 Ammonium sulphate precipitation of proteins

Biofilm exudates were treated with ammonium sulphate to precipitate proteins, as described by Bollag and Edelstein (1991). Briefly, exudates and AFSW were maintained at 5°C prior to protein precipitation. A total of 50 ml of biofilm exudates and AFSW were distributed equally to five 50 ml beakers and were placed in a cooling bath on top of a magnetic stirring plate. Proteins were precipitated by adding 50% (3.14 g) of ammonium sulphate to each beaker and stirring gently. This step was completed within 5–10 minutes. However, solutions were left for an additional 30 minutes at 5°C to aid thorough mixing of the salt. The suspensions were transferred to five centrifuge tubes and spun at 10,000 × g for 10 minutes at 5°C. The pellets and supernatants were withdrawn and pooled from all five centrifuge tubes. The pellets were re-dissolved in 50 ml of seawater and only10 ml of this solution was used for the settlement assay (the remaining 40 ml were discarded). Then, the re-dissolved pellets were de-salted through a 3 kDa centrifugal filter and centrifuged according to the manufacturer's instructions (Amicon® Ultra-15, Millipore, USA). The filtrate containing ammonium sulphate salts and molecules ≤ 3 kDa was discarded. The retentate containing the protein fraction (PF) ≥ 3 kDa was made up to 10 ml with AFSW and tested for induction of settlement. Similarly, 50 ml of the supernatant also was passed through a 3 kDa centrifugal filter. The retentate obtained from the centrifugal filter was made up to 50 ml with AFSW. This fraction was expected to contain lipids and carbohydrates (LCF), and 10 ml of this fraction was tested for settlement induction.

The remaining 40 ml of the LCF were subjected to the Bligh and Dyer method for lipid extraction in order to obtain 20 ml of aqueous phase and 20 ml of

organic phase, respectively. The organic phase containing the lipid fraction (LF) was tested for settlement. From 20 ml of the aqueous phase containing the carbohydrate fraction (CF), 10 ml were used to test for settlement and the remaining 10 ml were treated with periodic acid to degrade carbohydrates. The residual fraction (RF) obtained from this step also was tested for settlement induction.

6.3.7 Sequential fractionation

A total of 10 fractions were obtained through sequential fractionation from 100 ml of untreated exudates and AFSW (Fig. 6.4). All the fractions were tested for induction of settlement. The untreated exudates and AFSW (100 ml) were subjected to the Bligh and Dyer method for lipid extraction. The lipid extraction procedure resulted in a total of 50 ml of organic phase and 50 ml of aqueous phase, which were used for the subsequent fractionation procedures. From 50 ml of the organic phase containing the non-polar fraction (NpolF), 10 ml were tested for settlement induction, 30 ml were subjected to ethanol precipitation of proteins and the remaining 10 ml was subjected to periodic acid treatment to degrade non-polar carbohydrates. From 30 ml of NpolF subjected to ethanol precipitation, the pellets were obtained from 10 ml of the NpolF fraction and the supernatant was obtained from 20 ml NpolF. The pellet-containing lipoprotein fraction (LPF1) and supernatant (10ml) containing glycolipid fraction (GLF) were tested for induction of settlement. Periodic acid treatment was performed on the remaining 10 ml of NpolF and 10 ml of GLF to obtain the lipid and lipoprotein fraction 2 (LPF2) and the non-polar lipid fraction (NpolLF), respectively.

Similar fractionations also were carried out on 50 ml of the aqueous phase. Then, 10 ml from each of the resulting five fractions (see below) were tested for

larval settlement. The aqueous phase, which contained the polar fraction (PolF), was subjected to ethanol precipitation and periodic acid treatment. The ethanol precipitation of the PolF yielded pellets containing the protein fraction (PF), and supernatants containing the non-protein fraction (NPF). The periodic acid treatment on PolF and NPF yielded two fractions – residual fraction 1 (RF1) and residual fraction 2 (RF2).

6.3.8 Lipid fractionation

A lipid fractionation was obtained by two methods, namely acetone precipitation and column chromatography.

6.3.8.1 Acetone fractionation

Non-polar (organic) fractions from 20 ml of bacterial exudates and AFSW were obtained by the Bligh and Dyer method of lipid extraction, as described previously. The fractions were evaporated under nitrogen at 30–40°C and concentrated to 200–300 µl. To this, 5–6 ml (20 volumes) of cold acetone were added and transferred to a 15 ml centrifuge tube (Kates, 1986). Solutions were vortexed for 10 seconds, placed on ice for 1 hour and spun at 2500 × g for 3–5 minutes at 5°C. Pellets containing polar lipids were washed twice with 1 ml of cold acetone, cooled on ice and centrifuged, as described above. The washed pellets were then evaporated under nitrogen and desiccated over KOH. Supernatants obtained from each washing step were pooled and evaporated to dryness under nitrogen at 30°C. Solvent-free supernatant fractions and dried pellets were dissolved in a 2 ml of DMSO and topped up with 8 ml of AFSW and tested for induction of settlement.

6.3.8.2 Column chromatography

Micro-scale flash column chromatography was performed using a Pasteur pipette containing activated silicic acid to separate lipid fractions (Kates, 1986). 1ml of non-polar fraction from 30 ml of biofilm exudates and AFSW were prepared, as described above. The 1ml chloroform sample was loaded on to the column and eluted with 1 ml of chloroform. The eluent was collected and labelled "chloroform fraction". Similarly, 1ml of acetone followed by 1 ml of methanol was added to the column and the two fractions were individually collected. All three fractions were evaporated completely under nitrogen. To each of the three fractions, 2 ml of DMSO were added, followed by 8 ml of AFSW, and the resulting solution tested for settlement induction.

6.3.9 Enzyme treatment of biofilm exudates

The enzyme papain (BDH laboratory, 390 IU mg^{-1}) obtained from papaya juice was used to treat the biofilm exudates of *Macrococcus* sp. AMGM1. A concentration of 0.1 mg ml^{-1} was added to 10 ml of biofilm exudates and AFSW. The mixture was incubated at 34°C for 1 hour. For the settlement assay, 1 ml of the treatment was added to a 9 ml of AFSW containing larvae and the final enzyme concentration was 0.01 mg ml^{-1}.

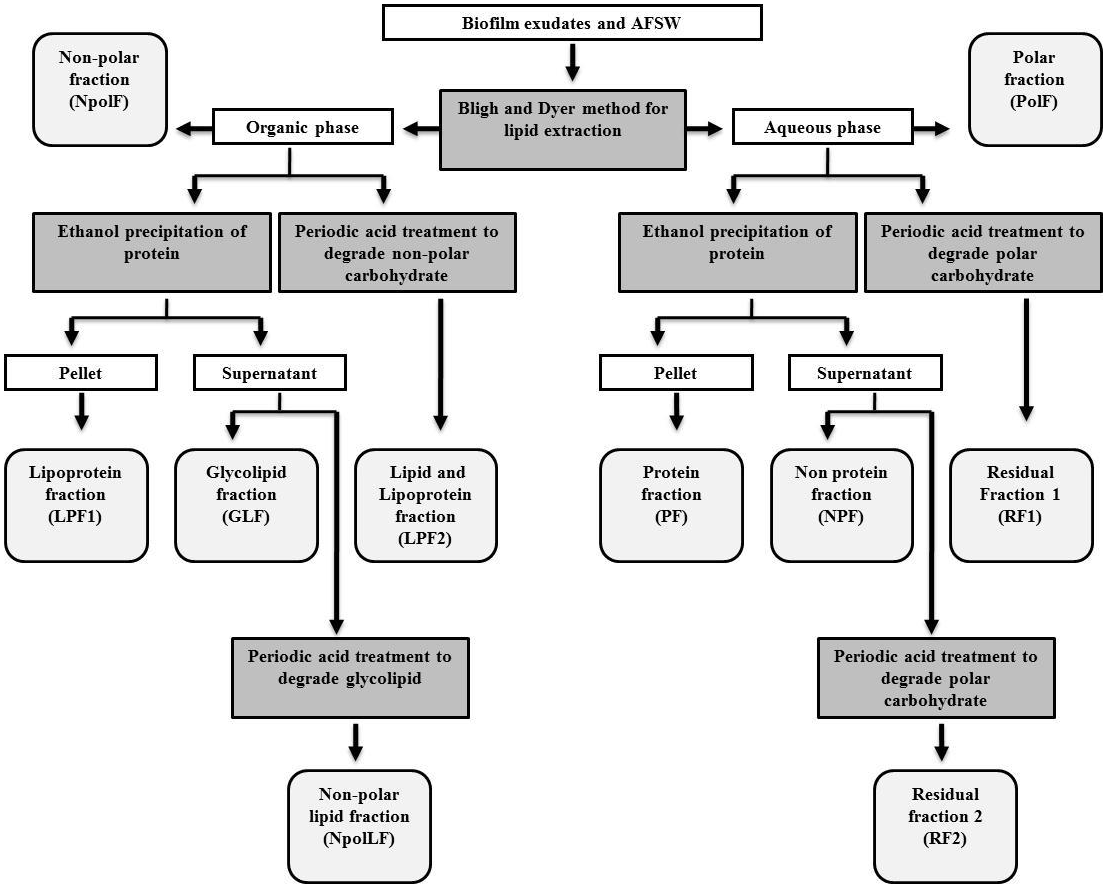

Figure 6.4: Protocol for sequential fractionation of biofilm exudates. Ten fractions (non-polar, lipoprotein fraction 1, Glycolipid fraction, lipoprotein fraction 2, non-polar lipid, polar, protein, non-protein, residual fraction 1 and residual fraction 2) were tested for their ability to induce larval settlement.

6.3.10 Larval settlement assays

Swimming larvae (19–21 days old) were obtained from Shellfish Production and Technology New Zealand Limited (SPATnz), Nelson, New Zealand, and were used in the settlement experiments. Larval maintenance and transport is as described in chapter 2. Briefly, polystyrene Petri plates (60 mm diameter × 14 mm depth) were used as the settlement substrates, with 10 replicates per treatment plus controls. Each plate received a final density of 20–30 larvae ml^{-1} and was maintained at 17°C throughout the assay. The treatment plates received 8 ml of AFSW, 1 ml of larvae, and 1 ml of treated biofilm exudates or treated AFSW. One set of controls consisted

of 9 ml of AFSW with 1 ml of swimming larvae. Another set of controls consisted of 8 ml of AFSW, 1ml of untreated biofilm exudates and 1ml of swimming larvae.

Settlement was observed after a period of 48 hours under a dissection microscope with 20 × magnification. Larvae were scored live-settled, live-unsettled and dead as described in chapter 2.

Data for the percentages of live settled, live unsettled, and dead larvae were arcsine transformed prior to statistical analyses. All statistics were performed using Predictive Analysis Software (PASW®) Statistics 18. Data obtained from molecular weight, heat treated, acetone precipitation, column chromatography and enzyme treated fractions of biofilm exudates were tested for and met all parametric assumptions. Then, these data were evaluated using one-way ANOVAs and *post-hoc* Tukey tests. Data obtained from crude, sub-sample and sequential fractionation of biofilm exudates also satisfied parametric assumptions, and were subjected to separate two-way ANOVAs (chemical treatments and exudates as fixed factors) for each bacterium. *Post-hoc* Tukey tests were conducted for pair-wise comparisons.

6.4 **Results**

6.4.1 Molecular weight fractionation

Three molecular weight fractions of 0–3, 3–10 and 10–30 kDa obtained from biofilm exudates of *Macrococcus* sp. AMGM1 were found to significantly induce greater (over 70%) settlement compared with sterile controls (Fig. 6.5 A; Tables 6.1 and 6.2). Also, a Tukey test resulted in non-significant differences ($p > 0.001$) between the three (0–3, 3–10 and 10–30 kDa) molecular weight fractions and unfractionated biofilm exudates of *Macrococcus* sp. AMGM1. The settlement

percentages (mean% ± S.E) of 30–50 and > 50 kDa molecular weight fractions of *Macrococcus* sp. were as low as 5.3 ± 1.0 and 7.3 ± 1.5, respectively, whereas that of unfractionated biofilm exudates and < 30 KDa fractions ranged from 78.8–83.5%. A Tukey test revealed non-significant differences between controls and 30–50 and > 50 kDa fractions of *Macrococcus* sp., indicating that fractions over 30 kDa from *Macrococcus* sp. did not induce larval settlement. Further characterisation of the 0–3 kDa fraction of *Macrococcus* sp. biofilm exudates (data not shown) indicated that the inductive fraction was between 1–3 kDa (80.5 ± 2.1) and not < 1 kDa (7.8 ± 0.9). The 1–3 kDa fraction and unfractionated *Macrococcus* sp. biofilm exudates significantly induced greater settlement compared with the sterile controls (ANOVA; $F_{3,36} = 535.6$, $p < 0.001$).

Two settlement inducing fractions of 10–30 and 30–50 kDa were obtained by fractionating biofilm exudates of *Bacillus* sp. AMGB1 (Fig. 6.5 B; Tables 6.1 and 6.2). A Tukey test resulted in non-significant ($p > 0.001$) differences between 10–30 and 30–50 kDa fractions and unfractionated biofilm exudates of *Bacillus* sp. Also, non-significant ($p > 0.001$) low settlement levels (< 12%) were observed between controls and molecular weight fractions between 0–3 kDa, 3–10 kDa and over 50 kDa.

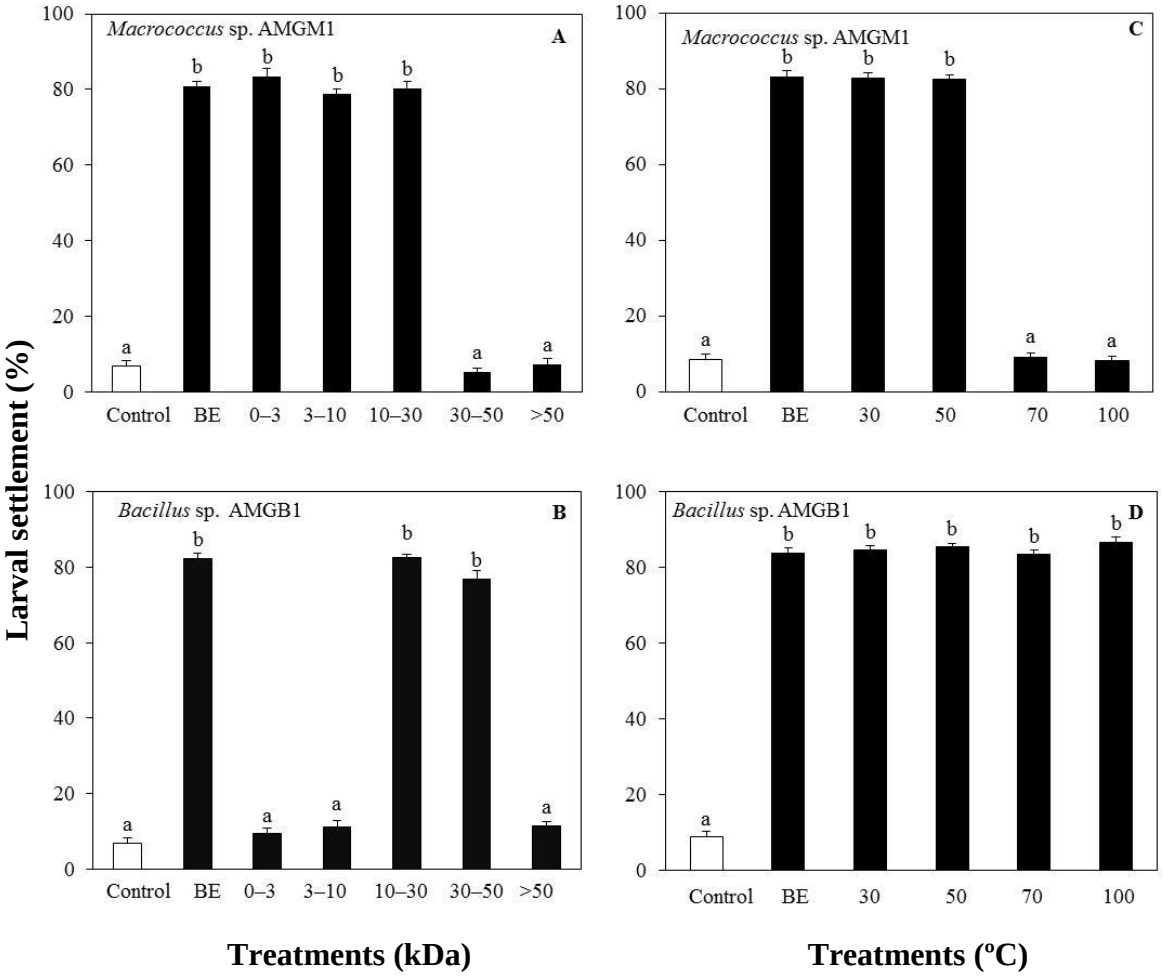

Figure 6.5: Effect of fractionated (physical) exudates on larval settlement. Data represent mean (±SE, n = 10) larval settlement of *P. canaliculus* after 48 hours on polystyrene Petri plates containing molecular weight fractions (0–3, 3–10, 10–30, 30–50 and > 50 kDa) and heat-treated (30, 50, 70 and 100°C) biofilm exudates (BE) of *Macrococcus* sp. AMGM1 (top row) and *Bacillus* sp. AMGB1 (bottom row). The open bars represent sterile control plates and shaded bars represent the treatment plates. Non-significant Tukey tests are denoted by the same letter over the bars.

6.4.2 Crude fractionation

Results from the crude fractionation of *Macrococcus* sp. AMGM1 suggest that the settlement cue is likely to be a protein, as the protein fraction (PF), non-lipid fraction (NLF) and non-carbohydrate fraction (NCF) induced significantly greater (over 70%) settlement (Fig. 6.6 A; Tables 6.1 and 6.3). Tukey tests resulted in non-significant ($p > 0.05$) differences between untreated exudates (controls) when compared with PF, NLF and NCF. Low settlement rates of 6.6 ± 1.8 were observed in the non-protein fraction (NPF) compared with 78.2 ± 2.5 in the PF. Furthermore, the lipid fraction (LF) of *Macrococcus* sp. exudates significantly (ANOVA; $\alpha = 0.05$; $p = 0.007$) induced greater settlement compared with NPF, indicating the possibility that a lipid and/or a lipoprotein cue induced larval settlement. However, LF induced only to 60% settlement and was significantly different ($p < 0.05$) when compared with controls, PF, NLF and NCF.

Conversely, the settlement cue from *Bacillus* sp. AMGB1 was likely to be a lipid and/or a carbohydrate molecule, with the highest settlement rates of 80.3 ± 1.8% observed in LF and the lowest settlement rate of 6.9 ± 1.5 for NCF (Fig. 6.6 B; Tables 6.1and 6.3). The settlement rates for PF and NPF were 7.0 ± 1.4 and 79.2 ± 1.5, respectively, which negated the possibility of the inductive cue being a protein. Tukey tests revealed non-significant ($p > 0.05$) differences between controls, NLF and NPF, and also between PF, NLF and NCF.

6.4.3 Sub-sample fractionation

Settlement cues with molecular weight over 3 kDa were shown to induce a maximum settlement rate of 82.7 ± 1.3 in the protein fraction (PF) for *Macrococcus*

sp. AMGM1, indicating the relevance of protein molecules for larval settlement (Fig. 6.6 C; Tables 6.1and 6.3). A Tukey test revealed non-significant ($p > 0.05$) differences between controls and PF. Moreover, another settlement cue or a part of the same molecule was found in the lipid fraction (LF) of the biofilm exudates, which induced a settlement rate of 55 ± 2.5. However, Tukey tests detected significant ($p < 0.05$) differences between LF when compared with controls and PF. These results indicated the presence of protein molecules as mediators for larval settlement. The lipid carbohydrate fraction (LCF), carbohydrate fraction (CF) and residual fraction (RF) of the exudates induced only up to 20% settlement. Hence, the settlement cues obtained from *Macrococcus* sp. AMGM1 may not be a carbohydrate, glycolipid and other residual molecules (e.g., humic substances).

The LCF and LF of ≥ 3 kDa from *Bacillus* sp. AMGB1 induced greater (over 70%) settlement, indicating the possibility of the cue being a carbohydrate and or a lipid molecule (Fig. 6.6 D; Tables 6.1and 6.3).Tukey tests revealed non-significant ($p > 0.05$) differences between controls when compared with LCF and LF. Significant ($p < 0.05$) low (< 20%) settlement rates were observed in PF, CF and RF, when compared to controls. Hence, the cue may not be a protein, polar carbohydrate or other residual molecules.

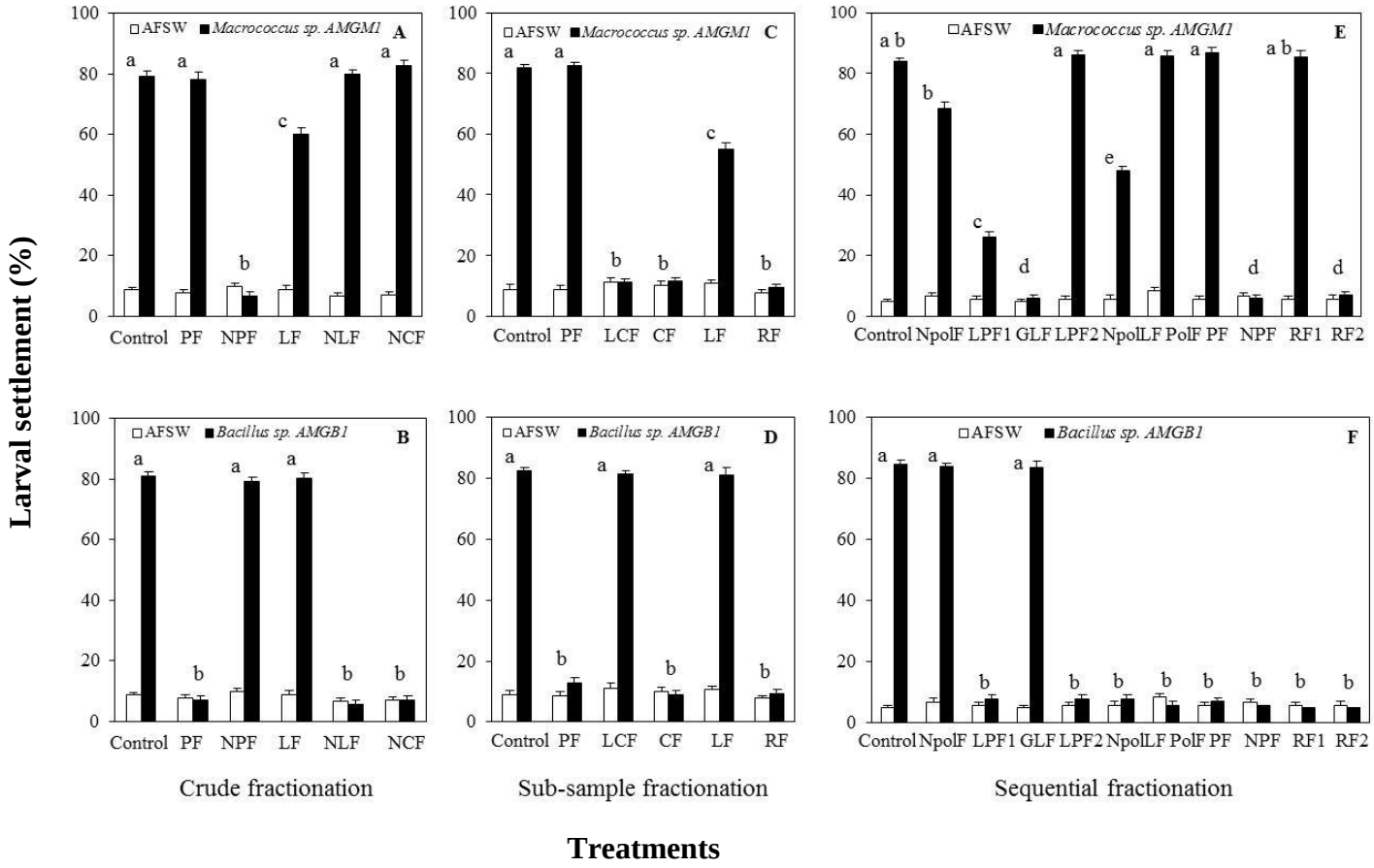

Figure 6.6: Effect of fractionated (chemical) exudates on larval settlement. Data represent mean (±SE, n = 10) larval settlement of *P. canaliculus* after 48 hours on polystyrene Petri plates containing autoclaved filtered seawater (AFSW) together with biofilm exudates of *Macrococcus* sp. AMGM1(top row) and *Bacillus* sp. AMGB1 (bottom row) subjected to crude fractionation (graphs on left), sub-sample fractionation (graphs in middle), and sequential fractionations (graphs on right). The open bars represent AFSW and shaded bars represent the biofilm exudates subjected to the above chemical treatments. The control plates contained untreated AFSW and biofilm exudates. Non-significant Tukey tests within (biofilm exudates) are denoted by the same letter over the bars.

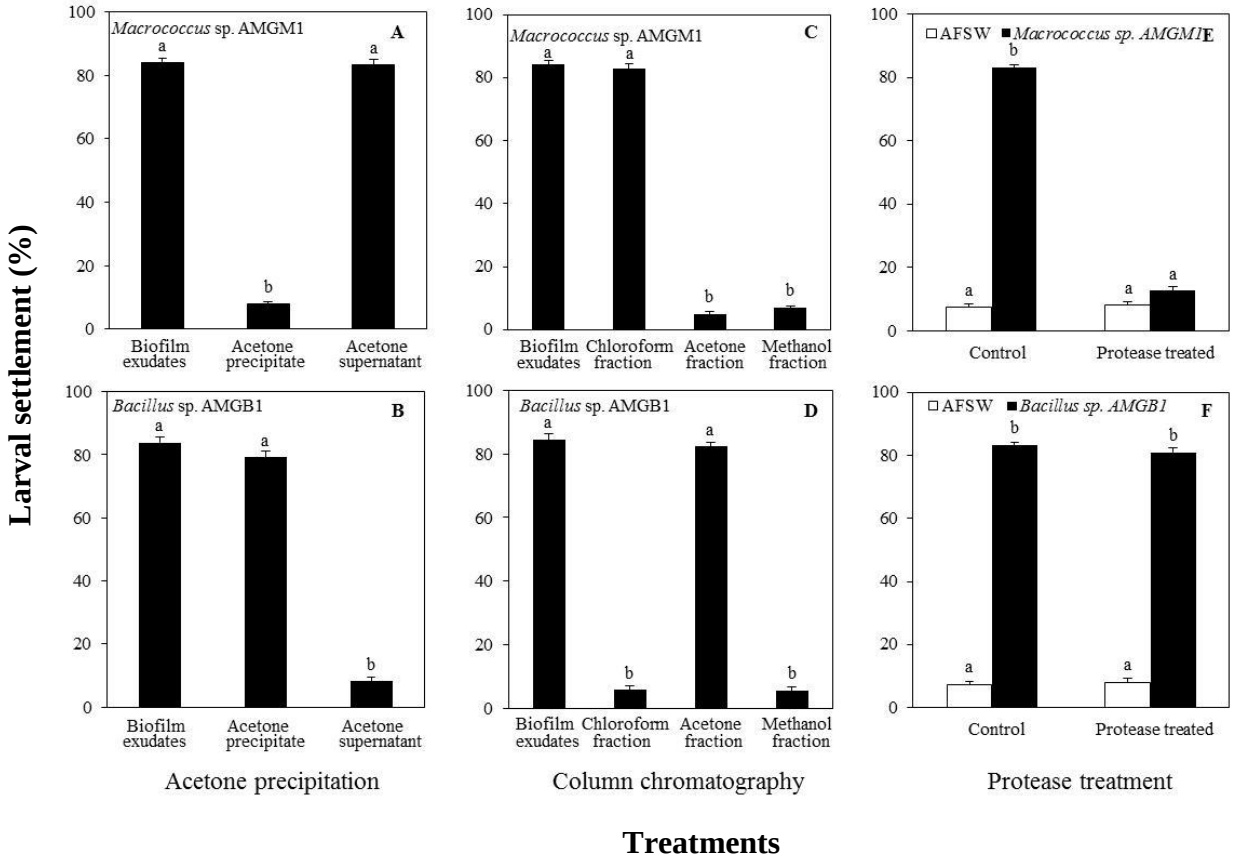

Figure 6.7: Effect of fractionated (chemical) exudates on larval settlement. Data represent mean (±SE, n = 10) larval settlement of *P. canaliculus* after 48 hours on polystyrene Petri plates containing biofilm exudates of *Macrococcus* sp. AMGM1 (top row) and *Bacillus* sp. AMGB1 (bottom row) subjected to acetone precipitation (graphs on left), column chromatography (graphs in middle) and protease treatment (graphs on right). For the protease treatment graphs (E & F), the open bars represent autoclaved filtered seawater (AFSW) and shaded bars represent the biofilm exudates. The control plates contain untreated AFSW and biofilm exudates. Non-significant Tukey tests within (biofilm exudates) are denoted by the same letter over the bars.

Table 6.1: Results for the physical and chemical methods for characterising biofilm exudates of *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1. Larval settlement rates of up to 80% were observed unless stated otherwise.

Methods	Settlement inducing fractions for *Macrococcus* sp. AMGM1	Settlement inducing fractions for *Bacillus* sp. AMGB1
Physical Methods		
Molecular weight fractionation	• Fractions with1−3, 3−10 and 10–30 kDa induced settlement.	• Fractions with 10–30 and 30–50 kDa induced settlement.
Heat treatment	• Biofilm exudates treated at 30º and 50ºC induced settlement. • Exudates treated at 70 and 100ºC did not induce settlement. • **Inductive cue may be a protein.**	• Biofilm exudates treated at temperature from 30 up to 100ºC induced settlement. • **Inductive cue may be a carbohydrate and/or a lipid.**
Chemical Methods		
Crude fractionation	• Protein fraction, non-lipid fraction and non-carbohydrate fraction induced settlement. • Lipid fraction induced over 60% settlement. • **Inductive cue is likely to be a protein and/or a lipid.**	• Non protein fraction and lipid fraction induced settlement. • Non-carbohydrate fraction did not induce settlement. • **Inductive cue is likely to be a carbohydrate and/or a lipid.**
Sub-sample fractionation	• Protein fraction induced settlement. • Lipid fraction induced over 60% settlement. • **Inductive cue is likely to be a protein, lipid and/or a lipoprotein.**	• Lipid-carbohydrate fraction and lipid fraction induced settlement. • **Inductive cue likely a carbohydrate, lipid and/or a glycolipid.**
Sequential fractionation	• Lipid and lipoprotein fraction 2 induced settlement. • Non-polar lipid (65%), lipoprotein fraction 1 (>20%) and non-polar lipid fraction (50%) induced settlement. • Polar fraction, protein fraction and residual fraction 1 induced settlement. • **Inductive cue is a non-polar lipoprotein and polar protein.**	• Non-polar fraction and glycolipid fraction induced settlement. • **Inductive cue is a glycolipid.**
Lipid fractionation (Acetone precipitation)	• Acetone supernatant fraction induced settlement. • **Inductive cue is a neutral lipid (e.g., lipoprotein)**	• Acetone precipitate fraction induced settlement. • **Inductive cue is a polar lipid (e.g., glycolipid).**
Lipid fractionation (Column chromatography)	• Chloroform fraction induced settlement. • **Inductive cue may be a lipoprotein.**	• Acetone fraction induced settlement. • **Inductive cue may be a glycolipid.**
Protease treatment	• Papain treated exudates did not significantly induce settlement. • **The inductive cue is a protein.**	• Papain treated exudates had no effect on settlement. • **The inductive cue is not a protein.**

6.4.4 Sequential fraction

The lipid and lipoprotein fraction 2 (LPF2) and protein fraction (PF) obtained from biofilm exudates of *Macrococcus* sp. AMGM1 were determined as two potential cues for mussel larval settlement (Fig. 6.6 E; Tables 6.1 and 6.3). Results show that both LPF2 (organic phase) and PF (aqueous phase) induced over 70% settlement, and were not significantly ($p > 0.05$) different from controls. However, settlement rates of LPF2 were significantly ($p < 0.05$) different from the lipoprotein fraction 1 (LPF1) present in the organic phase, indicating major differences in the extraction procedures. Also, the settlement rate for LPF2 (86.2 ± 1.4) was significantly ($p < 0.05$) greater than the original non-polar fraction (NpolF; 68.2 ± 2.2) indicating the possible presence of analogous carbohydrate molecules in NpolF that may have affected settlement. The non-polar lipid fraction (NpolLF) achieved a greater settlement rate (47.8 ± 1.6) when compared with the glycolipid fraction (GLF) which achieved a lower settlement rate (6.0 ± 1.3) prior to the removal of carbohydrates. The non-polar lipid fraction (NpolLF) induced a settlement rate of 47.8 ± 1.6, which was significantly different ($p < 0.05$) from the rates in LPF2 and controls. This indicated that non-polar lipids may have a limited role as inducers for larval settlement. On the other hand, the aqueous phase containing polar proteins, as observed in polar fraction (PolF), protein fraction (PF) and residual fraction (RF1), were able to induce significantly ($p < 0.05$) greater (over 70%) settlement when compared with non-protein fraction (NPF) and residual fraction (RF2). Tukey tests revealed non-significant ($p > 0.05$) differences between controls and PolF, PF, RF1, indicating the relevance of polar proteins in exudates of *Macrococcus* sp. AMGM1 as potential inducers of larval settlement.

Conversely, the glycolipid fraction (GLF) obtained from *Bacillus* sp. AMGB1 significantly induced greater settlement rates of 83.6 ± 2.1 (Fig. 6.6 F; Tables 6.1 and 6.3). Tukey tests revealed non-significant differences ($p > 0.05$) between GLF when compared with its original NpolF and controls. However, GLF induced significantly ($p < 0.05$) greater settlement compared with corresponding organic phase fractions (LPF1, LPF2 and NpolF) and aqueous phase fractions (PolF, PF, NPF, RF1 and RF2). These results clearly indicate that lipoproteins, lipids and polar compounds obtained from exudates of *Bacillus* sp. have no role in inducing settlement of larvae.

6.4.5 Lipid fractionation

Results from the acetone precipitation of biofilm exudates from *Macrococcus* sp. AMGM1 clearly indicate that a neutral lipid (e.g., lipoproteins) is a potential cue for larval settlement. In addition, the acetone supernatant fraction induced significantly ($p < 0.001$) greater settlement rates of 83.4 ± 1.6 when compared with the acetone precipitate fraction with 7.8 ± 0.9 (Fig. 6.7 A; Tables 6.1 and 6.4). Furthermore, a Tukey test revealed non-significant differences ($p > 0.001$) between untreated biofilm exudates and the acetone supernatant fraction. However, the acetone precipitate fraction of *Bacillus* sp. AMGB1 significantly induced greater (over 70%) settlement compared with the acetone supernatant fraction, which induced < 20% settlement (Fig. 6.7 B; Tables 6.1 and 6.4). Non-significant differences ($p > 0.001$) were observed in acetone precipitate fraction and untreated biofilm exudates. These results indicate the presence of polar lipids (e.g., glycolipids) as potential cues for larval settlement.

Results obtained from column chromatography experiments were in accordance with results from the acetone precipitation for both species of bacteria. The chloroform fraction of *Macrococcus* sp. AMGM1, significantly induced greater (> 70%) settlement ($p < 0.001$) when compared with corresponding acetone and methanol fractions, which induced settlement by up to 20% (Fig. 6.7 C; Tables 6.1 and 6.2). Non-significant ($p > 0.001$) settlement rates were observed between the chloroform fraction and untreated biofilm exudates of *Macrococcus* sp. However, acetone fractions of *Bacillus* sp. AMGB1 significantly induced settlement rates of 82.3 ± 1.4, when compared with corresponding chloroform and methanol fractions (< 6% settlement) (Fig. 6.7 D; Tables 6.1 and 6.4). Moreover, a Tukey test detected non-significant ($p > 0.001$) differences between the acetone fraction and untreated biofilm exudates of *Bacillus* sp.

6.4.6 Protease treatment

Protease treated biofilm exudates of *Macrococcus* sp. AMGM1 significantly ($p < 0.001$) induced lower settlement rates (12.5 ± 1.4) when compared with untreated biofilm exudates, which achieved higher (83.1 ± 0.9) settlement rates (Fig. 6.7 E; Tables 6.1 and 6.4). These results highlight the relevance of proteins as cues for larval settlement. In addition, low settlement rates (8.1 ± 1.1) were observed in autoclaved filtered seawater (AFSW), indicating that the enzyme papain has no role in larval settlement induction for this species. However, untreated and protease treated biofilm exudates of *Bacillus* sp. AMGB1 significantly induced greater settlement (over 70%) compared with untreated and treated AFSW (Fig. 6.7 F; Tables 6.1 and 6.4). A Tukey test revealed non-significant ($p > 0.001$) differences between untreated and enzyme treated biofilm exudates of *Bacillus* sp. AMGB1.

6.4.7 Larval mortality

Larval mortality of < 15% was observed throughout all the experiments. For each bacterium (*Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1), individual *post-hoc* Tukey tests confirmed non-significant differences between treatments and controls for each experiment (Tables 6.2–6.4; data not shown).

6.5 **Discussion**

Chemically mediated ecological interactions are prevalent in marine environments, and larval settlement is no exception (Rittschof & Bonaventura, 1986; Steinberg et al., 2002; Zimmer & Butman, 2000). Chemical settlement cues of natural origin emanate from a wide variety of sources, such as multi-species biofilms (Hung et al., 2009), host algae (Swanson et al., 2006), conspecifics (Matsumura et al., 1998) and prey species (Hadfield & Pennington, 1990), to name a few. Chemical settlement cues from laboratory-grown mono-species bacteria, such as *Pseudoalteromonas* sp., which induced settlement in coral larvae, have been successfully characterised (Tebben et al., 2011). However, no study to date has attempted to characterise chemical settlement cues of bacterial origin for marine bivalves. In this study, settlement-inducing chemical cues for *P. canaliculus* larvae from exudates of mono-species biofilms of *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1 were characterised to the lowest possible class of molecules based on the physical and chemical methods used.

Chemical cues in biofilm exudates often are present in very low concentrations, which makes it difficult to obtain sufficient amounts to conduct larval settlement assays (Hadfield & Paul, 2001). In addition, the purification and characterisation of the specific molecules responsible for the cues is extremely

challenging (Hadfield & Paul, 2001). To address these issues, the current study describes an innovative, comprehensive step-by-step approach to identify classes of molecules responsible for induction of larval settlement. The identity of the specific molecules can then be obtained by advanced techniques, such as liquid chromatography-mass spectrometry and protein sequencing of the particular chemical fractions. However, it is important to note that even if a biofilm inductive molecule is chemically well-defined, it is difficult to interpret which chemoreception pathway is involved in the resulting settlement process.

Previous studies have indicated that pediveliger larvae of *P. canaliculus* settle in response to bacterial biofilms and biofilm exudates of mono-species marine bacteria (Ganesan et al., 2010). Those results highlighted the importance of water soluble chemical cues as settlement inducers for *P. canaliculus* larvae. Studies with other marine invertebrate species have resulted in similar findings (Bao et al., 2007b; Fitt et al., 1990; Khandeparker et al., 2006; Neumann, 1979; O'Connor & Richardson, 1998; Satuito et al., 1997). In the present study, the settlement inducing fractions for *P. canaliculus* larvae were found to be of 1–3, 3–10 and 10–30 kDa for *Macrococcus* sp. AMGM1, and that of *Bacillus* sp. AMGB1 were of 10–30 and 30–50 kDa molecular size. The positive results with different fractions suggest that they may be either dissociable cues from a larger molecule, or separate inductive cues (Steinberg et al., 2002). Similarly, results from the studies with the mussel *Mytilus galloprovincialis* suggested that the settlement inducing fraction from *Alteromonas* sp.1was < 3 kDa and > 5 kDa for bacterium C1 (from the *Pseudomonas-Alteromonas* group) (Bao et al., 2007a; Satuito et al., 1997). Such studies highlight

the importance of specific molecular weight fractions in bacterial exudates as inducers of larval settlement across mussel species.

Apart from molecular weight analyses, another common physical method to partially characterise settlement cues is based on susceptibility of the inductive cue to high temperatures. Many studies have suggested that the reduction in the inductive capacity of heat-treated bacterial cues may indicate the relevance of bacterial proteins for larval settlement (Fitt et al., 1990; Satuito et al., 1997; Unabia & Hadfield, 1999). However, inferences drawn from such experiments may not indicate the loss of protein function due to heating, since some proteins of bacterial origin are known to withstand high temperatures (e.g., over 90°C; Somera,1995). Larman et al. (1982) reported the presence of protein molecules, stable at temperatures above 100°C, isolated from adult barnacles and causing settlement of conspecific larvae. However, in this study, when biofilm exudates of *Macrococcus* sp. AMGM1 were heated above 70°C, they did not induce settlement of *P. canaliculus* larvae, and this may indicate that the cue is one or more of the heat-labile protein molecules. Similar results were observed in the waterborne conspecific adult cue of the shore crab, *Hemigrapsus sanguineus*. The metamorphosis inducing cue was heat-labile and later found to contain protein molecules (Anderson & Epifanio, 2009; Anderson et al., 2010). Unlike *Macrococcus* sp. AMGM1, the settlement cues from exudates of *Bacillus* sp. AMGB1 were heat-stable at 70 and 100°C, and later confirmed to contain carbohydrate molecules. Generally, heat-stable compounds (i.e., carbohydrates, lipids) obtained from bacteria serve as deterrents for larval settlement (Holmstrom et al., 2002; Lau & Qian, 2000; Zapata et al., 2007). For example, Dobretsov and Qian (2004) identified that heat-stable

carbohydrate molecules from *Vibrio* sp. and from an α-Proteobacterium inhibited settlement of both polychaete (*Hydroides elegans*) and bryozoan (*Bugula neritina*) larvae. On the other hand, settlement cues for the mussel, *Mytilus galloprovincialis* have been suggested to be associated with 'glycoprotein or exopolysaccharide' of the bacterium *Alteromonas* sp. 1, although further experiments are essential to confirm these results (Bao et al., 2007b). The term 'bacterial exopolysaccharide' often can be misleading, as it not only refers to carbohydrate molecules released by bacteria but also to the extracellular material/exudates of bacterial biofilms (Costerton et al., 1995). For example, Szewzyk et al. (1991) suggested that settlement cues obtained from 'exopolysaccharide' (exudates) of *Pseudomonas* sp. strain 9 for ascidian larvae was likely to be a protein and not a carbohydrate, as incorrectly reported by Hung et al. (2009). Other than the current study, no other bacterial exudates containing heat-stable carbohydrate molecules have been reported to induce settlement of marine bivalve larvae. Nonetheless, microalgal cues that induce settlement of queen conch, (*Strombus gigas*) and polychaete (*Hydroides elegans*) larvae have been found to comprise heat-stable carbohydrate molecules (Boettcher & Targett, 1996; Lam et al., 2005).

While the physical methods used in this study were crude techniques compared with the chemical methods, this multi-technique approach was essential to partially elucidate the settlement cues for *P. canaliculus*. In addition, the incorporation of three complementary fractionation pathways to deduce chemical cues from the biofilm exudates provides a useful approach for future studies. Results from these characterisation methods were in agreement with one another. Based on the chemical characterisation methods, two settlement cues isolated from

Macrococcus sp. AMGM1 were characterised as a non-polar lipoprotein and a polar protein. From an ecological perspective, protein molecules have been known to facilitate various behavioural responses, including settlement, owing to their frequent occurrence in marine environments (Rittschof, 1990; Rittschof & Cohen, 2004). For example, naturally occurring polar peptides isolated from crude extracts of adult polychaetes (*Hydroides elegans*) and bryozoans (*Bugula neritina*) were able to induce settlement in corresponding larvae (Harder & Qian, 1999). Also, amino acids of natural and synthetic origin have been analysed for their ability to induce settlement of larvae of oysters (Zimmer-Faust & Tamburri, 1994) polychaetes (Beckmann et al., 1999; Jin & Qian, 2005; Zimmer-Faust & Tamburri, 1994) and barnacles (Mishra & Kitamura, 2000). Moreover, protein molecules of natural origin containing carbohydrate moieties also have been known to induce settlement of marine larvae (Dreanno et al., 2006; Larman et al., 1982). However, results from the current study clearly indicate that carbohydrates and/or their associated molecules obtained from *Macrococcus* sp. AMGM1 did not induce settlement in larvae of this mussel species and resulted in the lowest settlement rate (< 20%), similar to sterile controls. This result was evident in non-protein fractions (NPF) containing carbohydrate molecules, obtained by crude fractionation procedures. A similar result was noted in the lipid-carbohydrate (LCF), carbohydrate (CF) fractions (sub-sample fractionation), and the aqueous phase containing the non-protein fraction (sequential fractionation), which also failed to induce over 20% settlement in larvae.

An interesting observation is the occurrence of lower settlement rates (between 20–70%) in all organic phase fractions, except for the lipoprotein fraction 2 and untreated exudates (sequential fractionation). One possible explanation for these

results is that there could have been antagonistic substances present, such as non-polar carbohydrate molecules (i.e., glycolipids), which may have interfered with larval settlement. Although evidence of such antagonistic compounds within natural biofilms has been discussed in the past (Hung et al., 2009), potential producers of these compounds have not been identified thus far. The non-polar fraction (NpolF) obtained from the sequential fractionation induced up to 70% larval settlement. Conversely, NpolF treated with periodic acid to degrade glycolipids resulted in a lipid and lipoprotein fraction 2 (LPF2), which induced up to 80% settlement, indicating the absence of antagonistic carbohydrate molecules in LPF2. Similarly, the non-polar lipid fraction (NpolLF) induced up to 50% larval settlement, whereas the glycolipid fraction (GLF) prior to degradation of carbohydrate molecules induced less than 20% settlement. Another potential reason for differences in settlement rates between LPF1 and LPF2 (sequential fractionation) could be the dissociation of molecules as a result of differences in chemical procedures adopted during sequential fractionation. During the separation procedure, lipoprotein molecules from the original NpolF would have dissociated into non-polar protein (LPF1) and lipid (NpolLF) molecules, and would have remained stable in the LPF2 fraction. This interpretation is in agreement with the settlement rates result of 30% (LPF1) and 50% (NpolLF), which add up to the 80% observed in LPF2. The dissociation of molecules also was evident in the lipid fraction (LF) obtained from both crude and sub-sample fractionation, which induced up to 60% settlement when compared with the up to 80% settlement rates observed in untreated exudates. Dissociation of lipoproteins due to differences in chemical extraction protocols have been reported in the past (Scanu & Edelstein, 1971). Nonetheless, with the aid of protease treatment and column chromatography, protein and associated molecules from

biofilm exudates of *Macrococcus* sp. AMGM1 were found to be important for the settlement of *P. canaliculus* larvae.

In contrast, protein molecules of *Bacillus* sp. AMGB1 demonstrated no role in settlement of mussel larvae. The settlement cue was consistently found in the non-protein, lipid and carbohydrate fractions, strongly indicating the relevance of glycolipid molecules for larval settlement. Similarly, a glycolipid from a marine bacterium belonging to the family Micrococcaceae was found to induce stolon settlement of the jelly fish, *Aurelia aurita* (Schmahl, 1985). Furthermore, glycolipids from the green algae *Ulvella lens*, were found to induce settlement in the sea urchin, *Strongylocentrotus intermedius* larvae (Takahashi et al., 2002). Apart from glycolipids, carbohydrate molecules obtained from microalgae and associated biofilms have been known to induce larval settlement in sea slugs (Krug & Manzi, 1999), polychaetes (Lam et al., 2005) and sea urchins (Williamson et al., 2000).

Overall, the characterisation techniques presented in this study provide strongly corroborated evidence that the settlement of *P. canaliculus* larvae varied in response to unique chemical fractions from biofilm exudates of mono-species bacteria. A non-polar protein and a polar protein from *Macrococcus* sp. AMGM1, and a glycolipid from *Bacillus* sp. AMGB1, were found to induce larval settlement. Further studies could be undertaken to test amino acid and glycolipid sequences and/or to further characterise the inductive cues with the aid of high-end analytical techniques. However, for most applied aquaculture purposes, this level of cue characterisation may be sufficient to develop cultivation enhancement techniques. Finally, the combination of techniques used in this study provides a novel and cost-

effective approach to characterise larval settlement cues from a range of natural products.

Table 6.2: Statistical analyses (one-way ANOVA with Tukey test; $\alpha = 0.05$) for *P. canaliculus* larval settlement on biofilm exudates of *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1, subjected to molecular weight fractionation and heat treatment. Significant tests ($p < 0.001$) are in bold.

	Molecular weight fractionation				Heat treatment			
	df	MS	*F*	*p*	*df*	MS	*F*	*p*
Macrococcus sp. AMGM1								
% Settlement	6	7624.2	264.8	**0.001**	5	7245.1	391.2	**0.001**
% Mortality	6	29.0	1.7	0.143	5	42.3	1.1	0.347
Bacillus sp. AMGB1								
% Settlement	6	6543.3	258.5	**0.001**	5	4266.2	321.8	**0.001**
% Mortality	6	36.2	1.2	0.229	5	3.9	0.1	0.996

Table 6.3: Statistical analyses (two-way ANOVA with Tukey test; α = 0.05) for *P. canaliculus* larval settlement on biofilm exudates of *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1, subjected to crude, sub-sample and sequential fractionation. Significant tests ($p < 0.05$) are in bold.

	Crude fractionation				Sub-sample fractionation				Sequential fractionation			
	df	MS	*F*	*p*	*df*	MS	*F*	*p*	*df*	MS	*F*	*p*
Macrococcus sp. AMGM1 (% Settlement)												
Chemical treatment (CT)	5	1841.3	70.3	**0.001**	5	2597.8	116.9	**0.001**	10	3052.1	104.3	**0.001**
Exudates (E)	1	41932.9	1599.9	**0.001**	1	14211.5	639.5	**0.001**	1	60746.1	2076.6	**0.001**
CT×E	5	2304.5	87.9	**0.001**	5	2830.3	127.4	**0.001**	10	3000.7	102.6	**0.001**
Error	108	26.2			108				198	29.3		
Macrococcus sp. AMGM1 (% Mortality)												
CT	5	7.1	0.2	0.973	5	54.0	1.5	0.186	10	8.7	0.2	0.998
E	1	0.1	0.0	0.955	1	20.2	0.6	0.450	1	17.7	0.4	0.548
CT×E	5	38.5	0.9	0.473	5	27.0	0.8	0.577	10	12.0	0.2	0.991
Error	108	42.0			108	35.3			198	49.0		
Bacillus sp. AMGB1(% Settlement)												
CT	5	4182.9	143.6	**0.001**	5	3540.8	108.3	**0.001**	10	2985.8	92.9	**0.001**
E	1	15474.6	531.2	**0.001**	1	17112.4	523.2	**0.001**	1	12191.2	379.2	**0.001**
CT×E	5	3450.8	118.5	**0.001**	5	3239.5	99.0	**0.001**	10	3197.3	99.4	**0.001**
Error	108	29.1			108	32.7			198	32.2		
Bacillus sp. AMGB1(% Mortality)												
CT	5	6.2	0.1	0.985	5	46.9	1.2	0.303	10	7.9	0.2	0.998
E	1	4.0	0.1	0.773	1	3.4	0.1	0.766	1	22.8	0.5	0.480
CT×E	5	27.0	0.6	0.727	5	29.6	0.8	0.572	10	11.0	0.2	0.992
Error	108	47.8			108	38.3			198	45.5		

Table 6.4: Statistical analyses (One-way ANOVA with Tukey test; α = 0.05) for *P. canaliculus* larval settlement on biofilm exudates of *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1, subjected to acetone fractionation, column chromatography and protease treatment. Significant tests ($p < 0.001$) are in bold.

	Acetone precipitation				Column chromatography				Protease treatment			
	df	MS	*F*	*p*	*df*	MS	*F*	*p*	*df*	MS	*F*	*p*
Macrococcus sp. AMGM1												
% Settlement	2	8467.7	745.8	**0.001**	3	9282.8	670.9	**0.001**	3	5932.2	446.2	**0.001**
% Mortality	2	18.4	0.5	0.640	3	0.6	0.01	0.998	3	6.4	1.0	0.442
Bacillus sp. AMGB1												
% Settlement	2	8087.4	342.5	**0.001**	3	9572.0	366.6	**0.001**	3	8129.1	698.5	**0.001**
% Mortality	2	17.6	0.5	0.641	3	1.1	0.02	0.995	3	10.5	1.1	0.351

Chapter 7.

THE EFFECTS OF FRACTIONATED BIOFILM EXUDATES OF *PSEUDOALTEROMONAS* SP. AMGP1 ON THE SETTLEMENT OF *PERNA CANALICULUS* LARVAE

7. The Effects of Fractionated Biofilm Exudates of *Pseudoalteromonas* sp. AMGP1 on the Settlement of *Perna canaliculus* Larvae

7.1 Abstract

The biofilm exudates of the bacterium *Pseudoalteromonas* sp. AMGP1, previously shown to cause high (over 70%) larval mortality were selected for the present study. The chemical identity of the biofilm toxin was investigated by subjecting the biofilm exudates to physical (molecular weight fractionation and heat treatment) and chemical fractionation methods (crude fractionation, sequential fractionation, lipid fractionation and enzyme treatment) and testing the fractions for settlement of *P. canaliculus* larvae. Using this comprehensive multi-technique approach, the bacterial biofilm toxin was identified as a heat labile (> 70°C) polar protein molecule of < 1kDa in size. During the settlement assays, a fraction was found to induce over 70% larval settlement when compared with sterile controls and untreated exudates. Based on the fractionation methods, the settlement inducing molecule was found to be a heat labile non-polar lipoprotein of 30–50 kDa. These results indicated the presence of both settlement and toxic cues within the exudates of this mono-species bacterial biofilm. However the lack of settlement when larvae were exposed to the untreated exudates indicates that there is an antagonistic effect between the toxin and the settlement inducting molecule. Such complex and diverse (settlement inductive and toxic effects) properties of the biofilm exudates were revealed through the systematic comprehensive approach described in the present study. The ecological relevance of these molecules in bacterial-larval interactions is discussed for *Pseudoalteromonas* sp. AMGP1.

7.2 Introduction

Chemical cues are critical mediators of interactions among-species in the marine environment (Steinberg et al., 2002; Zimmer & Butman, 2000). In fact, bacteria rely on chemical cues to successfully colonise a newly submerged marine substrata (Dobretsov et al., 2009). Bacterial colonies often aggregate with a wealth of micro-organisms (i.e., fungi, protozoa and microalgae) to form distinct biofilms on surfaces (Pasmore & Costerton, 2003; Wahl, 1989). These biofilms have an important role in shaping the subsequent settlement of macro-algal spores and invertebrate larvae (Wahl, 1989). For many sessile invertebrate larvae, settlement is a key process that results in the transition between planktonic and benthic life phases. This settlement inductive process often appears to be mediated by signalling molecules which not only determine settlement success but may determine the individual's survival (Hadfield & Paul, 2001).

Bacteria are a major constituent of biofilm communities and are widely known to initiate larval settlement processes (Hadfield, 2011). Conversely, the bacterial biofilms of several species have been shown to inhibit settlement of many invertebrate larvae, such as polychaetes (Dobretsov & Qian, 2004), barnacles (Maki et al., 1990) and mussels (Alfaro et al., 2011b; Ganesan et al., 2010). These bacteria belong to a variety of genera (e.g., *Alteromonas*, *Bacillus*, *Vibrio* and *Micrococcus*), and their antifouling properties may not necessarily correlate with their phylogenetic relationship (Dobretsov & Qian, 2004; Lau et al., 2002). However, several closely related species of *Pseudoalteromonas* have been reported to produce diverse antifouling compounds (Egan et al., 2000). For example, *P. tunicata* have been analysed extensively for their settlement inhibiting properties on microalgae, fungi,

invertebrate larvae and other species of bacteria (Egan et al., 2001b; Holmström et al., 1998; Holmström & Kjelleberg, 1999; Rao et al., 2007). Also, *Pseudoalteromonas citrea* and *P. ulvae* have been shown to inhibit settlement of polychaete (*Hydroides elegans*) and barnacle (*Balanus amphitrite*) larvae (Holmström et al., 2002). However not all species of *Pseudoalteromonas* inhibit larval settlement (Lau et al., 2002; Lee & Qian, 2003). Indeed, *P. luteoviolacea* was found to enhance by more than 80% the rate of metamorphosis in the sea urchin (*Heliocidaris erythrogramma*) larvae when compared with controls (Huggett et al., 2006). However the chemical nature of the inductive cues and/or toxins has not been investigate to any great extent. This lack of information presents a gap in the current understanding of how the specific characteristics of these molecules and/or settlement- inducing and inhibiting cues operate to determine larval settlement behaviour.

The ability of certain *Pseudoalteromonas* species to induce or inhibit larval settlement has been attributed to their pigmentation. Thus only the deep-pigmented bacterial strains have been suggested to contain antifouling properties (Egan et al., 2002). For example, Huang et al. (2011) reported that the red-pigmented *Pseudoalteromonas* sp. sf57 inhibited settlement (< 20%) of polychaete (*Hydroides elegans*) larvae whereas, the corresponding white-pigmented mutant strain induced up to 80% larval settlement. Also, purified pigments (e.g., cycloprodigiosin, violacein, tambjamines) from *Pseudoalteromonas* sp. have been suggested to have anti-bacterial and anti-larval properties (Franks et al., 2005; Soliev et al., 2011). Nonetheless, anti-bacterial compounds also have been isolated from various non-

pigmented *Pseudoalteromonas* strains, indicating that antifouling compounds are not restricted to pigmented strains alone (Vynne et al., 2011).

Like bacterial pigments, other chemicals from bacterial exudates are produced during their biofilm phase, and have been found to contain ecologically relevant molecules for marine invertebrate larvae (Egan et al., 2001a; Egan et al., 2002). For example, Tebben et al (2011) isolated the chemical tetrabromopyrrole from biofilm exudates of *Pseudoalteromonas* sp. J010, which induced coral (*Acropora millepora*) larval metamorphosis. In addition, the biofilm exudates of *Pseudoalteromonas tunicata* were found to contain heat-stable, polar carbohydrate molecules of < 500 kDa which inhibited larval settlement of the barnacle, *B. amphitrite* and the ascidian, *C. intestinalis* (Holmström et al., 1998; Holmström et al., 1992). Since bacterial exudates contain a range of macromolecules, such as proteins, carbohydrates, lipids, nucleic acids and humic substances (Stoodley et al., 2002), a settling organism (e.g., bacteria, algal spores, larvae) may settle in response to a molecule with a specific chemistry (Hadfield, 2011). Moreover, the data provided in Table.7.1 clearly indicate that antifouling compounds obtained from *Pseudoalteromonas* sp. are likely to share their chemical characteristics and every bacterial-larval interaction has a unique chemical language that needs to be unravelled. This resulted in the need to systematically characterise biofilm exudates of *Pseudoalteromonas* sp. AMGP1 and to identify the chemistry of biofilm toxin that induces larval mortality. The characterisation procedures adopted in this study have been previously used to analyse the effect of fractionated biofilm exudates of *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1 on the settlement of *Perna canaliculus* larvae (Ganesan et al., 2012).

To elucidate the role of various bioactive compounds in exudates of *Pseudoalteromonas* sp. AMGP1, a unique set of characterisation procedures were used to test the effect of these characterised fractions on the settlement of *P. canaliculus* larvae. Biofilm exudates were initially fractionated based on physical methods by molecular weight analysis and susceptibility to temperatures between 30–50°C. To understand the chemical nature of the bacterial cue various chemical fractionation methods (i.e., crude fraction, sequential fractionation, lipid fractionation, and protease treatment) were also adopted. All characterisation processes were carried out using routine, inexpensive techniques in order to generate sufficient fraction amounts to conduct larval settlement assays. Such methods can also be used to elucidate the chemistry of signalling molecules in bacteria-mediated fouling or antifouling processes.

7.3 Materials and Methods

The marine bacterium *Pseudoalteromonas* sp. AMGP1 was isolated from the green seaweed *Ulva lactuca*, and it was identified using the 16S rRNA techniques as described in (chapter 3). The biofilm exudates from the biofilm formed by this bacterial species were characterised broadly based on physical (molecular weight fractionation and heat treatment) and chemical (crude fraction, sequential fractionation, lipid fractionation and protease treatment) fractionation methods.

Biofilm exudates from *Pseudoalteromonas* AMGP1 were prepared as described in (chapter 6). In order to obtain 100 ml of biofilm exudates, biofilm cells of *Pseudoalteromonas* sp. AMGP1 were cultured on 100 polystyrene Petri plates. Each plate contained 1 ml of log-phase bacteria (10^6 to 10^7 cells ml^{-1}) and 9 ml of (0.5%) peptone-enriched autoclaved filtered seawater (AFSW). The plates were

maintained on a rotary incubator (100 rpm at 37°C to aid biofilm formation. After 24 hours, the unattached cells were removed by washing thrice with a total of 30 ml of AFSW. The attached cells were removed by scraping with a glass cover slip (22 × 22 mm). The bacterial cells were pooled and finally suspended in 100 ml of AFSW. The biofilm cells were separated from their exudates by a two-step filtration (0.45 and 0.22 µm) process, and the exudates were used for further analyses. Detailed description of these laboratory protocols can be found in chapter 6. The volume of biofilm exudates that were exposed to various physical and chemical fractionation treatments ranged between 10–100 ml. However, for the settlement assay, a total of 10 ml of treated and untreated exudates were tested for their ability to induce settlement of *P. canaliculus* larvae.

7.3.1 Physical methods

Biofilm exudates of *Pseudoalteromonas* AMGP1 were subjected to centrifugal filtration in order to separate the cue based on molecular size. Centrifugal filters of 1 kDa (Macrosep®, Omega^{TM} membrane; Pall Corporation, U.S.A), and 3, 10, 30 and 50 kDa (Amicon® Ultra-15, Millipore Corporation, U.S.A) were used for this purpose. A sequential centrifugation in a decreasing order of magnitude (from 50 to 1 kDa) was carried out and the resultant fractions (0–1, 1–3, 3–10, 10–30, 30–50 and > 50 kDa) were tested for settlement.

For the heat treatment, biofilm exudates were individually maintained in four water baths set to 30, 50, 70 and 100°C for 60 minutes respectively. The exudates were cooled to 17°C prior to their use in the settlement assays.

7.3.2 Chemical methods

Biofilm exudates of *Pseudoalteromonas* sp. AMGP1 were exposed to various chemical (crude and sequential fractionation and trypsin treatment) to produce distinct fractions that were then incorporated in the larval settlement assay. To negate the possible settlement-inducing effect of these chemicals (i.e., chloroform, ethanol, trypsin etc.), AFSW was also exposed to similar treatments and used as controls.

7.3.2.1 Crude fractionation

Biofilm exudates and AFSW were subjected to protein precipitation (Bollag & Edelstein, 1991), lipid extraction (Bligh & Dyer, 1959) and carbohydrate degradation during this process, and the resultant fractions were tested for induction of larval settlement. Ethanol was used to precipitate proteins from 20 ml of exudates, which resulted in 10 ml protein fraction (PF) and 10 ml of non-protein fraction (NPF). The Bligh and Dyer method was used to separate lipids from 20 ml of exudates to achieve 10 ml of lipid fraction (LF) and 10 ml of non-lipid fraction (NLF). Periodic acid was used to degrade carbohydrates present in 10 ml of exudates to achieve a 10 ml of non-carbohydrate fraction (NCF).

7.3.2.2 Sequential fractionation

A sequential fractionation procedure on biofilm exudates and AFSW resulted in a total of 10 fractions (10 ml per fraction) that were tested for their ability to induce settlement of mussel larvae. Lipid extraction on 100 ml of biofilm exudates and AFSW resulted in 50 ml of non-polar fraction (NpolF) and 50 ml of polar fraction (PolF). From the 50 ml of NpolF and polF, 10 ml of the fractions were used for the settlement assay, 30 ml were subjected to ethanol precipitation and the

remaining 10 ml were treated with periodic acid. Ethanol precipitation on NpolF resulted in the pellet containing lipoprotein fraction 1 (LPF1) and the supernatant containing glycolipid fraction (GLF). The GLF and NpolF were exposed to periodic acid treatment to achieve non-polar lipid fraction (NpolLF), and lipid and another lipoprotein fraction 2 (LPF2) respectively.

Similarly, ethanol precipitation on 50 ml of PolF (from 100 ml of exudates) resulted in the pellet and the supernatant containing protein fraction (PF) and non-protein fraction (NPF) respectively. Periodic acid treatment on PolF resulted in residual fraction 1 (RF1) and on NPF resulted in residual fraction 2 (RF2).

7.3.2.3 Lipid fractionation

The organic (non-polar) phase fractions obtained from lipid extraction (Bligh and Dyer method) procedures were assigned to lipid fractionation by acetone precipitation and column chromatography (Kates, 1986). Acetone precipitation resulted in the precipitate (non-polar or neutral lipids) and the supernatant fractions (moderately polar and polar lipids) that were tested for settlement. Also, column chromatography procedures resulted in chloroform (non-polar lipids), acetone (moderately polar lipids) and methanol fractions (polar lipids) that were tested for settlement.

7.3.2.4 Enzyme treatment of biofilm exudates

The enzyme trypsin (beef pancreas, BDH laboratory, and 0.5 Anson units g^{-1}) was added to 10 ml of exudates and AFSW to achieve an initial concentration of 0.1 mg ml^{-1}. The enzyme treated mixtures were incubated at 34°C for 1 hour. For the

settlement assay, each treatment plate contained enzyme with a final concentration of 0.01 mg ml^{-1}.

7.3.3 Larval settlement assays

The settlement assays were conducted as reported in chapter 2. The treatment plates received 8 ml of AFSW, 1 ml of larvae and 1 ml of the treated exudates or treated AFSW (heat treated, molecular weight fractionated, chemical fractionated and enzyme treated). One set of controls consisted of 8 ml of AFSW and 1 ml of larvae and 1 ml of untreated biofilm exudates, and another set contained 9ml of AFSW and 1 ml of larvae. Settlement and mortality of the mussel larvae were recorded for all plates after 48 hours, using a dissection microscope (20 × magnification) as mentioned in chapter 2. All percent larval settlement and mortality data were arcsine-transformed prior to statistical analyses using the Predictive Analysis Software (PASW®) Statistics 18. The data obtained from physical treatment, lipid fractionation and enzyme treatment were subjected to one-way ANOVAs followed by *post-hoc* Tukey tests. Separate two-way ANOVAs (chemical treatments and exudates as fixed factors) along with *post-hoc* Tukey tests were conducted on the data obtained from chemical treatments (crude and sequential fractionation).

7.4 **Results**

7.4.1 Molecular weight fractionation

The biofilm exudates of *Pseudoalteromonas* sp. AMGP1 and all molecular weight fractionated exudates, except the 30–50 kDa fraction induced up to 20% settlement rates for *P. canaliculus* larvae (Fig. 7.1 A and C; Table 7.2). The 30–50

kDa fraction significantly ($p < 0.001$) induced higher settlement (over 70%) when compared with other fractionated exudates (0–30 and > 50 kDa), un-fractionated exudates, and sterile controls. However, the mortality rates (Mean% ± S.E) for the 0–3 kDa fraction and unfractionated exudates were found to be 86.0 ± 2.4 and 81.0 ± 2.4, respectively, and were significantly ($p < 0.001$) greater than the mortality rates of 8–16% observed in all fractions over 3 kDa and sterile controls (Fig. 7.1 B; Table 7.2). Initial fractionation analysis suggested the toxic molecule was ≤ 3 kDa and a Tukey test suggested non-significant differences ($p = 0.774$) between 0–3 kDa and unfractionated exudates. Subsequent fractionation (Fig. 7.1 D; Table 7.2) of the *Pseudoalteromonas* exudates revealed that the molecular weight of the toxin was between 0–1 kDa (81.9 ± 2.4) and not 1–3 kDa (8.2 ± 0.8). Moreover, a Tukey test showed significant differences ($p < 0.001$) between 0–1 kDa fraction when compared with the 1–3 kDa fractions and sterile controls, indicating that fractions > 1 kDa may have no role as toxicity for mussel larvae.

7.4.2 Heat treatment

The settlement rates for heat treated and untreated biofilm exudates of *Pseudoalteromonas* sp. AMGP1 were between 8–14%, and were not significantly ($p > 0.001$) different from the controls (Fig. 7.1 E; Table 7.2). The mortality rates for exudates subjected to 70 and 100°C were significantly lower (< 10%) when compared with higher (> 75%) mortality rates observed in untreated exudates and exudates treated at 30°C and 50°C (Fig. 7.1 F; Table 7.2). Furthermore, a Tukey test revealed non-significant differences between controls and exudates treated at 70°C ($p = 0.321$) and 100°C ($p = 0.146$), indicating that the toxic molecule was susceptible to temperatures over 70°C.

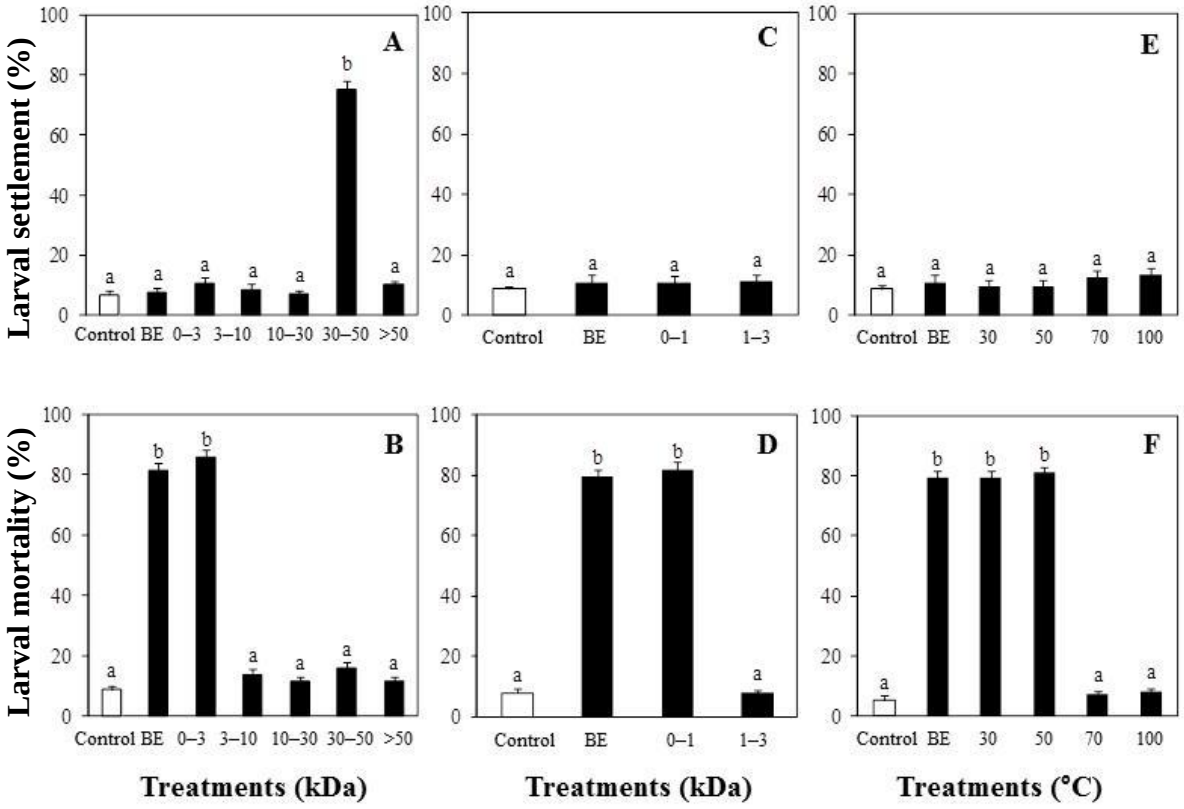

Figure 7.1: Effect of fractionated (physical) exudates on larval settlement. Data represent mean (±SE, n = 10) larval settlement (top row) and mortality (bottom row) of *P. canaliculus* after 48 hours on polystyrene Petri plates containing biofilm exudates (BE) of *Pseudoalteromonas* sp. AMGP1 subjected to molecular weight fractionation (A–D), and heat treatment (E&F). The open bars represent sterile control plates and shaded bars represent the treatment plates. Non-significant Tukey tests are denoted by the same letter over the bars.

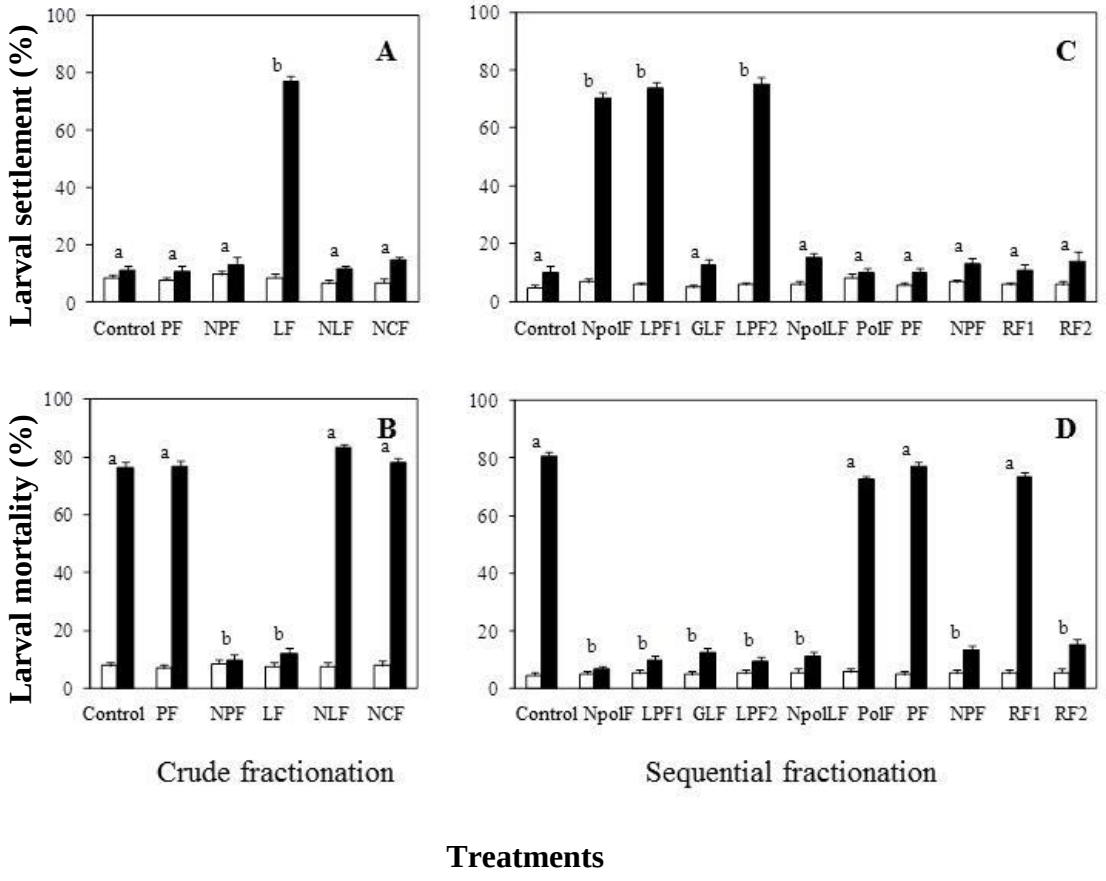

Figure 7.2: Effect of fractionated (chemical) exudates on larval settlement. Data represent mean (±SE, n = 10) larval settlement (top row) and mortality (bottom row) of *P. canaliculus* after 48 hours on polystyrene Petri plates containing autoclaved filtered seawater (open bars) along with biofilm exudates of *Pseudoalteromonas* sp. AMGP1 (shaded bars) subjected to crude fractionation (A&B) and sequential fractionations (C&D).The control plates contain untreated AFSW and biofilm exudates. Non-significant Tukey tests within (biofilm exudates) are denoted by the same letter over the bars.

7.4.3 Crude fractionation

The biofilm exudates of *Pseudoalteromonas* sp. AMGP1 subjected to the crude fractionation resulted in a lipid fraction (LF), which contained the cue for larval settlement (Fig. 7.2 A; Table 7.3). Also, significantly ($p < 0.05$) greater settlement rates were observed in LP (76.9 ± 1.8) when compared with four other fractions and controls (untreated exudates), which induced only up to 15% larval settlement. The mortality results suggested that the toxin was likely a protein and not

a lipid or carbohydrate molecule (Fig. 7.2 B; Table 7.3). The results were supported by the mortality rates (over 70%) observed in the protein fraction (PF), non-lipid fraction (NLF) and non-carbohydrate fraction (NCF). Tukey tests detected non-significant differences between controls when compared with PF ($p = 0.996$), NLF ($p = 0.906$) and NCF ($p = 1.000$). Low settlement (13.2 ± 2.5) and mortality (9.6 ± 1.8) rates were observed in the non-protein fraction (NPF), indicating that the protein molecules were responsible for both larval settlement and mortality. Non-significant ($p > 0.05$) differences were observed between NPF and LF, and both these fractions were significantly ($\alpha = 0.05$; $p = 0.001$) different from PF, NLF, NCF and controls.

7.4.4 Sequential fraction

The settlement results from sequential fractionation of biofilm exudates from *Pseudoalteromonas* sp. AMGP1 suggest that the non-polar fraction (NpolF) contained the settlement cue, and was found to be a lipoprotein molecule (Fig. 7.2 C; Table 7.3). The NpolF, lipoprotein fraction 1 (LPF1) and lipid and lipoprotein fraction 2 (LPF2) induced up to 80% larval settlement, and were significantly ($p < 0.05$) different from the controls and all other fractions. Tukey tests showed non-significant ($\alpha = 0.05$; $p = 1.000$) differences between NpolF, LPF1 and LPF2, indicating the presence of the settlement cue in all these fractions. Moreover, low settlement rates were observed in non-polar lipid fractions (NpolLF; 15.1 ± 1.7) and glycolipid fraction (GLF; 12.6 ± 2.1), which indicated that lipids and glycolipids from exudates of *Pseudoalteromonas* sp. AMGP1 may have no inductive role in the settlement of mussel larvae. Furthermore, all polar phase fractions induced between 10–14% settlement rates, and were not significantly ($p > 0.05$) different from the controls containing untreated exudates.

Results from mortality assays suggested the role of polar proteins as potential toxins for *P. canaliculus* larvae (Fig. 7.2 D; Table 7.3). Mortality rates up to 80% were found in the polar fraction (PolF), protein fraction (PF) and residual fraction 1 (RF1) and these were not significantly different ($p > 0.05$) from the untreated-exudates. Although PolF contained the toxic molecule, fractions obtained from PolF, such as non-protein fraction (NPF) and residual fraction 2 (RF2) showed lower mortality rates of 13.3 ± 1.7 and 13.9 ± 3.2, respectively. These fractions (NPF and RF2) were significantly different from the controls ($\alpha = 0.05$; $p = 0.001$) indicating that molecules other than polar proteins do not kill larvae. In accordance with the above result, all non-polar fractions induced mortality rates between 6–12%, and were significantly different from the control, PolF, PF and RF1.

7.4.5 Acetone precipitation (lipid fractionation)

Greater settlement rates (73.8 ± 1.9) were observed in the acetone supernatant fraction of the biofilm exudates, indicating the relevance of neutral lipid or non-polar lipids (e.g., lipoproteins) as potential larval settlement cues (Fig. 7.3 A; Table 7.2). Unfractionated exudates and acetone precipitate fraction were significantly different from the acetone supernatant fraction ($p < 0.001$), supporting the above results. Lower mortality rates of 9.6 ± 1.6 and 11.6 ± 1.5 were observed in acetone precipitate and acetone supernatant fractions, respectively when compared with significantly ($p < 0.001$) greater (80.7 ± 1.5) mortality rates in the untreated exudates (Fig. 7.3 B; Table 7.2). A Tukey test detected non-significant differences between acetone precipitate and supernatant fractions ($p = 0.433$) indicating that these fractions do not induce larval mortality.

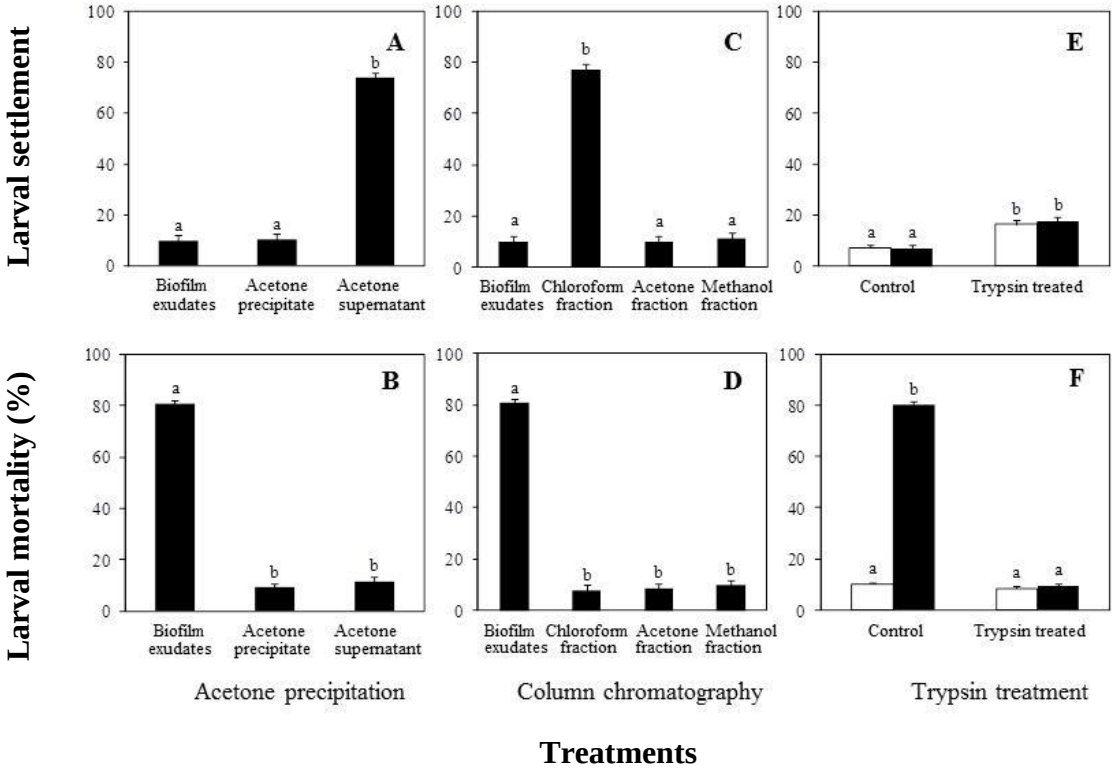

Figure 7.3: Effect of fractionated (chemical) exudates on larval settlement. Data represent mean (±SE, n = 10) larval settlement (top row) and mortality (bottom row) of *P. canaliculus* after 48 hours on polystyrene Petri plates containing biofilm exudates of *Pseudoalteromonas* sp. AMGP1 subjected to acetone precipitation (A&B), column (C&D) chromatography and trypsin treatment (E & F). For trypsin treatment graphs, the open bars represent autoclaved filtered seawater (AFSW) and shaded bars represent the biofilm exudates. The control plates contain untreated AFSW and biofilm exudates. Non-significant Tukey tests within (biofilm exudates) are denoted by the same letter over the bars.

7.4.6 Column chromatography (lipid fractionation)

Results from acetone precipitation tests showed that the chloroform fraction (containing non-polar lipids) of biofilm exudates from *Pseudoalteromonas* AMGP1 significantly ($p < 0.001$) induced greater (over 70%) settlement when compared with settlement rates of 9–12% observed in unfractionated exudates, acetone and methanol fraction (Fig. 7.3 C; Table 7.2). However, all fractionated exudates were not toxic to larvae, and had significantly ($p < 0.001$) lower mortality rates (up to 10%) when compared with unfractionated exudates (Fig. 7.3 D; Table 7.2). Tukey

tests revealed non-significant differences between all three fractions ($p > 0.001$) suggesting that do not induce larval mortality.

7.4.7 Trypsin treatment

Although trypsin treated exudates did not show greater (over 70%) settlement rates, the enzyme trypsin was found to induce larval settlement of up to 18% as observed in trypsin treated seawater and biofilm exudates of *Pseudoalteromonas* sp. AMGP1(Fig. 7.3 E; Table 7.2). A Tukey test detected significant differences between trypsin-treated and untreated seawater and biofilm exudates ($p < 0.001$) indicating that the enzyme trypsin may have some role in the mussel larval settlement. However, results from the mortality assay clearly indicate the presence of protein molecule in the toxin, as trypsin-treated exudate resulted in significantly ($p < 0.001$) lower (9.6 ± 0.8) mortality rates when compared with higher (80.3 ± 1.2) rates observed in untreated exudates (Fig. 7.3 F; Table 7.2). A Tukey test detected non-significant differences between trypsin-treated exudates when compared with trypsin-treated ($p = 0.965$) and untreated ($p = 0.729$) seawater. These results suggested that both settlement inducing and toxin molecules of *Pseudoalteromonas* sp. AMGP1 were protein molecules and their activity was inhibited in the presence of the serine-protease enzyme, trypsin.

Table 7.1: Bioactive compounds from marine bacteria and their effects on invertebrate larval settlement.

Bacteria	**Source**	**Bioactive compound**	**Invertebrate species**	**References**
Acinetobacter sp.	Ascidian, *Stomozoa murrayi*.	6-bromoindole-3carbaldehyde	Barnacle, *Balanus amphitrite*	Olguin-Uribe et al. (1997)
Alteromonas sp.	Sponge, *Halichondria okadai*	Ubiquinone	*B. amphitrite*	Kon-ya et al. (1995)
Alteromonas sp.	Natural and artificial substrates	< 3.5 kDa, heat stable polar protein	Tunicates, *Ciona intestinalis* and *Pyura praeputialis*	Zapata et al. (2007)
Bacillus amyloliquefaciens	Gorgonian, *Junceella juncea*	24-membered ring lactone Macrolactin V	Bryozoan, *Bugula neritina*	Gao et al. (2010)
Pseudoalteromonas issachenkonii	Deep sea sediment	Protease	*B. neritina*	Dobretsov et al. (2007a)
Pseudoalteromonas piscida	Sponge, *Mycale adherens*	Unknown substances from biofilms	Polychaete, *Hydroides elegans*	Lee and Qian (2003)
Pseudoalteromonas sp.	Adult tunicate, *Ciona intestinalis.*	Unknown substances from biofilms	Polychaete, *Hydroides ezoensis*	Ma et al. (2009)
Pseudoalteromonas sp. AMGP1	Sea weed, *Ulva lactuca*	< 1 KDa, heat labile polar protein	Mussel, *Perna canaliculus*	**Present study**
Pseudoalteromonas sp.4	Sponges, *Haliclona* sp.	< 1 KDa, polar carbohydrate	*H. elegans* and *B. neritina*	Dobretsov (2005)
Pseudoalteromonas tunicata	Adult tunicate, *C. intestinalis.*	< 0.5 kDa, heat stable, polar carbohydrate	*B. amphitrite* and *C. intestinalis*	Holmström et al. (1998; 1992)
Pseudoalteromonas tunicata and *Phaeobacter* sp.	*Ulva australis*	Unknown substances from biofilms	*B. neritina*	Rao et al. (2007)
Pseudomonas sp.	Nudibranch sponge	Phenazine-carboxylic acid, hydroxyphenazine, heptyl-quinol-one and pyolipic	*B. amphitrite*	Burgess et al. (2003)
Pseudomonas rhizosphaerae	Deep sea sediment	Diketopiperazine like compounds	*B. amphitrite* and *B. neritina*	Qi et al. (2009)
Shewanella oneidensis	Sea water	Cis-9-oleic acid	*B. amphitrite* and mussel, *Mytilus* sp.	Bhattarai et al. (2007)
Streptomonas. albidoflavus sp.	Deep Sea sediments	2-furanone ring with straight alkyl side chain	*B. amphitrite*, *H. elegans* and *B. neritina*	Xu et al. (2010)
Streptomyces fungicidicus	Deep seawater	Diketopiperazines	*B. amphitrite*	Li et al. (2006)
Vibrio alginiyticus, *Vibrio* sp. 4 and unidentified α-*Proteobacterium*	Sea weed, *Ulva reticulata* and Coral, *Dendronephthya* sp.	< 100 kDa, polar carbohydrate	*H. elegans* and *B. neritina*	Dobretsov (2005)
Vibrio alginolyticus and *Vibrio proteolyticus*	Sea weed, *Ulva reticulata*	> 200 kDa polysaccharides	*B. amphitrite*, *H. elegans* and *B. neritina*	Qian et al. (2006)
Vibrio sp. and unidentified α-*Proteobacterium*	Coral, *Dendronephthya* sp.	> 100 kDa, heat stable polar polysaccharides	*H. elegans* and *B. neritina*	Dobretsov and Qian. (2004)
Vibrio sp. 2	Sea weed, *Ulva reticulata*	> 100 kDa compounds	*H. elegans*	Dobretsov and Qian (2002)
Winogradskyella poliferorum	Novel sponge	Organic extracts	*B. amphitrite* and *H. elegans*	Dash et al. (2009)

Table 7.2: Statistical analyses (one-way ANOVA with Tukey test; α = 0.05) for *P. canaliculus* larval settlement on biofilm exudates of *Pseudoalteromonas* sp. AMGP1, subjected to molecular weight fractionation, heat treatment, acetone precipitation, column chromatography and trypsin treatment. Significant tests ($p < 0.001$) are in bold.

Treatments	Molecular weight fractionation (0–50 kDa)				Molecular weight fractionation (0–3 kDa)				Heat treatment			
	df	MS	*F*	*p*	*df*	MS	*F*	*p*	*df*	MS	*F*	*p*
% Settlement	6	2874.011	72.506	**0.001**	3	8.7	0.2	0.879	5	26.8	0.5	0.761
% Mortality	6	5459.0	132.0	**0.001**	3	7764.5	340.0	**0.001**	5	7402.1	271.4	**0.001**
Treatments	Acetone precipitation				Column chromatography				Trypsin treatment			
	df	MS	*F*	*p*	*df*	MS	*F*	*p*	*df*	MS	*F*	*p*
% Settlement	2	5896.7	126.2	**0.001**	3	4873.9	101.0	**0.001**	3	294.6	16.5	**0.001**
% Mortality	2	6986.6	384.1	**0.001**	3	5817.7	133.2	**0.001**	3	5300.5	626.3	**0.001**

Table 7.3: Statistical analyses (two-way ANOVA with Tukey test; α = 0.05 for *P. canaliculus* larval settlement on biofilm exudates of *Pseudoalteromonas* sp. AMGP1, subjected to crude and sequential fractionation. Significant tests ($p < 0.05$) are in bold.

Treatments	Crude fractionation				Sequential fractionation			
	df	MS	*F*	*p*	*df*	MS	*F*	*p*
(% Settlement)								
Chemical treatment (CT)	5	1475.4	54.7	**0.001**	10	1767.2	48.1	**0.001**
Exudates (E)	1	3652.0	135.3	**0.001**	1	15595.5	424.8	**0.001**
CT×T	5	1436.7	53.2	**0.001**	10	1715.1	46.7	**0.001**
Error	108	27.0			198	36.7		
(% Mortality)								
CT	5	2616.6	69.1	**0.001**	10	2268.2	63.1	**0.001**
E	1	32317.9	852.9	**0.001**	1	28121.2	783.0	**0.001**
CT×T	5	2808.2	74.1	**0.001**	10	2307.7	64.2	**0.001**
Error	108	37.9			198	35.9		

7.5 **Discussion**

Earlier studies established the role of bacterial cell suspensions, bacterial biofilms and biofilms exudate of *Pseudoalteromonas* sp. AMGP1 on the settlement of *Perna canaliculus* larvae (Ganesan et al., 2010; Ganesan et al., 2012a). Those results indicate that biofilms and exudates of *Pseudoalteromonas* sp. AMGP1 induce less than 20% larval settlement, which was similar to sterile controls and were highly toxic (over 70% mortality rates) to the mussel larvae. In addition, the biofilm toxin for larvae was found to be absent in washed-biofilm cells (without exudates) and when bacteria were cultured as planktonic populations. Results from these experiments asserted the role of biofilm exudates as potential cause for larval mortality, but larval responses to specific chemical molecules (i.e., proteins, lipids, carbohydrates etc.) in biofilm exudates of *Pseudoalteromonas* sp. AMGP1 were not identified during this process. Thus, a comprehensive multi-step characterisation processes was carried out to identify the chemistry of *Pseudoalteromonas* sp. AMGP1 exudates that modified larval settlement behaviour in-vitro.

To date, only a few chemical compounds from biofilm exudates of *Pseudoalteromonas* have been successfully characterised and reported to induce (Tebben et al., 2011) or inhibit settlement of larvae (see Table 7.1). However, the presence of both inductive and toxic bioactive compounds in exudates of a bacterium that modulates larval settlement has been reported only in the present study. During the multi-step characterisation process, the larval toxin from biofilm exudates of *Pseudoalteromonas* sp. AMGP1 was identified as a heat labile, polar protein molecule of < 1 kDa in size. Incidence of such low molecular weight polar substances from *Pseudoalteromonas* sp. that inhibit settlement of larvae has been

reported previously (Dobretsov, 2005; Holmström et al., 1992; Zapata et al., 2007). These results contradicted an earlier hypothesis that all antifouling compounds from marine organisms are mostly non-polar (Steinberg et al., 2002). However, the information presented in Table 7.1 clearly indicates that bacteria are able to produce a variety of antifouling compounds with dissimilar chemical signatures.

Pseudoalteromonas sp. AMGP1 may specifically generate larval toxins as polar compounds for a number of reasons. In the wild, polar molecules can be detected by an organism from a distance and elicit an avoidance response (Kristensen et al., 2008). This could perhaps be one of the means by which epiphytic *Pseudoalteromonas* bacteria effectively safeguards their host seaweed (e.g., *Ulva lactuca* and *U. reticulata*) from fouling organisms (Dobretsov & Qian, 2002; Egan et al., 2000). Release of antifouling substances by *Pseudoalteromonas* sp. to the external environment are via the type II secretion (T2S) pathway, a protein secretion systems conserved in all Gram-negative γ-proteobacteria (Gerlach & Hensel, 2007). For example, the T2S pathway of *Pseudoalteromonas tunicata* is believed to play an essential role in exporting antifouling substances to inhibit settlement of other fouling organisms and solely associate with their eukaryotic host (Evans et al., 2008). Since, *Pseudoalteromonas* sp. AMGP1 is a Gram-negative γ-proteobacterium, it is highly likely that a similar T2S system may be responsible for exporting the polar protein molecules to inhibit larval settlement and to protect *U. lactuca* from other fouling organisms. Such protection in turn assists *Pseudoalteromonas* sp. from sharing their space, nutrients as well as protecting themselves from eukaryotic grazers (Harder, 2009; Holmström & Kjelleberg, 1999).

The effect of extracellular polar proteins obtained from *Pseudoalteromonas* sp. AMGP1 as a potential anti-larval compound was shown in exudates that were subjected to sequential fractionation and enzyme treatment. Sequential fractionation of the exudates presented the polar fraction (PolF) and the protein fraction (PF) that showed higher (over 70%) rates of larval mortality, confirming the above results. Also, the residual fraction-1 (RF1) obtained by removing polar carbohydrates from PolF induced similar mortality rates, indicating that the polar carbohydrates of *Pseudoalteromonas* sp. AMGP1 may not contain larval toxins. Nonetheless, data presented in Table 1 clearly indicate that relevance of polar carbohydrates obtained from *Pseudoalteromonas* sp. 4 and *P. tunicata* as settlement inhibiting and toxic substance for invertebrate larvae (Dobretsov, 2005; Holmström & Kjelleberg, 1999; Holmström et al., 1992). The role of proteins as toxins for *P. canaliculus* larvae was corroborated by treating the exudates of *Pseudoalteromonas* sp. AMGP1 with a protease enzyme (trypsin). Trypsin treated exudates significantly reduced larval mortality, indicating that a specific peptide sequence altered by trypsin was essential for this process. Trypsinated peptides are known to contain lysine and arginine in their C-terminal (Olsen et al., 2004), and these amino acids have been shown to induce settlement in oyster and barnacle larvae (Tegtmeyer & Rittschof, 1989; Zimmer-Faust & Tamburri, 1994). However, such peptide sequences may have no role in the settlement of *P. canaliculus* larvae as trypsin-treated exudates of *Pseudoalteromonas* sp. AMGP1 showed induction in the settlement of *P. canaliculus* larvae.

With the aid of the physical and chemical characterisation procedures, a settlement inducing molecule in exudates of *Pseudoalteromonas* sp. AMGP1 was

identified as a 30–50 kDa, heat labile, non-polar lipoprotein. Column chromatography and acetone precipitation were performed exclusively on the organic phase fraction of the exudates to confirm the molecular class of the inductive cue. Although several *Pseudoalteromonas* sp. have been shown to induce larval settlement, the chemical nature of most of these inductive cues is still poorly understood (Huang et al., 2007; Huggett et al., 2006; Lee & Qian, 2003; Negri et al., 2001). For example, the extracellular lipids (lipopolysaccharide and/or lipoprotein) from biofilms of *Pseudoalteromonas espejiana*, (a.k.a. *Alteromonas espejiana*) have been reported to induce metamorphosis of the hydroid, *Hydractina echinata* larvae, while the exact chemistry of the metamorphic cue remains elusive (Leitz & Wagner, 1993). Lipoproteins for Gram-negative bacteria are a key component of the outer membrane protein and have been suggested to be responsible for cell-cell and cell-host communication processes (Davey & O'toole, 2000; Kuehn & Kesty, 2005; Pathak et al., 2012). These bacterial lipoproteins serve as adhesive molecules which bind to their host's extracellular matrices in order to effectively mediate cross-kingdom communication (Gerlach & Hensel, 2007). For example, a 32 kDa lipoprotein LipL32 identified in the genome of *P. tunicata* was believed to be responsible for the effective interaction with the extracellular matrix of their tunicate host, *C. intestinalis*. However, their role in larval settlement remains unknown (Hoke et al., 2008; Thomas et al., 2008). Therefore, it can be presumed that *Pseudoalteromonas* sp. AMGP1 could have secreted similar 30–50 kDa, lipoprotein molecules in order to adhere to their host, *U. lactuca* (*in-vivo*) or to the polystyrene Petri plate (*in-vitro*), and the mussel larvae could have responded to those adhesive molecules.

In summary, the current study reports on the presence of two protein molecules with contrasting properties obtained from *Pseudoalteromonas* sp. AMGP1 for the mussel larvae. The multi-step characterisation process revealed the general identity of a settlement inductive cue (heat labile, 30–50 kDa, non-polar lipoprotein) and a toxic molecule (heat labile, 0–1 kDa, polar protein) for *P. canaliculus* larvae. From an ecological perspective, such larval toxins produced by epiphytic *Pseudoalteromonas* sp. AMGP1 may enable this epiphyte to solely associate with their eukaryotic host, *U. lactuca* and protect them from subsequent fouling.

This study highlights the need for a comprehensive approach to understand the cumulative and individual chemicals within biofilm exudates that affect larval settlement. In addition, our findings provide insights on the importance of marine bacterial biofilms as mediators of larval settlement processes and the potential antifouling characteristics of *Pseudoalteromonas* sp.

Chapter 8.

ATTACHMENT AND RETENTION OF MUSSEL JUVENILES (SPAT) IN RESPONSE TO BACTERIAL BIOFILMS

8. Attachment and Retention of Mussel Juveniles (Spat) in Response to Bacterial Biofilms

8.1 Abstract

A layer of multi-species biofilm obtained during the conditioning of farmed ropes is believed to enhance retention on mussel *Perna canaliculus* spat. However, the nature of such biofilms and their ability to induce settlement and retention remains largely unknown. In an attempt to understand the process of biofilm-mediated settlement and retention of juvenile mussels, the present study examined the role of three mono-species biofilm, *Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1 on settlement substrates, such as Petri plates, glass and coir rope through controlled laboratory assays. Results from these settlement studies indicated that mussel spat were settled equally on both filmed (for all three bacterial species) and un-filmed Petri plates. However, significantly greater settlement was observed on glass slides and coir ropes containing biofilms of *Macrococcus* sp. and *Bacillus* sp. compared with their corresponding controls. Conversely, biofilms of *Pseudoalteromonas* sp. on polystyrene and glass substrates induced low levels of settlement, and similar to sterile controls. These results demonstrated that juvenile mussels may actively choose their settlement substrates, based on the physicochemical properties and/or cues associated with bacterial biofilms. During the initial 24-hour period, biofilms of *Macrococcus* sp. and *Bacillus* sp. significantly improved retention of spat on glass slides and coir ropes compared with controls. From 48-hour period onwards, retention of spat was observed to be similar on both filmed and un-filmed surfaces. Future research is required to

determine more precisely what physical characteristics of the settlement substrate and properties of biofilms are effective in inducing settlement and retention of spat.

8.2 Introduction

Mussel farming is the backbone of New Zealand's primary growing aquaculture industry. In 2011 alone, the volume of Greenshell™ mussel exported were 38,097 tones, estimated be over NZ$ 220 million in total value (Ministry of Fisheries, 2011a). The success of the mussel industry majorly depends on the supply of wild-caught spat that contributes to over 80% of the mussel seed requirement. The remaining supply of mussel spat is obtained by placing spat-catching ropes near mussel farms. However intermittent supplies of naturally occurring spat have been shown to affect production of this commercially important shellfish (Alfaro et al., 2010), and there has been a constant need to replenish the seed required for mussel production. Given the economic value of this bivalve species, it is therefore imperative to understand the underlying mechanisms of mussel juvenile settlement and retention.

Past studies have indicated that the plantigrades (juveniles/spat/seed) of mussel (*P. canaliculus*) preferentially settle on filamentous substrates, such as macro-algae and hydroids (Alfaro & Jeffs, 2002, 2003; Buchanan & Babcock, 1997). In addition, the processes of initial attachment and settlement have been investigated with regard to the substrate physical structure (Alfaro & Jeffs, 2002, 2003; Buchanan & Babcock, 1997), chemical make-up (Alfaro et al., 2006; Young et al., 2011) and bacterial biofilms (Ganesan et al., 2010; Ganesan et al., 2012a). However, during initial stages of settlement, juvenile mussels can actively explore their settlement site and re-attach themselves several times until they find a suitable settlement substrate

(Alfaro, 2006a; Alfaro & Jeffs, 2002; Buchanan & Babcock, 1997). Furthermore, mussel spat (up to 6 mm) may produce mucous strands to transport themselves to suitable rocky substrate (Buchanan & Babcock, 1997; Jeffs et al., 1999). This secondary settlement behaviour of mussels is thought to be one of the major causes of spat loss after they are initially seeded on farm ropes. Spat losses are a common occurrence in mussel farms and range from 50% to 90%, sporadically (Hayden, 1995). Apart from settlement behaviour, some spat losses are caused by competition for space and nutrients from biofouling organisms (Woods et al., 2012), viral disease (Jones et al., 1996), predation (Jenkins, 1985; Van de Ven, 2007), and desiccation and starvation during and prior to seeding (Carton et al., 2007; Sim-Smith & Jeffs, 2011; Webb & Heasman, 2006). Other factors that hinder settlement and retention include water velocity, water flow and oxygen concentration (Alfaro, 2005, 2006a; Hayden & Woods, 1997). Apart from these extrinsic factors (e.g., predation, diseases), determining the intrinsic factors that modulate substrate choice for settlement and recruitment may considerably improve our understanding of mussel juvenile retention.

Initial work by Buchanan & Babcock, (1997) demonstrated that juvenile spat (< 0.5 mm) preferred finely-branched macro-algae, whereas older spat (0.5–5 mm and > 5mm) favoured moderate to coarsely-branched macro-algae. This settlement preference of mussel spat was confirmed by using artificial aquarium plants with varying degrees of branching and tested for on the corresponding mussel size (Alfaro & Jeffs, 2002). Information on mussel spat settlement preferences have aided spat collectors to create suitable ropes that mimic the filamentous nature of algae and thereby enhance spat retention. Currently, filamentous braided ropes of natural (coir)

or synthetic (polypropylene) material are deployed in open waters in an attempt to capture young pelagic mussels (Alfaro & Jeffs, 2003; Food and Agriculture Organization, 2012). These ropes are usually placed in specific locations where there is an abundance of larvae or spat recorded at specific times of the year. Aquaculture hatcheries also use similar ropes as substrates for the settlement and metamorphosis of mussel larvae within rearing tanks. Prior to the incorporation of these ropes into hatchery tanks, they are conditioned in natural seawater for a period of time (routine hatchery practice). Conditioning of substrates in seawater have shown to increase spat growth of mussels (e.g., *Mytilus galloprovincialis*) and oysters (*Crassostrea belcheri*) (Peteiro et al., 2007; Tanyaros, 2011). While, the effects and induction mechanisms of natural biofilms have not been investigated to date, biofilms of diatom *Amphora* sp., in combination with bacteria, *Halomonas* sp., have been shown to increase juvenile settlement and retention of the Chilean scallop, *Argopecten purpuratus* (Leyton & Riquelme, 2008).

Thus far, the effects of single-species bacterial biofilm have not been investigated for their ability to induce settlement and retention of juvenile green-lipped mussels. Since the settlement inducing properties of the three mono-species biofilms (*Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1) have been previously described for *P. canaliculus* larvae (refer chapters 3–5), these bacterial biofilms were selectively chosen for the spat settlement and retention study. Effects of these biofilms were investigated on Petri plates, glass slides and coir ropes as the former two substrates have been used routinely in laboratory-guided settlement assays and the latter is currently been used as a settlement substrate in a mussel hatchery. Thus, the effect of these substrates in

modulating the bacterial biofilms for the settlement and retention of mussel plantigrades were examined.

8.3 Materials and Methods

8.3.1 Source of mussel juveniles

Wild mussel juveniles (spat) of 0.5–5.0 mm in size were provided by Kaitaia spat collectors, Ninety Mile Beach, northern New Zealand. Spat still attached to macro-algae (Fig. 1.2; chapter 1) were collected from the surf zone and transported to the aquaculture facility at AUT. Clumps of seaweed containing several thousands of spat were placed in a polyethylene bag and were kept cool and moist during transportation. Upon arrival at AUT, the spat attached to the various macro-algal species were immediately placed in a 200 litre circular tank containing seawater. The tank was maintained at 17 ± 1°C with a constant flow of water and the outlet was connected to a re-circulating system. The spat that attached to the walls of the system were handpicked and transferred to another tank. Macro-algae also were removed to avoid degradation by bacteria and other potential interactions with the spat. Individuals were fed *ad libitum* with mixed microalgal diet of *Pavlova lutheri* and *Isochrysis galbana* as described in Young (2009). For the settlement and retention experiments, organisms between 1–3 mm in size were selected. These organisms were either handpicked or obtained by sieving seawater with a mesh over 1mm in size. Spat that attached to the substrata were found suitable for the settlement experiments, as un-attached spat were mostly dead, and hence discarded.

8.3.2 Preparation of substrates with mono-species bacterial biofilms

Bacterial biofilms of three mono-species marine bacteria (*Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1) were prepared by obtaining their respective biofilm cell suspensions (BCx) as described in chapter 3. Briefly, colonies from five replicate marine agar plates were withdrawn and mixed with 10 ml of autoclaved filtered seawater (AFSW). Cells in AFSW were washed thrice by centrifugation (3000 × g) and the supernatant was discarded at the end of each washing step. Washed cells were re-suspended in 10 ml of AFSW to achieve BCX with 10^6 to 10^7 cells ml^{-1}.

Biofilms were prepared on polystyrene Petri Plate (60 mm diameter × 14 mm depth), microscope glass slides (76 mm length × 26 mm width) and coir ropes (60 and 100 mm, Cawthron Institute, Nelson, New Zealand). Biofilms on Petri plate were prepared as described in chapter 5. Individual Petri plates contained 1 ml of BCx (10^6–10^7 cells ml^{-1}) and 5 ml of sterile 0.5% peptone in AFSW. All plates were incubated on a rotary incubator (50 rpm) at 34°C for 24 hours. The unattached biofilm cells on the plate were removed by washing thrice with a total of 30 ml of AFSW.

Growth of biofilms on glass slides was achieved by placing each slide on a Petri plate (90 × 15 mm) containing in 10 ml of 0.5% peptone AFSW and 1 ml of BCx (10^6–10^7 cells ml^{-1}). The plates containing glass slides were incubated on a rotary incubator (50 rpm at 34°C) for 24 hours. The glass slides containing biofilms were washed thrice with a total of 30 ml of AFSW to remove the unattached cells.

Individual coir ropes were cut to a desired length (60 and 100 mm) and each rope was inserted into a 10 ml test tube containing peptone (0.5%) AFSW. Tubes

with ropes were sterilised by autoclaving and cooled down to room temperature prior to the inoculation of BCx. About 1 ml of BCx (10^6–10^7 cells ml^{-1}) from the respective mono-species bacteria were inoculated to the test tubes which were placed in a beaker on a rotary incubator set to 50 rpm at 34°C for 24 hours. The ropes were removed from the tubes and un-attached cells were removed by washing the rope thrice with a total of 30 ml of AFSW.

For the spat settlement assay, mono-species bacterial biofilms of *Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1 were tested on Petri plates, glass and slides. Based on results from the settlement assay, the ability of *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1 separately on glass slides and coir ropes were examined for their ability to retain mussel spat.

8.3.3 Spat settlement assay (Petri plate, glass and coir rope)

For the spat settlement assay, Petri plates with bacterial biofilms were used as the primary substrate. Each glass and coir rope (60 mm) containing biofilms was placed in the centre of a secondary substrate (polystyrene Petri plate; 90 mm diameter × 15 mm depth). Respective sterile un-filmed Petri plates, slides and ropes were used as controls. Individual treatment and control plates received 10 ml of AFSW and 20 spat (1–3 mm). The spat were randomly added to Petri plates but ten spat were placed on either side of the rope and glass substrate maintaining a distance of about 30 mm from each substrate. The experiment was conducted in replicates of ten per treatment and controls for each of these substrates and was incubated at 17 ± 1°C under ambient light conditions for 24 hours. After the incubation period, the settled spat on the substrates (Petri plates, glass slides and coir ropes) were counted

by dipping them into individual beakers containing fresh AFSW. The number of spat still attached to settlement substrates was recorded as settled.

8.3.4 Spat retention assay (glass and coir rope)

The retention of spat on glass substrates was observed over a period of five days. Biofilms were achieved on glass slides as per the protocol described above. Biofilm containing slides were placed on a clean tray and mussel spat were added to the treatments. After 24 hours, the slides with attached mussels were tested for their ability to retain spat on the surface. The slides containing 30–40 attached spat were hung vertically in a 400 ml beaker containing AFSW. The slides were suspended in the water inside beakers and the set up ensured that the glass slides were not in contact with the walls of the beaker. The spat that fell off during the first four hours were not taken into consideration across all the treatments. The time after the 4 hour acclimatisation period was recorded as the starting point of the assay. In addition, density of spat on glass slides at this point was documented as the starting density and considered 100% retained. The number of spat that remained on the slides and the number that fell off were noted every 24 hours up to 120 hours.

The retention of spat on coir ropes was observed over a period of six days. The coir ropes (100 mm in length) containing biofilms were placed in a clean tray and mussel spat were added. This was left for a period of 24 hours. The attached spat on the coir ropes were used to monitor retention over time. The ropes were standardised to contain 50–60 spat per rope by removing some spat from ropes that had high density of attached spat. Then the ropes were hung vertically in a 600 ml beaker containing AFSW ensuring that the coir ropes were not in contact with the walls or bottom of the beaker. The spat that fell off during the first four hours were

not taken into consideration across all the treatments. The density of the spat retained after the four hour period was recorded as the starting density and considered 100% retained. The spat retained on the ropes and the number that fell off the ropes was accounted over a period of 24 to 144 hours.

For the spat retention assay, experiments were performed in replicates of ten for both treatments and controls. The setup was maintained at 17 ± 1°C under ambient light conditions throughout the assay period. The system remained static and water was not changed during the experimental period. The spat were unfed throughout the assay.

Mortality of the spat was observed by noting movement of their foot and shell gaping. Individuals that displayed evidence of movement, attachment, or searching behaviour were recorded as live. Any individuals that did not exhibit any of the above signs and resisted manual closure of their shells were recorded as dead. However, no mortality was observed in any of the treatments included in this study.

8.3.5 Statistical analyses

All data for percent spat settlement and retention were arcsine-transformed and were analysed using IBM® SPSS® statistics version 19. Data that were tested for and met parametric assumptions were subjected to independent sample t-test to compare the treatment effects in this study.

8.4 **Results**

8.4.1 Spat settlement assay

Results from spat settlement on Petri plates indicated that mussel spat did not preferentially settle on filmed (mono-species biofilm) or un-filmed (controls) substrates, and that settlement was uniformly between 50–70% across all substrates (Figs. 8.1 A–C). Independent t-tests showed non-significant differences between control and treatment for all three bacteria ($p > 0.05$; Table1). However, significantly low settlement levels of spat settlement ($p < 0.05$) were observed on un-filmed glass slides compared with glass slides with biofilms of *Macrococcus* sp. AMGM1 (Figs. 8.1 D; Table 8.1). Similarly *Bacillus* sp. AMGB1 was able to significantly induce ($p < 0.05$) between 45–50% settlement, whereas controls achieved < 40% settlement for mussel spat (Figs. 8.1 E; Table 8.1). Conversely, biofilms of *Pseudoalteromonas* sp. AMGP1 showed non-significant differences ($p > 0.05$) in settlement patterns between controls and treatments (Figs. 8.1 F; Table 8.1). These results indicated that mussel spat were able to differentiate cues from mono-species biofilm and differentially settled on glass slide with and without mono-species biofilm. Likewise, spat were able to differentiate from biofilm cues on coir ropes containing mono-species bacterial biofilms. Settlement responses were considerably low (< 10%) on sterile controls compared with ropes containing biofilms of *Macrococcus* sp. and *Bacillus* sp., each achieved over 30% settlement (Figs. 8.1 G & H; Table 8.1). In addition, independent t-tests showed significant differences between control and treatments for each of these biofilms ($p < 0.05$). However, biofilms of *Pseudoalteromonas* sp. had < 10% spat settlement, which was not significantly different ($p > 0.05$) to sterile controls.

8.4.2 Spat retention assay

The biofilms of *Macrococcus* sp. AMGM1 significantly ($p < 0.05$) improved spat retention on glass substrates during the initial 24-hour period compared with sterile controls (Fig. 8.2 A). Likewise, biofilms of *Bacillus* sp. AMGB1 induced over 40% spat retention, whereas sterile controls induced < 20% retention during the initial 24-hour period (Fig. 8.2 B). Independent t-tests revealed significant ($p < 0.05$) differences between control and treatment during the initial 24-hour period (Table 8.2). However, spat retained on glass substrates gradually declined from 100% to almost 0% during the five day assay period. At every 24 hour observation point from 48–120 hours, mean spat retention on control and treatment slides was similar for both species of biofilms with no statistical differences observed at these stages.

Over the six day assay period proportion of spat retained on coir ropes declined linearly from 100% to about 30% in treatment and control ropes for both *Macrococcus* sp. and *Bacillus* sp. (Fig. 8.3 A & B; Table 8.3). Mean spat retained were similar for controls and treatment ropes for both species of bacterial biofilm for the 48–144 hour period. However, biofilms of *Macrococcus* sp. significantly ($p < 0.05$) enhanced over 10% retention during the initial 24-hour period compared with un-filmed ropes. Similarly, ropes containing *Bacillus* sp. induced 76% retention, whereas controls achieved only 65% spat retention. These results indicated that biofilms of both *Macrococcus* sp. and *Bacillus* sp. were able to induce retention of spat on ropes at least during the initial 24-hour period.

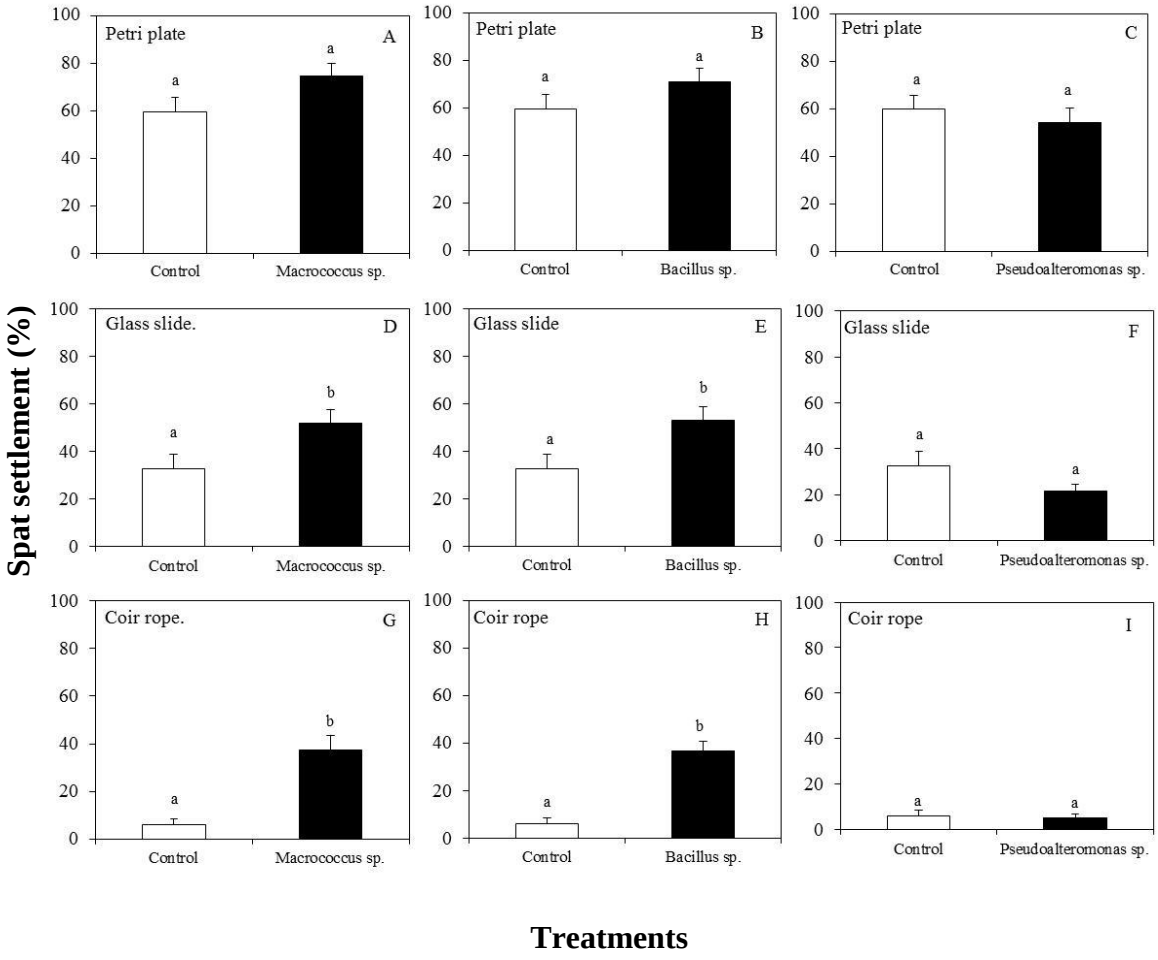

Figure 8.1: Data represent mean (±SE, n = 10) spat settlement of *P. canaliculus* after 48 hours on bacterial biofilms of *Macrococcus* sp. AMGM1 (A, D & G), *Bacillus* sp. AMGB1 (B, E & H) and *Pseudoalteromonas* sp. AMGP1(C, F & I) on Petri plates (top row), glass slides (middle row) and coir ropes (bottom row). The open bars represent sterile control plates and shaded bars represent the treatment plates. Non-significant t-tests between controls and treatments are denoted by the same letter over the bars.

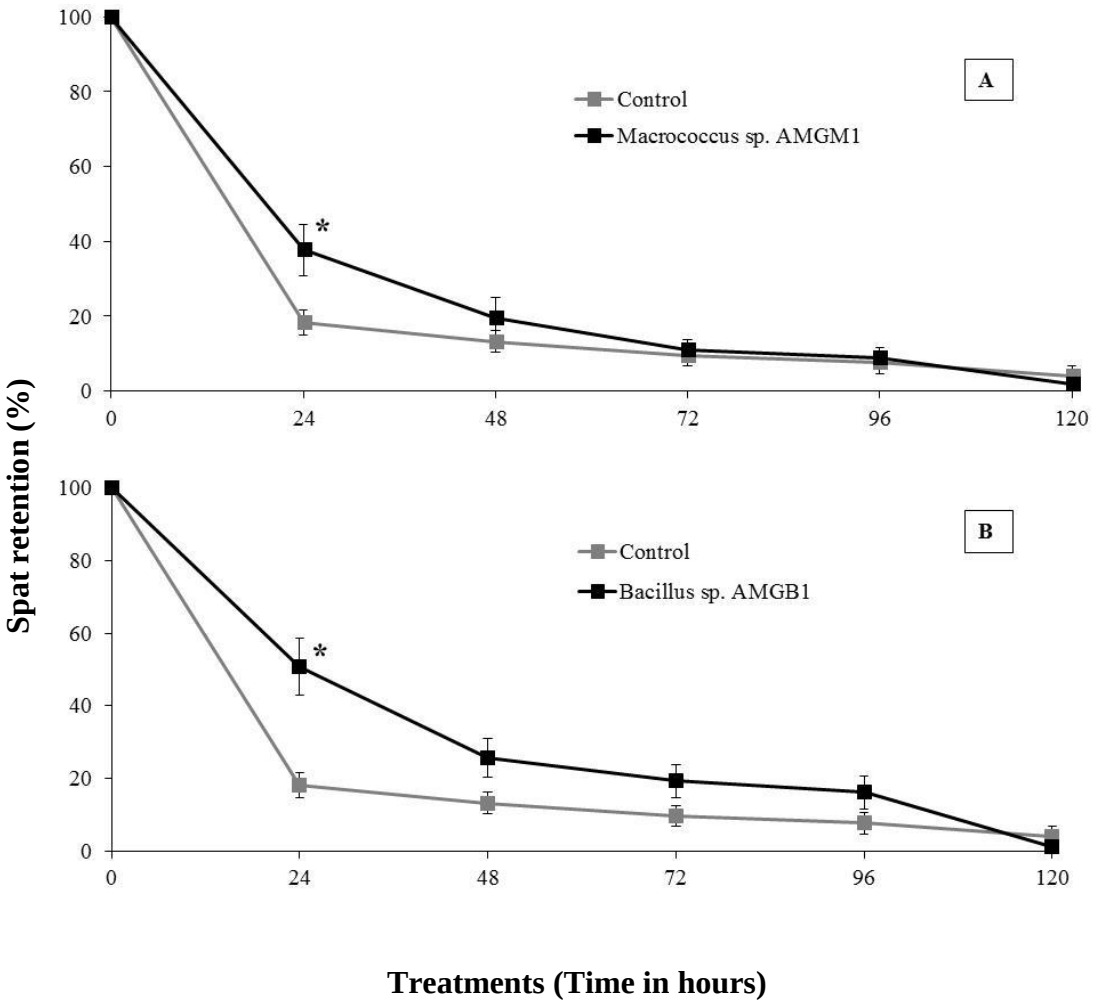

Figure 8.2: Data represent mean (±SE, n = 10) spat retention of *P. canaliculus* from 0–120 hours on glass slides containing bacterial biofilms of *Macrococcus* sp. AMGM1 (A) and *Bacillus* sp. AMGB1 (B). The grey bars represent sterile control plates and black bars represent the treatment plates. Non-significant t-tests between controls and treatments at each time interval are denoted by * over the bars.

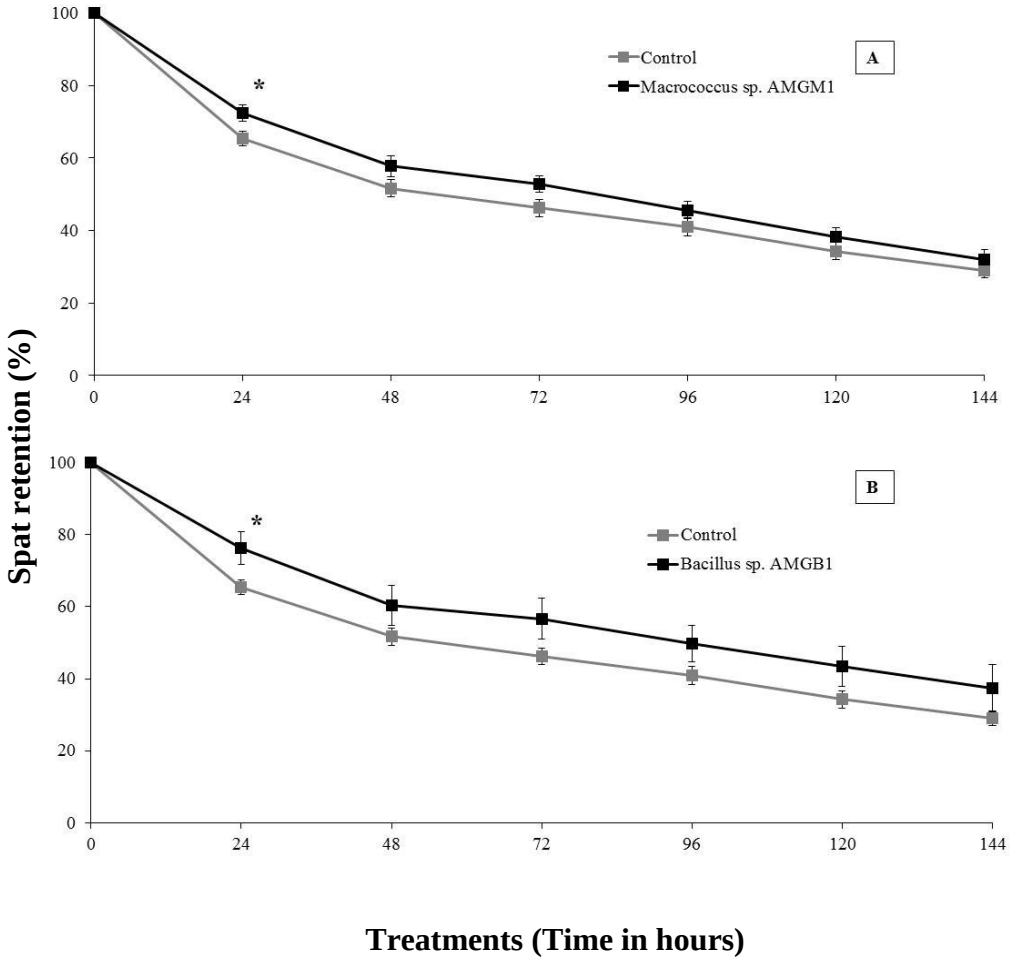

Figure 8.3: Data represent mean (±SE, n = 10) spat retention of *P. canaliculus* from 0–144 hours on coir ropes containing bacterial biofilms of *Macrococcus* sp. AMGM1 (A) and *Bacillus* sp. AMGB1 (B). The grey bars represent sterile control plates and black bars represent the treatment plates. Non-significant t-tests between controls and treatments at each time interval are denoted by * over the bars.

Table 8.1: Statistical analyses (arcsine transformed data and independent t-test) for *P. canaliculus* spat settlement on Petri plates, glass slides and coir rope containing biofilms of *Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1. Significant tests ($p < 0.05$) are in bold.

Treatment	Control mean ± S.D. (arcsine)	Treatment mean ± S.D. (arcsine)	Pooled DF	*t*-value	*p*-value
Petri plates					
Macrococcus sp. AMGM1		61.68 ± 13.70	18	1.94	0.068
Bacillus sp. AMGB1	50.84 ± 11.17	59.32 ± 14.26	18	1.48	0.156
Pseudoalteromonas sp. AMGP1		47.55 ± 11.06	18	0.66	0.516
Glass slides					
Macrococcus sp. AMGM1		47.17 ± 10.70	18	2.47	**0.024**
Bacillus sp. AMGB1	34.42 ± 12.34	46.25 ± 11.04	18	2.26	**0.037**
Pseudoalteromonas sp. AMGP1		27.14 ± 6.90	18	1.63	0.121
Coir ropes					
Macrococcus sp. AMGM1		37.30 ± 11.51	18	5.45	**0.001**
Bacillus sp. AMGB1	10.23 ± 10.68	36.89 ± 8.31	18	6.23	**0.001**
Pseudoalteromonas sp. AMGP1		10.77 ± 7.70	18	0.13	0.898

Table 8.2: Statistical analyses (arcsine transformed data and independent t-test) for *P. canaliculus* spat retention on glass slides containing biofilms of *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1. Significant tests ($p < 0.05$) are in bold.

Time (hrs)	**Control mean ± S.D. (arcsine)**	**Treatment (*Macrococcus* sp.) mean ± S.D. (arcsine)**	**Pooled DF**	***t*-value**	***p*-value**
0	89.34 ± 0.14	89.22 ± 0.20	18	1.60	0.127
24	24.37 ± 8.20	36.90 ± 14.07	18	2.43	**0.026**
48	19.74 ± 9.20	23.84 ± 13.75	18	0.78	0.444
72	16.31 ± 8.67	17.69 ± 8.90	18	0.80	0.730
96	13.36 ± 9.98	14.87 ± 10.05	18	0.34	0.739
120	6.77 ± 10.22	3.72 ± 7.08	18	0.78	0.448
Time (hrs)	**Control mean ± S.D. (arcsine)**	**Treatment (*Bacillus* sp.) mean ± S.D. (arcsine)**	**Pooled DF**	***t*-value**	***p*-value**
0	89.34 ± 0.14	89.30 ± 0.11	18	0.69	0.498
24	24.37 ± 8.20	44.26 ± 18.72	18	3.08	**0.006**
48	19.74 ± 9.20	27.45 ± 15.65	18	1.34	0.196
72	16.31 ± 8.67	23.02 ± 14.01	18	1.29	0.214
96	13.36 ± 9.98	20.45 ± 13.79	18	1.32	0.204
120	6.77 ± 10.22	3.10 ± 5.66	18	1.00	0.333

Table 8.3: Statistical analyses (arcsine transformed data and independent t-test) for *P. canaliculus* spat retention on coir ropes containing biofilms of *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1. Significant tests ($p < 0.05$) are in bold.

Time (hrs)	**Control mean ± S.D. (arcsine)**	**Treatment (*Macrococcus* sp. AMGM1) mean ± S.D. (arcsine)**	**Pooled DF**	***t*-value**	***p*-value**
0	89.63 ± 0.04	89.60 ± 0.03	18	2.03	0.057
24	54.02 ± 3.90	58.40 ± 4.53	18	2.32	**0.032**
48	45.97 ± 4.28	49.57 ± 5.36	18	1.66	0.114
72	42.83 ± 4.26	46.67 ± 4.11	18	2.05	0.055
96	39.73 ± 4.60	42.48 ± 4.44	18	1.36	0.190
120	35.74 ± 4.53	38.15 ± 4.57	18	1.18	0.252
144	32.48 ± 4.16	34.32 ± 5.30	18	0.90	0.399
Time (hrs)	**Control mean ± S.D. (arcsine)**	**Treatment (*Bacillus* sp. AMGB1) mean ± S.D. (arcsine)**	**Pooled DF**	***t*-value**	***p*-value**
0	89.63 ± 0.04	89.60 ± 0.03	18	2.03	0.057
24	54.02 ± 3.90	62.73 ± 12.31	18	2.13	**0.047**
48	45.97 ± 4.28	52.61 ± 14.46	18	1.39	0.181
72	42.83 ± 4.26	50.44 ± 14.89	18	1.55	0.138
96	39.73 ± 4.60	45.04 ± 9.76	18	1.56	0.137
120	35.74 ± 4.53	41.32 ± 11.02	18	1.48	0.155
144	32.48 ± 4.16	37.55 ± 12.93	18	1.18	0.253

8.5 **Discussion**

This study improves the understanding of how the combination of substrate type (polystyrene Petri plates, glass slides, and coir rope) and bacterial biofilms can affect settlement and retention of mussel spat in laboratory-guided assays. The results from these experiments showed striking differences across the three substrates tested. During a 24-hour settlement assay, un-filmed Petri plates were able to induce similar settlement percentages (60–70%) compared with bio-filmed plates. In contrast, biofilms of *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1 induced significantly greater settlement on glass slide and coir rope surfaces compared with un-filmed controls. These findings suggest that the combination of substrate and biofilm is important and that mussel spat show specific settlement behaviours depending on the surface they encounter. These settlement patterns of spat contrast those observed for larvae (chapter 2), as mussel larvae did not respond to the physical cues of polystyrene Petri plate. However, the physicochemical properties (e.g., surface charge, wettability) of the Petri plates could have enhanced settlement of mussel spat. This suggests that larvae and spat interact differently with their settling environment.

Although, several studies have suggested the effect of surface wettability on attachment preferences of invertebrates to artificial substrata (i.e., glass and polystyrene), there is substantial ambiguity in those results. This is because some organisms selectively choose to settle on hydrophobic substrates, while others select hydrophilic substances (Brewer, 1984; Crisp et al., 1985; Holm et al., 1997; Maki et al., 1989; O'Connor & Richardson, 1994; Rittschof & Costlow, 1989; Roberts et al., 1991). Young & Crisp (1982) have argued that in the case of hydrophobic surface, it

is easier for adult mussels to secure attachment to surfaces with their byssus threads by simply displacing water and spreading over a larger area compared with hydrophilic surfaces. Nonetheless, adult mussels attach to settlement surfaces by their byssus thread, which contain catecholamine molecules such as DOPA (L-3,4, dihyroxyphenylalanine), whereas juveniles (0.5–1.5mm) attach by secreting mucous containing weakly acidic mucopolysaccharides and proteins (Gao et al., 2007; Lee et al., 2011). Similarly, pediveligers of the mussels, *P. canaliculus* (Petrone et al., 2008) and *Mytilus edulis* (Lane & Nott, 1975) and oyster, *Ostrea edulis* (Cranfield, 1973) produce proteins and acid mucopolysaccharides during their initial settlement process. The bryozoan, *Bugula neritina* larvae also produce similar chemical molecules (Loeb & Walker, 1977) during their attachment, and they preferentially settle on un-filmed polystyrene plate over glass substrates (Maki et al., 1989). Moreover, it has been reported that the physicochemical reaction between bryozoan mucous and substrate enable larvae to preferentially choose low wettable, hydrophobic surface with low surface energy (Maki et al., 1989), which is in accordance with the results from this study. However, inferences drawn from that study are speculative, as the physical properties of the settlement substrates were not investigated. Nevertheless, mucopolysaccharides contain a large number of negatively charged molecules on their surface which can attract positively charged molecules, and this can be a plausible reason for increased spat settlement of mussels on positively charged surfaces (Young, 2009). From an ecological perspective, plantigrades of *P. canaliculus* may secrete mucous containing mucopolysaccharides for temporary adhesion on to substrates so that they can actively explore the substrate and, if necessary, migrate to another settlement site (Buchanan & Babcock, 1997). The ability of plantigrades to subsequently settle on a secondary settlement

site may actually be due to the instability of mucopolysaccharides in seawater. In principle, mucopolysaccharides do not function as ideal adhesives due to their ability to swell and disentangle in water (Vincent, 1990). Thus, the lack of spat retention on ropes may perhaps be due the instability of mucous rather than a behavioural response.

Bacterial biofilms have been shown to modulate wettability and surface charge of substrates (Fletcher & Loeb, 1979; Loosdrecht et al., 1989), and also provide an ideal settlement site for invertebrate larvae (Faimali et al., 2004; Maki et al., 2000; Maki et al., 1989; Rittschof & Costlow, 1989). This may be a reason for enhanced spat settlement of *P. canaliculus* on filmed glass and coir substrates compared with the respective un-filmed controls. If wettability and/or surface charge alone was the principle factor that determined mussel spat settlement, then coir rope and glass slides covered with biofilms should have induced the same settlement percentages as controls. This is especially true as Petri plates were used as the secondary substrate to hold the primary substrates (coir and glass) during the settlement assay. Thus, mussel spat had an additional substrate (Petri plate) to choose in those assays. However, spat were observed to move towards the biofilm present on glass slides and ropes instead of retaining on the Petri plates. In addition, no spat were directly added on slides and ropes and spat settled on these surfaces were alone scored. Thus, these results explicitly demonstrated the role of *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1 biofilms as potential inducers of spat settlement. Furthermore, biofilms of *Pseudoalteromonas* sp. AMGP1 induced only up to 10 and 20% spat settlement on glass slides and coir rope, respectively, indicating that spat were able to differentiate cues from mono-species biofilm and

actively respond to these biofilm cues, in the context of the solid substrate. Surprisingly, biofilms of *Pseudoalteromonas* sp. AMGP1 that were previously known to induce mortality of *P. canaliculus* larvae (see chapters 3 to 7), did not induce mortality of mussel spat. The differences in toxic responses between mussel larvae and spat could be due to differences in age and development rather than the biofilm chemicals. In addition, the difference in settlement assay duration (48 hours for larvae and 24 hours for spat) could have contributed to the dissimilarity in larvae and spat mortality. Additional observations were consistent with the low levels of spat settlement on control glass slides (< 30%) and coir ropes (< 15%) compared with the enhanced settlement levels on control Petri plates (< 50%). These differences in spat settlement could have been due to their inherent physicochemical properties. Although these properties were not analysed in this study, in theory, glass slides have net negative charge on their surface. Similarly charged surface have been previously shown to decrease settlement of *P. canaliculus* spat (Young, 2009).

Although biofilms did not considerably enhance retention of spat on coir ropes and glass slides after the 48-hour period, they improved retention on the substrates during the initial 24-hour period. The reduction in spat retention from the 48-hour period also may be due to the consumption of the biofilms by spat (refer chapter 9) during the initial retention period, which could have minimised the continued efficiency of biofilms to retain spat. Another potential influence in this experiment could have been the anoxic conditions that may have developed in the assay medium due lack of water exchange. In addition, oxygen was not supplied to these spat and depletion of oxygen has been shown to negatively influence settlement of mussel spat (Alfaro, 2005). Since mussel spat were unfed throughout

the assay, lack of nutrition also may have effected their retention ability, as it has been shown previously (Carton et al., 2007). Incorporation of microalgae in future spat retention experiments may improve retention levels. However, the presence of another organism in the assay may present confounding effects. Indeed, macro-algae in combination with bacteria have been shown to enhance retention of scallop (*Argopecten purpuratus*) spat (Leyton & Riquelme, 2008). Interestingly, unlike spat settlement, retention of spat on glass slides was considerably low (0–20%) on un-filmed glass slides compared with coir ropes, which achieved between 30–60% during the assay period. These differences could be due to the topography of the settlement substrates, which may be a crucial factor for spat settlement. Thus, it is explicit that biofilms are required to enhance initial settlement of spat on substrates, but physical topography of the settlement substrate (e.g., coir rope) may help retain them after a 24-hour period. Similarly, a number of studies have demonstrated the importance of physical structure of the substrata to improve retention of bivalve spat (Bourgeois et al., 2006; Cashmore et al., 1998; Harvey et al., 1997; Myrand et al., 2012; Taylor et al., 1998). Conversely, only a few studies have dealt with the addition of specific mono-species biofilms to enhance spat retention (Leyton & Riquelme, 2008; Yang et al., 2012). Nonetheless, some studies have emphasised the need to condition settlement substrates in seawater prior to their use in hatcheries in order to enhance spat retention (Tanyaros, 2011; Taylor et al., 1998). Substrates immersed in seawater may incorporate a wide range of micro-and macro-organisms (Wahl, 1989), and some of these organisms have been shown to interfere with retention of spat (Woods et al., 2012). It is, therefore, ideal to select specific and appropriate mono-species bacterial biofilms to enhance settlement and retention of spat.

In summary, biofilms of *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1 were able to induce greater settlement of mussel spat on ropes and glass slides compared with their respective controls. Conversely, biofilms of *Pseudoalteromonas* sp. AMGP1 did not induce spat settlement and achieved settlement patterns similar to those of controls. However, mussel spat appeared to be unable to differentiate un-filmed and biofilmed surfaces of polystyrene Petri plates. Previous retention studies have indicated that feeding of spat during the assay and topography of the settlement substrata (rope) might play an important role in improving the rate of retention over a period of time. This study also demonstrated that mussel spat actively responded to biofilm cues on two of the three settlement substrates tested. Future work on identification of appropriate substrates with ideal surface charge, topography, wettability combined with bacterial biofilms and microalgal diet will improve our understanding and application of settlement and retention of spat in hatchery settings.

Chapter 9.

ROLE OF MARINE BACTERIA IN NUTRITION OF MUSSEL LARVAE AND JUVENILES

9. Role of Marine Bacteria in Nutrition of Mussel Larvae and Juveniles

9.1 Abstract

In hatcheries, larvae of the green-lipped mussels have been shown to survive best and successfully metamorphose when fed a diet of microalgae, which often host a suite of bacterial populations. Despite the presence of bacteria in their environment, the ability of mussel larvae to obtain nutrition from bacteria remains largely unknown. The present study investigated the ability of three mono-species marine bacteria (*Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1) to provide nutrition for mussel larvae and juveniles. The nutrition assays included the incorporation of heavy metal strontium (Sr) and ytterbium (Yb) labelled mono-species bacteria in treatment culturing plates and no bacteria in control plates. Mussel larvae and spat were incubated in treatment and control plates and allowed to feed on the bacteria. The results indicated that controls without bacteria also contained Sr. A follow up investigation revealed that significant amounts of Sr were present in the shells of larvae and juveniles, which confounded the results. Thus, Sr was regarded as an inefficient marker for nutrition assays due to its presence in seawater. Larvae and spat that fed on treatment plates containing Yb-labelled bacteria, showed enhanced concentration of Yb, indicating that both mussel larvae and juveniles fed on the bacteria during the assay period. In addition, survival and metamorphosis of pediveliger larvae were tested over a 10-day period on a diet of microalgae (*Chaetoceros calcitrans* and *Isochrysis galbana* T-Iso clone), bacteria alone and mixed diet containing bacteria and algae. These results indicated that larvae were able to develop into healthy juveniles even on a sole diet

of biofilm cell suspensions (BCx) from *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1. In addition, survival and metamorphosis remained constant when the two species of bacteria were mixed with the routine microalgal diet. These results support the potential use of mono-species marine bacteria as an alternate or added value additive for hatchery-reared mussels. Further examination of the preparation of large-scale mixed diets, including microalgae and selected bacterial populations, and their stability is required.

9.2 Introduction

New Zealand's endemic green-lipped mussel is a dioecious broadcast spawner with relatively high fecundity of larvae. However, survival and the duration of the planktonic phase largely depends on constant availability of food (Bos et al., 2007; Costa et al., 2012; Rym et al., 2010). Thus, nutrition is a crucial parameter for settlement and recruitment success of larvae and juveniles (Buchanan, 1998; Carton et al., 2007). Indeed microalgae have been conventionally used as the primary food source for bivalve larvae under hatchery conditions (Benemann, 1992; Brown, 2002; Ragg et al., 2010; Rodhouse et al., 1983). However, there has been a constant demand for alternative food sources to replace traditional microalgal diets (Coutteau & Sorgeloos, 1993; Knauer & Southgate, 1999). Typical reasons include the high costs involved in production of live microalgae (Borowitzka, 1997; Urban Jr & Langdon, 1984) and their short shelf life (Heasman et al., 2000). In addition, some microalgal feeds lack specific nutrients, such as amino acids and fatty acids (Nell & Wisely, 1983; Numaguchi & Nell, 1991), and are difficult to maintain in axenic cultures (Nicolas et al., 2004). Apart from microalgae, other microorganisms, such as yeasts and bacteria have been incorporated in mixed diets to enhance growth and

development of bivalve larvae and juveniles (Brown et al., 1996; Coutteau et al., 1994; Douillet & Langdon, 1993; Nell et al., 1994). Bacterivory alone has been shown to sustain populations of several invertebrates, especially during their early life stages (Adams & Angelovic, 1970; Douillet, 1993; Gooselin & Qian, 1997; Gosselin & Qian, 2000; Reiswig, 1975; Rieper, 1978). For example, larvae of the echinoderm, *Porania antartica* were reported to ingest bacteria preferentially over phytoplankton (Rivkin et al., 1986). Conversely, the gastropod, *Crepidula fornicata* larvae preferred diatoms (*Chaetoceros gracilis*) over bacteria when supplied with the two mixed sources (Leroy et al., 2012). Nonetheless, based on their cell size *C. fornicata* larvae were suspected to consume bacteria. Generally, the capacity to understand larval dietary preferences can be heavily challenged due to the small size of larvae and their food. However, several techniques, such as the incorporation of labelled-diets have been used to evaluate their feeding habits.

Previously, isotope-labelled bacteria have been used as tracers to understand bacterivory of bivalve larvae (Baldwin & Newel, 1991; Moal et al., 1996; Rivkin et al., 1986). However, owing to the hazardous nature of radioisotopes, these have been largely replaced by stable isotopes (Koletzko et al., 1997). While stable isotopes may be ideal for trophic studies (Davenport & Bax, 2002), their detection sensitivity is lower compared with radioisotopes and hence, they may have limited application in larval nutritional analyses (Stürup et al., 2008). Alternatively, larval diets can be labelled with fluorescent dyes, or fluorescently labelled microspheres may be used to determine preference of particles of specific sizes (Leyton & Riquelme, 2008; Okaji et al., 1997). For example, Ayukai (1994) labelled bacteria with fluorochromes to establish the dietary requirements of the star fish *Acanthaster planci* larvae.

Nonetheless, both isotope and fluorescent labelling techniques can be expensive and time-consuming processes. Furthermore, the toxicity of such labels needs to be analysed prior to their use in larval nutrition assays. Inert metals, such as ytterbium and chromium have also been used as markers to analyse the digestive efficiency of feed in prawns (Deering et al., 2007) and abalone (Shipton & Britz, 2001), respectively. However, those studies examined the digestive ability of adult organisms based on the presence of heavy metal markers in their faeces. Working with adult organisms is less challenging compared with their juvenile or larvae owing to their smaller sizes. Thus, the use and efficiency of such inert markers to understand nutritional processes in larvae and juveniles remain largely unknown. Apart from labelled diets, some studies have visually observed the development of specific diets for the growth and metamorphosis of larvae through to their juveniles phase (Gooselin & Qian, 1997; Gosselin & Qian, 2000). This technique may perhaps provide more information on bacterivory as a means to provide sustenance and improve larval survival.

Thus far, the feeding ecology of cultured adult green-lipped mussel species has been investigated extensively (Hawkins et al., 1999; Ogilvie et al., 2004; Ogilvie et al., 2003; Ogilvie et al., 2000; Ren et al., 2006; Safi & Gibbs, 2003; Safi & Hayden, 2010; Zeldis et al., 2004). Previous studies have focused on detritivorous and phytoplanktivorous feeding habits of adult mussels. In addition, there has been evidence of cannibalism and zooplanktivory in wild adult populations of *P. canaliculus* (Alfaro, 2006b; Zeldis et al., 2004). Compared with adult mussels, there is limited knowledge on the nutritional aspects of mussel larvae and juveniles (Buchanan, 1998; Carton et al., 2007; Jenkins, 1985; Ragg et al., 2010; Sim-Smith &

Jeffs, 2011). Although specific bacterial strains have been identified as potential probiotics for D-stage larvae of *P. canaliculus* (Kesarcodi-Watson et al., 2010), their involvement in larval nutrition has not been discussed to date. Considering the high availability of wild larvae throughout the year (Alfaro et al., 2010), these larvae may be feeding on alternative diets (e.g., bacteria, dissolved organic matter), especially during times when there is low availability of phytoplankton in the water column. Thus, it is important to determine whether bacteria could contribute to feeding requirements of *P. canaliculus* larvae, especially during the settlement and early metamorphosis period. For this purpose, mono-species marine bacteria (*Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1) were tested for their ability to provide nutrition to mussel larvae and juveniles. These three bacteria were principally chosen since their settlement-inducing activity and toxic effects have been previously investigated (see, Chapters 3–7). In addition, it was essential to know if these settlement-inducing bacteria were able to provide nutrition to individuals during static laboratory assays over the 48 hour period, especially when mussel larvae remained unfed. The novel aspect of this study is the use of inert markers (strontium and ytterbium) to achieve labelled bacteria to analyse bacterivory of mussel larvae and juveniles. Furthermore this study determined whether mussel larvae can survive and metamorphose exclusively on diets of single species bacteria (*Macrococcus* sp. and *Bacillus* sp.) or in combination with microalgae. The results in the present study may help develop alternative diets for hatchery-grown larvae, thereby reducing the overall production costs required to generate mussel seeds.

9.3 Materials and Methods

9.3.1 Source of organisms

Pediveliger mussel larvae (19–21 days old) were sourced from SPATnz as described in chapter 2. Larvae were maintained cool and moist and were transported overnight to the aquaculture facility at AUT University. Upon arrival, larvae were immediately transferred to a beaker containing autoclaved filtered seawater (AFSW) and left undisturbed for initial acclimatisation. After about 1 hour, healthy larvae that were observed swimming in the water column were transferred to another beaker and used for the nutrition experiments.

Hatchery-grown mussel spat were provided by Cawthron Institute, Nelson, New Zealand. Coir ropes containing mussel spat of 0.5–1 mm in size were transported to AUT's aquaculture laboratory in a sealed polypropylene bag. The bag was maintained at 17°C in an insulated box during transport. At the AUT laboratory, the coir ropes containing spat were placed in a clean tray and immersed in fresh seawater. The tray was left undisturbed for 4–5 hours. The spat that attached to the corner of the trays (considered alive) were handpicked and transferred to a beaker containing AFSW maintained at 17°C for the nutrition experiments.

9.3.2 Marking bacteria with strontium

Bacterial cultures of *Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1 were transferred from respective marine agar plates to five 100 ml aliquots of marine broth for each species of bacteria. Preparation of marine broth has been described previously (chapter 4). Strontium chloride was incorporated in the medium prior to sterilisation to achieve 35 ppm of strontium. All

cultures were incubated on a rotary platform at 100 rpm for 24 hours. The bacterial cells were drawn from the culture flasks and centrifuged at 3000 × g for 10 minutes in separate centrifuge tubes. The supernatant from each tube was discarded and the cells were washed with 10 ml AFSW and vortexed for 20 seconds. Suspensions were centrifuged again for 3000 × g for 10 minutes. The process was repeated thrice to remove excess salts from the medium. The cells were finally re-suspended in 10 ml of AFSW to achieve a final concentration of 10^6–10^7 cells ml^{-1}.

To examine the concentration of Sr in bacteria, 2 ml from each of the five tubes were withdrawn and transferred to pre-weighed 1.5 ml Eppendorf vials. Tubes containing cells were centrifuged under the same conditions as before. The supernatant was discarded and the vials containing cell pellets were dried at 105°C for 16 hours. The tubes were removed and cooled prior to weighing, and the dry cell mass were calculated by subtraction.

Prior to the feeding assay, the contents of all five tubes containing 10^6–10^7 cells ml^{-1} were pooled to achieve an even suspension of bacterial cells with uniform concentration of heavy metals.

9.3.3 Marking bacteria with ytterbium

Ytterbium (Yb) was used as an additional marker and the levels of Yb incorporated in bacteria were analysed as a way to track bacteria. A procedure similar to Sr-labelled bacteria was used to obtain Yb-labelled bacteria (refer previous section). Briefly, colonies from *Macrococcus* sp. *Bacillus* sp. and *Pseudoalteromonas* sp. were grown in five 100 ml aliquots of marine broth containing ytterbium chloride to achieve 50 ppm of ytterbium. The cells were

harvested after 24 hours and washed thrice with AFSW to remove the excess salts and metals. The process ensured a final concentration of 10^6–10^7 cells ml^{-1} of Yb-labelled bacteria.

The concentration of Yb in bacteria was determined as described above for Sr. For the feeding assays the contents of the tubes were mixed to achieve homogeneous suspension of cells and a uniform concentration of ytterbium.

9.3.4 Nutrition assays

Bacteria were investigated as a source of nutrition for mussel larvae and spat in separate experiments. In order to determine if bacterial cells provided nutrition during the settlement assay period (48 hours), approximately 200–250 larvae and 50 spat were used for each nutrition assay. To study the effect of bacteria as a nutritional source, the bacterial cells were marked with heavy metals (see above), which were traced after the larvae and the spat were given time to feed.

For the nutrition assay, 2 ml of the labelled bacterial sample was added to 8 ml of seawater containing the larvae or the spat on a Petri plate (60 mm diameter × 14 mm depth). Bacteria were added to the treatment plates and the control plates had no bacteria. The experiments were performed in replicates of 5 per treatment and control. All plates were incubated at 17°C for 48 hours.

After the assay period, the larvae or spat were carefully removed from the plates. The larvae or spat were sieved through a nylon mesh (200 µm) that covered one end of a 50 mm polypropylene tube. The sieve containing the larvae or spat was washed thrice with a total of 30 ml AFSW to remove the bacteria attached to the animal surfaces. The sieve was slowly removed from the tubing and the contents

were transferred to sterile Eppendorf vials. All vials were dried in an oven at 60°C for 48 hours. The Eppendorf vials were weighed before and after the addition of larvae/spat to maintain a uniform weight of samples across the treatments.

9.3.5 Measuring the marker with ICP

The levels of strontium (Sr) and ytterbium (Yb) in labelled bacteria and mussel larvae and spat fed with corresponding heavy metal labelled-bacteria were measured using a *Varian* inductively coupled plasma-atomic emission spectrometer (Liberty AX Sequential ICP-AES).

Individual dried samples of bacteria, mussel larvae or spat were digested in 2 ml of concentrated nitric acid. The digested solution was transferred to a 15 ml screw-capped centrifuge tube (Polystyrene, Thermo Fischer Scientific, Auckland, New Zealand). To aid the digestion process, the samples were placed in a boiling water bath for 30 minutes. The solutions were cooled and diluted with 8 ml of distilled water. The samples were centrifuged and passed through a 0.22 µm filter if any suspended particles were observed in the digested solution. The samples were measured at wavelengths 346.446, 407.771 and 421.552 nm in the ICP. Three standards of 0.1, 0.5 and 1 and 2 ppm of strontium were used as reference to find the concentration of strontium in the unknown samples. Since the wavelength 346.446 nm was sensitive in providing the concentrations of Sr in the samples, this information was recorded.

The concentration of Yb in each sample also was measured using ICP spectrometry. The samples (n = 5) were digested in 2 ml of concentrated nitric acid and the solution was transferred to a 15 ml centrifuge tube. To aid the digestion

process, the sample was placed in a boiling water bath for 30 minutes. The solution was cooled and diluted with 8 ml of distilled water and was filtered through a 0.22 µm filter. The samples were measured at wavelengths 222.445 and 369.419 nm in the ICP. Three standards of 0.1, 0.5, 1 and 2 ppm of Yb were used as reference to find the concentration of Yb in the unknowns. The concentration of Yb across all samples was detected at 369.419 nm, and this information was recorded.

9.3.6 Larval survival and metamorphosis

The effects of bacterial biofilms from *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1, exclusively and in combination with microalgal diets of *Chaetoceros calcitrans* and *Isochrysis galbana* (T-Iso clone) on larval survival and metamorphosis were assessed over a ten-day assay period at SPATnz (Nelson, New Zealand).

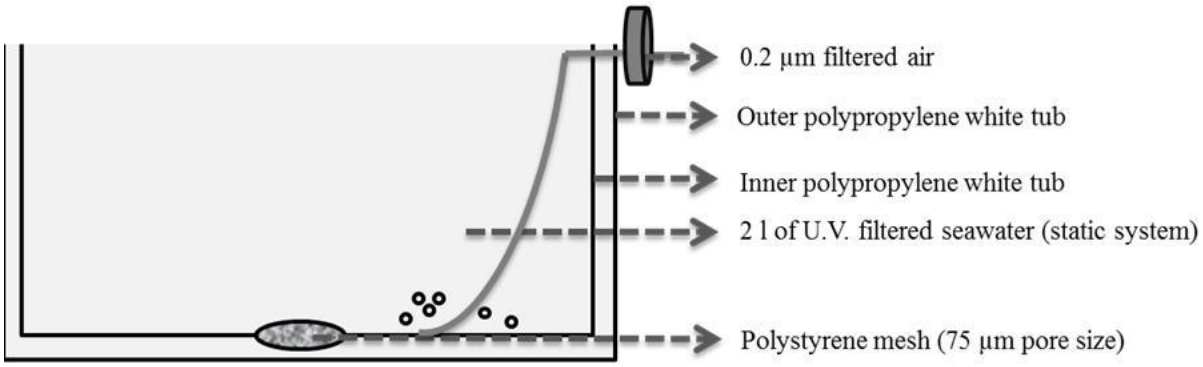

Figure 9.1: Schematic representation of the two-tub system (tank) for conducting nutrition experiments with pediveliger *P. canaliculus* larvae (17 days old) over a 10-day period.

Each treatment consisted of two 2-litre white polypropylene square tubs (Stowers, Auckland, New Zealand), stacked one inside the other (Fig. 9.1). The center of the inner tub contained a circular (50 mm diameter) opening that was

covered by a polystyrene mesh (0.75 µm) and the outer tub remained completely sealed. A tube with sterile filtered (0.22 µm) air was supplied to the inner tub to achieve a constant concentration of oxygen throughout the experiment. The two-tub system will be hereafter referred to as 'tank' throughout this chapter. Each tank received 2 litres of seawater that was previously sterilised by ultraviolet (U.V.) irradiation. Approximately 1000 larvae (pediveliger stage, 19–21 days old) were added to each tank containing U.V. filtered seawater. Water was exchanged every day by gently lifting the upper tub and re-filling the lower tub with fresh filtered seawater. The set-up eased the water exchange process without the need to constantly remove the larvae from the tanks. All fifteen tanks were maintained at 17°C in a flow through water bath, and each tank was placed as indicated in Figures 9.2 A & B. A total of twenty four tanks were used, of which nine tanks were used as blanks to randomise the effect of treatments. The remaining fifteen tanks used in this experiment included three replicates per treatment. The five treatment diets for *P. canaliculus* larvae included: microalgal diet (M) of *Chaetoceros calcitrans* and *Isochrysis galbana* (T-Iso clone), biofilm cell suspensions (BCx) of *Macrococcus* sp. AMGM1 (B_1), BCx of *Bacillus* sp. AMGB1 (B_2) and mixed diets containing microalgae and bacterium 1 (MB_1) and similarly with bacterium 2 (MB_2).

1	3	5	7	9	11	13	15	17	19	21	23
MB_2	X	B_1	B_2	X	M	MB_1	X	X	MB_2	B_1	B_2
2	4	6	8	10	12	14	16	18	20	22	24
X	M	MB_1	X	X	MB_2	B_1	B_2	X	M	X	MB_1

Symbols	Treatments	Tank code
M	Microalgal diet	4, 11 & 20
B_1	Bacterium 1	5, 14 & 21
B_2	Bacterium 2	7, 16 & 23
MB_1	Microalgae and bacterium1	6, 13 & 24
MB_2	Microalgae and bacterium 2	1, 12 & 19
X	Blank	2, 3, 8, 9, 10, 15, 17, 18 & 22

B

Figure 9.2: Figure on left hand side (A) represents the experimental setup of 24 white polystyrene tanks of which fifteen were used for the larval survival and metamorphosis assay. The black container beneath the tanks is the water bath to maintain all tanks at 17°C (photo courtesy, A. B. Rusk). Treatments in each tank have specific symbols and tank codes (B). The shaded squares in the diagram represent treatment tanks and open squares represent tanks that were used as blanks.

The biofilm cell suspensions (BCx) of respective mono-species bacteria were prepared at the AUT University School of Applied Sciences. A modified protocol (Chapter 3) was carried out to achieve a high concentration (10^7–10^8 cells ml^{-1}) of BCx for the nutrition experiments. Biofilm cells from respective mono-species bacteria were withdrawn from fifty replicate marine agar plates, pooled and re-suspended in 50 ml of AFSW. Colonies from agar plates were preferred over broth cultures to achieve greater concentration of cells and to avoid interference of salts in the liquid media. The cells were transferred to two 50 ml centrifuge tubes, mixed briefly by vortexing and centrifuged at 3000 × g for 10 minutes. Supernatants were discarded and pellets containing cells were re-suspended in 30 ml of fresh AFSW. The process was repeated thrice to achieve a final concentration on 10^7–10^8 cells ml^{-1} in 50 ml AFSW. BCx of the respective mono-species bacteria were stored in two 50 ml sterile screw-capped centrifuge tubes, and transported to the aquaculture

facility at SPATnz in a polystyrene foam bin containing ice packs. Individual microalgal species, *Chaetoceros calcitrans* and *Isochrysis galbana* (T-Iso clone) necessary for the nutrition experiments were provided by SPATnz.

The microalgal diet (M) tested in this study comprised of 20 ml of 1:1 (v/v) *Chaetoceros calcitrans* 15,000 cell $\mu l^{-1}$and *Isochrysis galbana* (T-Iso clone) 10,000 cell μl^{-1} harvested in the exponential phase of a 48-hour culture. B_1 comprised of 2 ml of 10^7–10^8 cells ml^{-1} of *Macrococcus* sp. AMGM1 and B_2 included 2 ml of 10^7–10^8 cells ml^{-1} of *Bacillus* sp. AMGB1. Diets of MB_1 consisted of 10 ml of diet M and 1 ml of B_1, and similarly MB_2 consisted of 10 ml of diet M and 1 ml of B_2.

9.3.7 Statistical analyses

Raw data from the nutrition assays for larvae and spat were individually analysed using IBM® SPSS® statistics version 19. Data that were tested for and met parametric assumptions were subjected to independent sample t-test to compare the treatment effects in this study. Data that were non-parametric were analysed using the Mann-Whitney U test.

For the larval survival and metamorphosis assays, all percent survival and metamorphosis data were square root transformed since the range of percentages were between 0–20 percent. Values ≤ 10% were square root transformed using the formulae $\sqrt{(x+0.5)}$, where x represented the percentage data (Ahrens et al., 1990). Transformed data were tested for and met parametric assumptions were subjected to one-way Analysis of Variance (ANOVA) followed by *post-hoc* Tukey tests for pair-wise comparisons.

9.4 **Results**

9.4.1 Nutrition assays

Results from the nutrition experiments clearly indicate that both strontium (Sr) and ytterbium (Yb) were incorporated in the cells of mono-species bacteria (Fig. 9.3 A & B). It was observed that 2 ml of bacteria with 10^6–10^7 cells ml^{-1} contained 0.0002 grams of dry weight with concentrations of about 1.0 ppm of Sr and 0.3 ppm of Yb. The mean (ppm) ± S.E. of the Sr concentration measured at 346.446 nm were 1.05 ± 0.16 for *Macrococcus* sp., 1.30 ± 0.17 for *Bacillus* sp. and 1.07 ± 0.19 for *Pseudoalteromonas* sp. Similarly, the Yb concentration of the bacteria *Macrococcus* sp., *Bacillus* sp. and *Pseudoalteromonas* sp. were 0.30 ± 0.07, 0.35 ± 0.08 and 0.32 ± 0.05, respectively.

For the larval and juvenile nutrition assay, each control and treatment plate contained 200–250 pediveliger mussel larvae with 4–5 mg of average dry mass, and for 50 (0.5–1 mm) spat were found to be between 7–8 mg. In addition, the concentration of strontium (Sr) in mussel larvae measured at 346.446 nm across treatment and control plates were about 0.5 ppm, and those of spat were between 0.8–1.0 ppm. The feeding assay with larvae and spat fed with Sr- labelled bacteria did not show any difference in the concentration of Sr for both treatment and control plates (Figs 9.4 A–F; Table 9.1). Individual independent t-test results revealed non-significant differences between control and treatments for all three bacteria and for both larvae and spat. Since relatively high concentrations of background Sr were observed in control plates, Sr was shown to be an inefficient marker for nutrition assays.

Larvae were inferred to feed on bacteria due to the presence of Ytterbium in larvae obtained from the treatment plates containing Yb-labelled bacteria. Larvae were shown to ingest *Macrococcus* sp., since larvae obtained from those treatment plates contained significant ($p < 0.05$) proportions of Yb compared with controls containing no bacteria (Fig. 9.5 A; Table 9.2). Similarly, larvae that fed on *Bacillus* sp. AMGB1 showed enhanced Yb concentration, which was significantly ($p < 0.05$) different from the controls (Fig. 9.5 B; Table 9.2). In addition, larvae that fed on Yb-labelled *Pseudoalteromonas* sp. AMGP1 showed significantly ($p < 0.05$) greater concentration of Yb compared with controls (Fig. 9.5 C; Table 9.2). However, the concentration of Yb observed in larvae that fed on Yb-labelled *Pseudoalteromonas* sp. was considerably lower (0.0001 ppm) compared with those of larvae that fed on *Macrococcus* sp. and *Bacillus* sp., and which were observed to contain over 0.03 ppm of Yb. These results may indicate that larvae might not have actually ingested *Pseudoalteromonas* sp. and the trace of Yb in their sample could have been due to handling effects. Mann-Whitney analyses showed significant differences ($p < 0.05$) between control and treatments for each of the three bacteria tested with mussel larvae.

Similarly, mussel spat obtained from treatment plates containing labelled bacteria were shown to contain a greater concentration of Yb than controls (Figs. 9.5 D–F; Table 9.2). Moreover, significant differences ($p < 0.05$) were observed between control and treatments for each of the three bacteria analysed in this study. In addition, enhanced proportion of Yb concentration was observed in spat that fed on *Macrococcus* sp. (0. 0714 ppm) and *Bacillus* sp. (0.0836 ppm) compared with only 0.0020 ppm in spat that fed on *Pseudoalteromonas* sp. These results indicate that the

former two species of bacteria were preferentially selected by larvae and spat during the feeding assay.

9.4.2 Larval survival and metamorphosis

Results from the larval survival and metamorphosis experiments (Figs. 9.6 A & B) clearly indicate that bacterial cell suspensions (BCx) of *Macrococcus* sp. AMGM1 and *Bacillus* sp. individually or in combination with microalgae resulted in similar rates of survival and metamorphosis for *P. canaliculus* larvae. Mean percentage of larval survival were between 4–6% across all treatments. In addition, the treatments with BCx of *Macrococcus* sp. AMGM1 (B_1), BCx of *Bacillus* sp. AMGB1 (B_2), microalgae and *Macrococcus* sp. (MB_1), microalgae and *Bacillus* sp. (MB_2) were not significantly different from the microalgal (M) treatment (ANOVA; $F_{4,10} = 0.406$, $p = 0.838$). Individual Tukey *post-hoc* tests revealed non-significant ($p > 0.05$) differences between the microalgal diet and other treatment diets.

Similarly, the metamorphosis experiment indicates that treatments B_1, B_2, MB_1 and MB_2 were not significantly different compared with diet M (ANOVA; $F_{4,10} = 0.034$, $p = 0.239$). Furthermore, *post-hoc* tests revealed non-significant ($p > 0.05$) differences between the microalgal diet and other treatment diets.

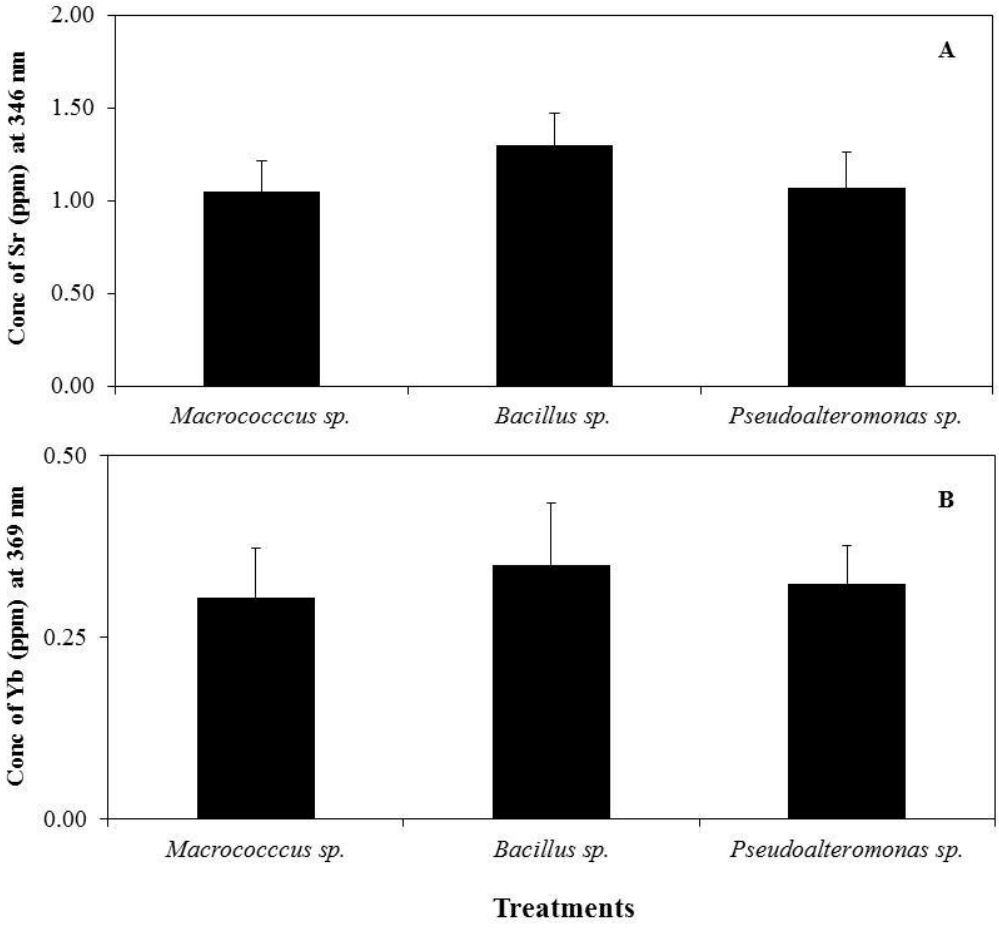

Figure 9.3: Concentration of strontium (A) and ytterbium (B) in labelled-bacterial cells of *Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1 prior to their use in the nutrition assays.

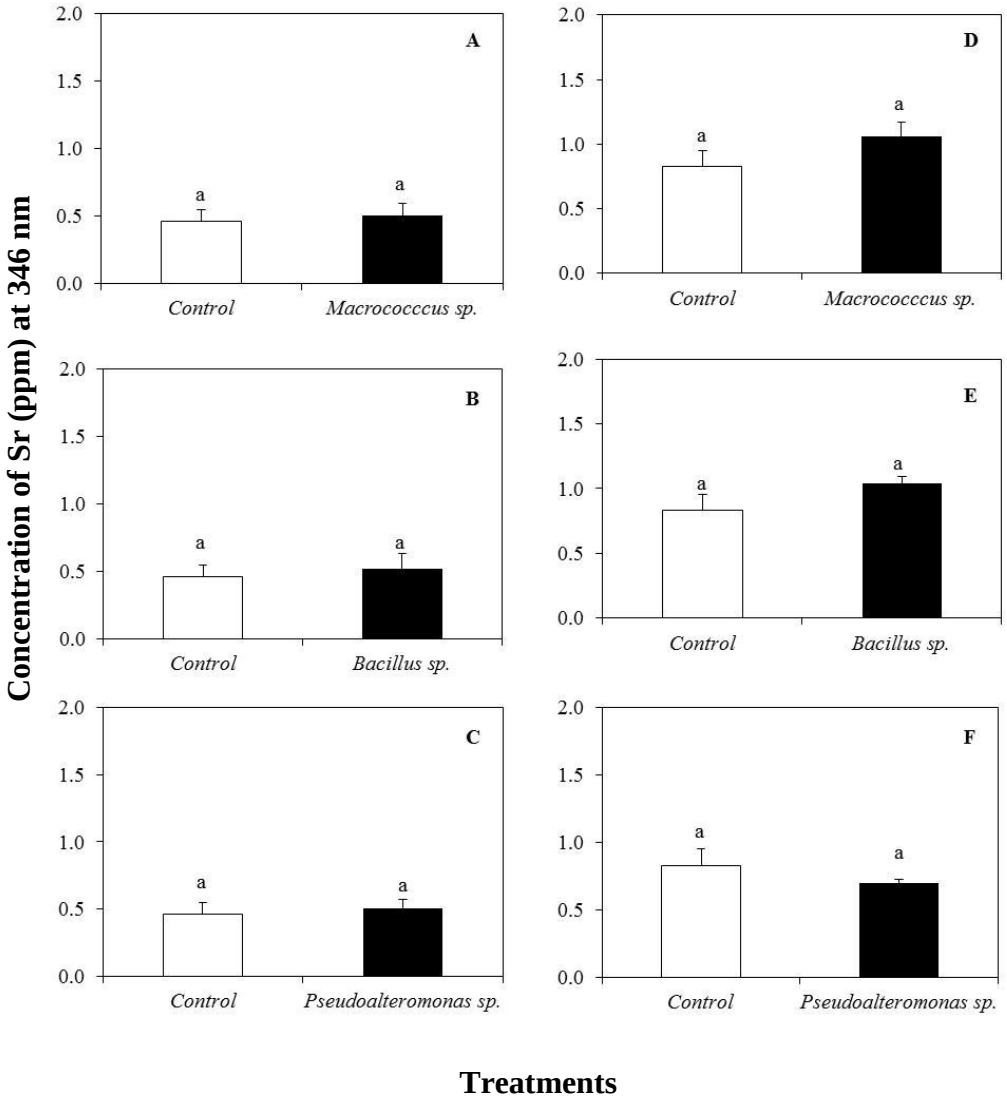

Figure 9.4: Mean (±SE, n = 5) concentration of strontium (Sr) measured at 346.446 nm for mussel larvae (A, B & C) and spat (D, E, F) on polystyrene Petri plates containing Sr-labelled cells of *Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1. The open bars represent sterile control plates and shaded bars represent the treatment plates. Non-significant independent t- tests are denoted by the same letter over the bars.

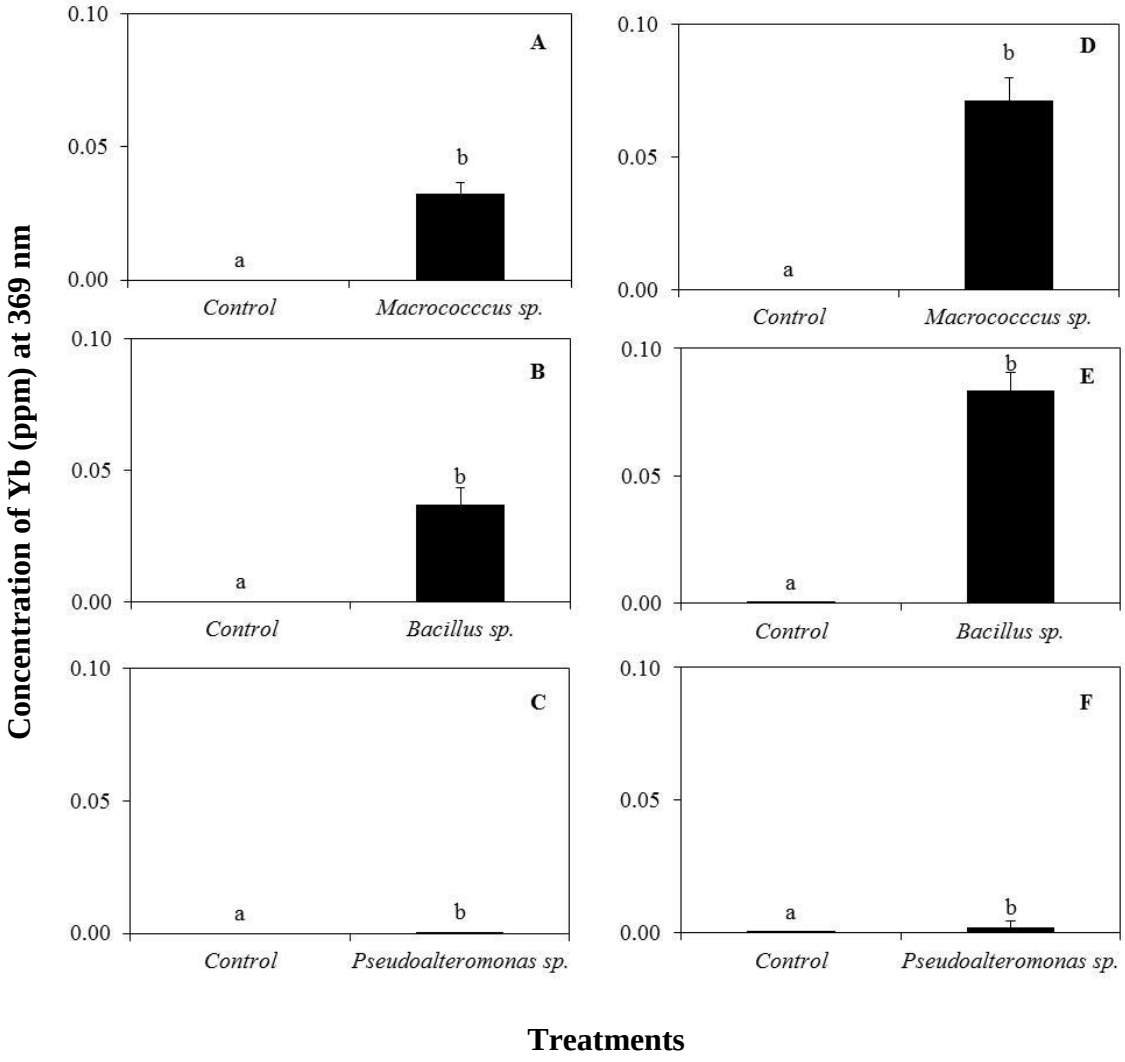

Figure 9.5: Mean (±SE, n = 5) concentration of ytterbium (Yb) measured at 369.419 nm for mussel larvae (A, B & C) and spat (D, E, F) on polystyrene Petri plates containing Yb-labelled cells of *Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1. Shaded bars represent the treatment plates and non-significant Mann-Whitney tests are denoted by the same letter over the bars.

Table 9.1: Statistical analyses (Square root transformed data and independent t- test) for *P. canaliculus* larvae and spat nutrition assay fed with Strontium-labelled *Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1. Significant tests ($p < 0.05$) are in bold

Treatment (Strontium-labelled bacteria)	**Control mean ± S.D. (square root)**	**Treatment mean ± S.D. (square root)**	**Pooled DF**	***t*-value**	***p*-value**
Larvae					
Macrococcus sp. AMGM1		0.50 ± 0.16	8	0.42	0.684
Bacillus sp. AMGB1	0.46 ± 0.15	0.52 ± 0.20	8	0.56	0.591
Pseudoalteromonas sp. AMGP1		0.51 ± 0.12	8	0.52	0.615
Spat					
Macrococcus sp. AMGM1		1.06 ± 0.19	8	1.78	0.113
Bacillus sp. AMGB1	0.83 ± 0.21	1.04 ± 0.10	8	1.98	0.083
Pseudoalteromonas sp. AMGP1		0.70 ± 0.05	8	1.36	0.212

Table 9.2: Statistical analyses (Raw data and Mann-Whitney U test) for *P. canaliculus* larvae and spat nutrition assay fed with Ytterbium-labelled *Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1. Significant tests ($p < 0.05$) are in bold.

Treatment (Ytterbium-labelled bacteria)	**Control mean ± S.D.**	**Treatment mean ± S.D.**	**U value**	***p*- value**
Larvae				
Macrococcus sp. AMGM1		0.0325 ± 0.0042	0.000	**0.005**
Bacillus sp. AMGB1	0.0000 ± 0.0000	0.0370 ± 0.0108	0.000	**0.005**
Pseudoalteromonas sp. AMGP1		0.0001 ± 0.0001	2.500	**0.019**
Spat				
Macrococcus sp. AMGM1		0.0714 ± 0.0087	0.000	**0.009**
Bacillus sp. AMGB1	0.0000 ± 0.0000	0.0836 ± 0.0680	0.000	**0.009**
Pseudoalteromonas sp. AMGP1		0.0020 ± 0.0022	1.000	**0.016**

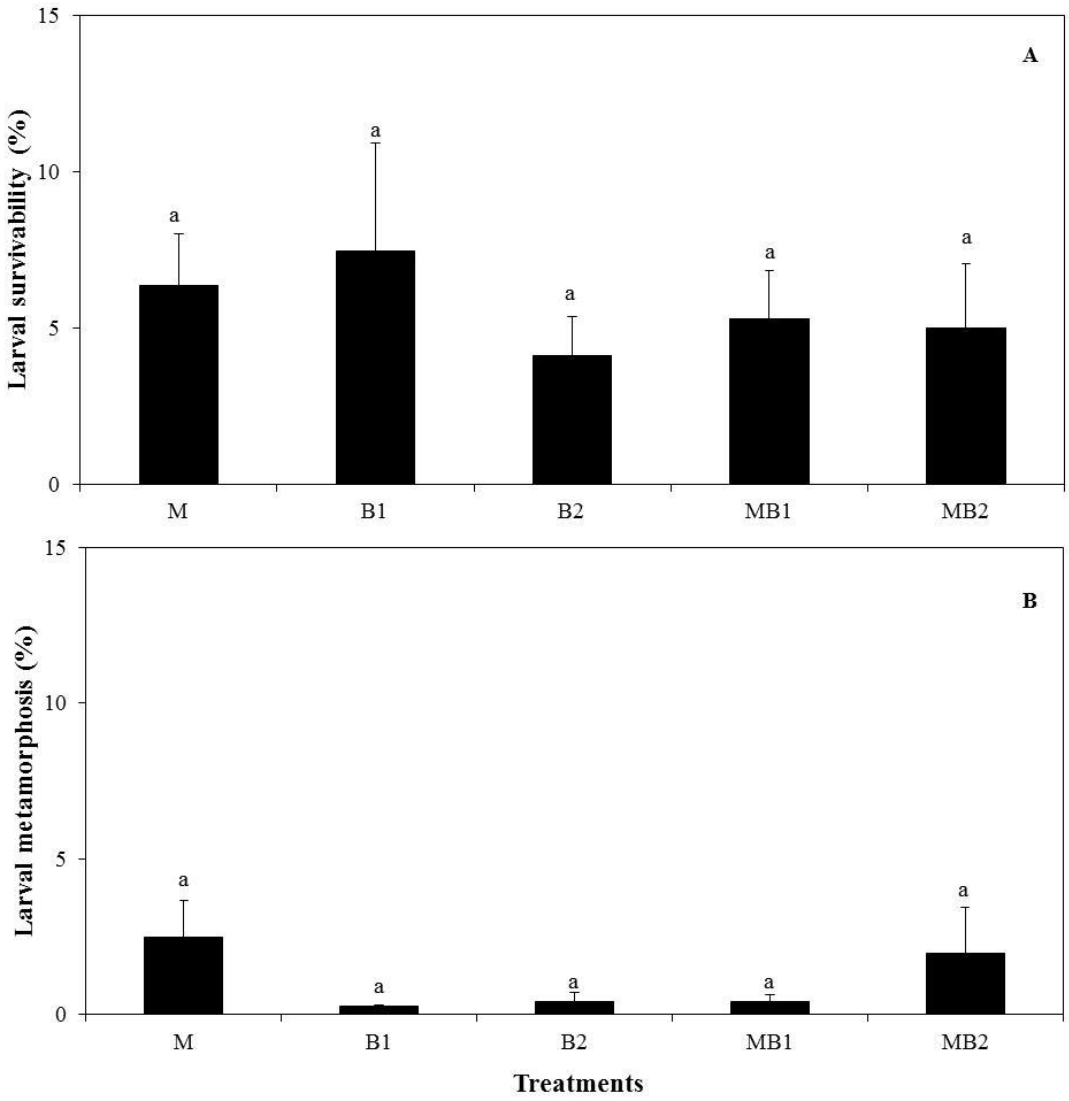

Figure 9.6: Mean (±SE, n = 3) percent larval survival (A) and metamorphosis (B) of *P. canaliculus* after 10 days on tanks containing diets of microalgae (M), biofilm cell suspensions (BCx) of *Macrococcus* sp. AMGM1 (B_1), BCx of *Bacillus* sp. AMGB1 (B_2) and mixed diets containing microalgae and *Macrococcus* sp. (MB_1) and mixed diet of microalgae and *Bacillus* sp. (MB_2). Non-significant Tukey tests are denoted by the same letter over the bars.

9.5 **Discussion**

This study is the first to examine the effects of mono-species bacteria as a potential source of nutrition for *Perna canaliculus* larvae and juveniles. Results from the nutrition assay using ytterbium as a marker clearly indicate that both mussel larvae and spat preferentially ingested bacterial cells of *Macrococcus* sp. AMGM1

and *Bacillus* sp. AMGB1 and did not ingest cells of *Pseudoalteromonas* sp. AMGP1. These results are in agreement with previous settlement assays, which showed that both mussel larvae (chapters 3–5) and spat (chapter 8) settled in response to cues from biofilms of *Macrococcus* sp. and *Bacillus* sp. In addition, biofilms of *Pseudoalteromonas* sp. AMGP1 were shown to induce greater (over 60%) larval mortality (chapters 3–5) and did not enhance settlement of mussel spat (chapter 8), a plausible reason for mussel larvae and spat to refrain from ingesting cells of *Pseudoalteromonas* sp. In addition, ytterbium (Yb) was shown to be a suitable marker compared with strontium (Sr) for nutrition assays. The presence of high concentrations (7.85 ± 0.03 ppm) of Sr in seawater (Bernat et al., 1972; Brass & Turekian, 1974; Veizer, 1989) may result in the incorporation of Sr in bivalves shells (Cathey et al., 2012). This is due to the divalent nature of Sr, which can substitute for calcium during the biogenic carbonate process in bivalve larvae and juveniles (Takesue et al., 2008). Thus, larvae and spat analysed from control plates containing seawater and no bacteria were observed to contain significant proportions of strontium.

Another important finding of this study is the observed ability of larvae to choose preferentially specific species of bacteria to obtain nutrition. Thus far, the genus *Macrococcus* has not been used in any nutritional studies for marine organisms, and there have not been any reported studies on their probiotic activities. Unlike *Macrococcus*, species of *Bacillus* and *Pseudoalteromonas* have been commonly examined for their ability to improve digestion in marine invertebrate larvae and to confer their host's heath (Gatesoupe, 1999; Irianto & Austin, 2002). For example, *Bacillus* spp. were found to improve the overall survival of larvae and

post larvae of white shrimp *Litopenaeus vannamei* (Silva et al., 2011). Similarly, *Pseudoalteromonas* sp. PP107 were shown to protect spiny lobster (*Panulirus ornatus*) larvae against *Vibro owenssii* infection (Goulden et al., 2012). However, *Pseudoalteromonas* sp. AMGP1 tested in this study did not provide nutrition to larvae and juveniles. Nonetheless, toxic effects of *Pseudoalteromonas* sp. have been extensively reported (Bowman, 2007; Ganesan et al., 2010; Holmström et al., 2002; Holmström et al., 1998; Rao et al., 2007) and the probiotic ability of bacteria may remain species-specific.

The most significant finding of this study is the ability of *P. canaliculus* larvae to successfully survive and metamorphose into juveniles on a sole diet of mono-species bacteria. These results are in agreement with those of larvae of several bivalve species which have been previously reported to digest bacteria and obtain nutrition from them (Baldwin & Newel, 1991; Douillet & Langdon, 1993; Douillet, 1993). The larvae of the European blue mussel, *Mytilus edulis* were demonstrated to consume mono-species diet of bacteria (Prieur, 1983). However, that study did not examine the effect of bacteria on metamorphosis and survival of larvae. Nonetheless, larvae of polychaetes *Hydroides elegans* were shown to grown into healthy juveniles on a sole diet of bacteria, indicating that bacteria can provide enough nourishment during early life stages for some species (Gooselin & Qian, 1997; Gosselin & Qian, 2000). The relatively small size of bacteria compared with microalgal cells and the presence of essential nutrients (e.g., vitamins, proteins and carbohydrates) in bacteria have enabled their use as a preferential diet for many invertebrates (Brown et al., 1996; Robert & Trintignac, 1997).

From an ecological perspective, settlement and recruitment of larvae and juveniles may not entirely depend on the availability of food source (Gooselin & Qian, 1997; Gosselin & Qian, 2000). For the New Zealand green-lipped mussel, larvae have been observed in the water column throughout the year (Alfaro et al., 2010). This is due to the apparent differences in spawning periods recorded between northern and southern adult mussel populations. The populations at Ninety Mile Beach, Northland, mostly spawn between June and December (Alfaro et al., 2001, 2003). Whereas, the northern South Island populations have been recorded to have two distinct spawning periods in early summer and autumn-spring (Buchanan, 1998; Flaws, 1975; Tortell, 1976). Hence, specific cohorts of mussel larvae may be exposed to varied seasonal patterns and differences in food availability. In addition, phytoplankton abundances have been shown to be relatively low especially during winter in New Zealand (Murphy et al., 2001). Thus, it is possible that mussel larvae may rely entirely on nutrition from bacteria in order to settle and metamorphose during some periods. Low or no spat arrivals at Ninety Mile Beach, northern New Zealand have previously been attributed to oceanic processes (Alfaro et al., 2010), but the potential effects of phytoplankton availability or depletion of nutrients in the sea have not been clearly investigated. In addition, to the general nutritional ecology of *P. canaliculus* larvae and juveniles, the present study has significant commercial implications for this mussel species. Although microalgal diets have been widely used for the culturing of *P. canaliculus* for over a decade, there is a constant search for optimal diets (Buchanan, 1998; Ragg et al., 2010). In addition, production of microalgae is a bottleneck, and a significant proportion (over 30%) of cost involved in rearing larvae could be spent on generating their feed by continuously culturing microalgae (Borowitzka, 1997; Coutteau & Sorgeloos, 1993; Knauer & Southgate,

1999; Urban Jr & Langdon, 1984). Thus, these bacterial species *Macrococcus* sp. AMGM1 and/or *Bacillus* sp. AMGB1 may be used as an alternative diet in *P. canaliculus* hatcheries. Moreover, mixed diets of bacteria and microalgae may be used to achieve similar rates of larval survival and metamorphosis and thereby reducing the overall cost involved in the hatcheries. Future work on the digestive capability, knowledge on the diet behaviour in the water column (i.e., dispersion, leaching) and the fate of bacteria in the gut of mussel larvae and juveniles will provide more information on the success or failure of bacterial diets. A diverse interdisciplinary team with knowledge on aquaculture, food science, microbiology and analytical chemistry may be needed to comprehensively examine the success of bacteria as a potential artificial diet for mussel larvae and juveniles.

Chapter 10.

GENERAL DISCUSSION

10. General Discussion

Research on the indigenous green-lipped mussel, *Perna canaliculus* (Gmelin 1791) has been constantly expanding for over forty years. Earlier studies focused on adult mussels with considerable attention to their population dynamics (Alfaro, 2006c; Alfaro et al., 2008; Hickman, 1991; Lachowicz, 2005; Paine, 1971), reproduction patterns (Alfaro, 2001; Alfaro & Jeffs, 2003; Flaws, 1975; Hickman & Illingworth, 1980; Jenkins, 1985; Tortell, 1976) and dietary requirements (Alfaro, 2006b; Gardner, 2002; Hatton et al., 2005; Hawkins et al., 1999; Safi & Hayden, 2010). Research on early life stages (larvae and juveniles) of this species was initially based on the effect of physical and biological factors on wild individuals (Alfaro, 2001, 2005, 2006a; Alfaro et al., 2006; Alfaro & Jeffs, 2002, 2003; Alfaro et al., 2004; Alfaro et al., 2010). The recent focus on larval and juvenile research for this species is on its physiology, behaviour and nutrition for applications to aquaculture practices (Ganesan et al., 2010; Ganesan et al., 2012a; Ganesan et al., 2012b; Young, 2009; Young et al., 2011). The novel aspect of this thesis is that it provides the first comprehensive study on the effect of bacterial biofilms as mediators of larval settlement behaviour for this species. Through extensive laboratory assays and by means of combining a variety of scientific disciplines (e.g., microbiology, chemistry, genetics), this work greatly contributes to the understanding of prokaryotic-mediated settlement and nutrition for mussel larvae and larvae of other marine invertebrates.

For the first time, *P. canaliculus* larvae were shown to settle in response to cues from single-species marine bacteria. The ability of mussel larvae to respond to

specific bacterial cues appears to facilitate the identification of suitable substrates for settlement. From an ecological perspective, it is clear that larval settlement is a systematic well-orchestrated process. In addition to morphological and chemical cues from macro-algae, larvae undergo settlement in response to unique bacterial signals. In this study, the settlement response to specific mono-species marine bacteria was investigated by still-water laboratory based settlement assays. Settlement experiments were conducted in a laboratory setting to rule out extraneous influences (e.g., temperature, salinity, pollution) that are routinely observed in field-based assays. Furthermore, larval settlement responses to discrete cues, especially from single species bacterial biofilm cannot be examined in field conditions owing to the ubiquitous nature and interference of other microbes. With previous knowledge of a laboratory-based settlement assays for *P. canaliculus* larvae (Alfaro et al., 2006; Young et al., 2011), the present study has optimised parameters, such as settlement substrata, settlement medium and duration of the assay, to achieve meaningful results with minimum variability among replicate plates. Previous studies also indicated that 48-hour incubation would be most suited to conducting settlement assays on mussel larvae. These experimental parameters are essential to eliminate or minimise the biases that often occur with other species, where there is no standard protocol for laboratory-based settlement assays. For instance, the duration of settlement assays for the bryozoan larvae, *Bugula neritina* can range between 30 minutes and up to 24 hours (Bryan et al., 1998; Dahms et al., 2004; Rittschof & Costlow, 1989), and without prior optimisation, it may be challenging to conduct reliable settlement assays. Therefore, this study has addressed the need to optimise settlement assays to standard robust experimental protocols, which can be applied to

future settlement, metamorphosis and survivability studies, and may be comparable across taxa.

Of the fourteen culturable marine bacteria isolated, screened and investigated for involvement in larval settlement in this study, three bacteria (*Macrococcus* sp. AMGM1, *Bacillus* sp. AMGB1 and *Pseudoalteromonas* sp. AMGP1) were chosen for their ability to produce distinct cues that modulated larval settlement compared with the other bacterial species and sterile controls. During the screening of bacterial biofilms, *P. canaliculus* responded to various bacterial cues and differentially settled in response to cues from mono-species bacteria. Furthermore, this and other studies have emphasised the importance of using mono-species bacteria over natural multi-species bacterial biofilms for larval settlement experiments (Alfaro et al., 2011b; Holmström et al., 2002; Huggett et al., 2006; Maki et al., 1990; Negri et al., 2001; Tebben et al., 2011). Biofilms of multi-species bacteria may be suitable for one-off settlement assays and it is most often impractical to achieve the same proportion and composition of bacterial populations for repeated analyses. Studies by Lau & Qian (1997) suggested that the ability of multi-species biofilms to induce settlement may actually be due to the presence of small proportions of settlement-inducing bacteria in the assays. Further work by Lau et al. (2002) revealed that certain mono-species bacteria were able to induce greater settlement for polychaete larvae (*Hydroides elegans*), compared with other species of bacteria obtained from the same source. These results were in accordance with the results from this study, as only two of the fourteen culturable bacteria were able to induce greater settlement of *P. canaliculus* larvae compared with sterile controls and other species of bacteria. The Gram-positive coccus, *Macrococcus* sp. AMGM1, sourced from seawater induced over

60% settlement compared with sterile controls. Similar settlement percentages were observed in treatments with *Bacillus* sp. AMGB1, a Gram-positive rod isolated from mussel guts. Conversely, the Gram-negative rod, *Pseudoalteromonas* obtained from *Ulva lactuca* induced only up to 20% larval settlement, which was similar to controls, but unlike the other bacterial species and controls, *Pseudoalteromonas* sp. induced over 60% larval mortality. Although species of *Bacillus* have been previously examined for their settlement inducing and inhibitory effects on other invertebrate larvae (Khandeparker et al., 2003; Lau & Qian, 2001; Unabia & Hadfield, 1999), the current isolate of *Bacillus* sp. designated AMGB1 has not been described for its ability to induce mussel larval settlement. Similarly, several studies have analysed a variety of *Pseudoalteromonas* strains for their larval settlement inducing and inhibitory properties (Holmström et al., 1998; Huggett et al., 2006; Lau et al., 2003; Tebben et al., 2011). However, this study is the first to analyse the effect of marine *Macrococcus* sp. for the settlement on invertebrate larvae. All three species of bacteria described in this study are worthy additions to the list of previously described mono-species bacteria tested for invertebrate larvae. From a microbiological perspective, it is interesting to note that traditional culturing techniques may still remain suitable for effective isolation of mono-species bacteria for settlement assays (Zobell, 1941). However biochemical techniques described for terrestrial microbes may not be appropriate for the identification of marine bacteria, as observed from this study. Thus, with the volume of research being focused on marine microbes, an area to investigate would be to develop biochemical kits that are specifically designed for the identification of marine bacteria. With the aid of sequence analyses, nomenclature of marine bacteria can be achieved at least to the genus level. Phylogenetic analysis may support identification to species level. From

an aquaculture point of view, the ability to determine the species of bacteria using in-expensive kit assays will enable identification of bacterial strains that benefit larvae, and also aid in control of other pathogenic microbes that reduce larval health and survival.

While settlement assays are aimed at examining the effect of mono-species bacteria, a range of substrata with varied physical properties need to be tested prior to choosing specific strains for subsequent analyses. This study clearly demonstrated that the physicochemical interaction of mono-species bacterial biofilm with different substrates can alter settlement inducing properties for mussel larvae. The results showed that substrates, such as polystyrene, glass and agar modulated the activity of six out of the fourteen bacteria tested in this study. These results have challenged our alleged bacterio-centric view, with respect to bacterially mediated larval settlement assays (Faimali et al., 2004; O'Connor & Richardson, 1994). Intriguingly, despite the physical properties of the settlement substrates, three key mono-species marine bacteria (*Macrococcus* sp. *Bacillus* sp. and *Pseudoalteromonas* sp.) induced similar larval settlement percentages, which indicated that the origin of the settlement cue was confined to the bacterial biofilm and not modulated by the physical substrates. Therefore, coating a layer of settlement inducing bacterial biofilms on any settlement substrata may enhance larval settlement. This knowledge may be applicable to development of spat-catching surfaces in the wild and/or improvement of larval production in hatcheries. However, managing extraneous factors, such as presence of other pathogens and monitoring water quality may be major issues that need to be addressed when conducting larval settlement experiments in large-scale hatchery settings.

While, testing individual bacterial cues within the biofilm architecture (cells and exudates), specific sources of settlement cues were identified. The inability of planktonic bacteria and washed biofilms (without exudates) to modulate larval settlement, discounted the role of surface-bound bacterial cues as potential inducers of larval settlement. With the aid of various microbiological techniques, biofilm exudates of the three bacteria were shown to contain cues that regulate larval settlement. In the sea, such waterborne settlement cues may guide larvae to choose their substrata, which may be some distance away (e.g., several centimetres). Biofilms may not only provide signals for larval settlement, but may provide a sticky matrix containing cells and exudates for larval attachment and nutrition. The chemical signature of the settlement and mortality cues in the biofilm exudates were examined *via* an innovative step-wise multi-technique approach, including physical (molecular weight fractionation and heat treatment) and chemical (crude fractionation, sub-sample fractionation, sequential fractionation, lipid fractionation and enzyme treatment) separation methods. This multi-disciplinary approach allowed for the demonstration that larvae responded to unique chemical signatures that were bacterium species-specific. Mussel larvae settled in response to polar protein and non-polar lipoprotein molecules from exudates of *Macrococcus* sp. AMGM1 and glycolipid molecules from *Bacillus* sp. AMGB1. Molecules from *Pseudoalteromonas* sp. AMGP1 and toxic to larvae were found to be polar proteins. These results indicated that mussel larvae may settle in response to the quorum sensing signals that bacteria generate to communicate among themselves and/or across species, genera and kingdoms. Since quorum sensing (QS) signals for Gram-positive bacteria are predominantly proteins (March & Bentley, 2004), *P. canaliculus* larvae could have settled in response to QS chemicals of *Macrococcus*

sp. AMGM1 since the settlement cue of this organism appeared to be proteinaceous. *Bacillus* sp. AMGB1 was also Gram-positive, yet its settlement cue was glycolipid in nature. Molecules of this type are likely to be adhesive exo-polymeric molecules secreted during the biofilm phase enabling bacteria to adhere themselves to substrates and not QS chemicals. QS signalling molecules for Gram-negative bacteria have been shown to be N-acyl homoserine lactones (Fuqua et al., 2001) Thus, the toxin from the Gram-negative *Pseudoalteromonas* sp. AMGP1 may not be produced for quorum sensing since it was found to be a small polar protein .

The effects of biofilm exudates (Alfaro et al., 2011b; Costa-Ramos & Rowley, 2004; Fitt et al., 1990) and surface-bound cues (Kirchman et al., 1982b; Lau et al., 2003; Szewzyk et al., 1991) of bacteria have been extensively investigated in the past. Compared with the number of bacterial biofilm-based chemical compounds that have been identified, only a handful of studies detail the chemical nature of these cues (Ganesan et al., 2012b; Holmström et al., 1998; Neumann, 1979; Szewzyk et al., 1991; Tebben et al., 2011). Therefore, the present study is an addition to the existing knowledge on the range of chemicals present in mono-species biofilm that are able to induce or deter settlement of larvae. Although the chemical structures of the inductive and toxic cues were not elucidated in this study, this information might not be required from an aquaculture point of view. This may be due to the fact that high throughput techniques (i.e., mass spectrometry) may aid in providing a catalogue of molecules that are present in the settlement inductive fraction. Furthermore, these high-end techniques might not be able to produce optimum quantities of fractionated chemicals to conduct larval settlement assays. Thus, this study provides knowledge on the chemical nature of the cue and demonstrates their

capacity to induce larval settlement by *in-vitro* settlement assays. The techniques used for the elucidation of the chemical fraction were routine and inexpensive, and these chemicals obtained from bacteria can potentially be incorporated in mussel hatchery procedures to enhance larval settlement. In a hatchery setup, metabolites of bacteria may be more advantageous than using biofilms since chemicals are less likely to interact with surrounding microbes and change their composition. Therefore, the effect of chemicals from mono-species bacteria in hatchery settings would be a possible direction for future investigation.

The retention experiment for juvenile mussels clearly demonstrated the differences in settlement pattern for the larvae and juvenile stages of mussels. The juveniles settled in response to the physical nature of the settlement cue with greater settlement on un-filmed polystyrene surfaces than glass and coir substrates. Further examination of the physicochemical properties of polystyrene substrate for spat settlement, and preparation of specific substrates with known surface charge and wettabilities could be an area to investigate in the future. Nonetheless, it was clear that coir ropes containing mono-species biofilm of *Macrococcus* sp. AMGM1 and *Bacillus* sp. AMGB1 were able to enhance mussel spat settlement. Furthermore, decreased settlement was observed for coir ropes containing *Pseudoalteromonas* sp. AMGP1, and this biofilm was not able to induce spat mortality. Retention of spat in a static assay indicated that mono-species biofilm of *Macrococcus* sp. and *Bacillus* sp. on glass and coir substrates improved retention during the first 24 hours. The decline in retention could be due to the fact that these organisms were kept unfed throughout the assay and the system was also static. Future experiments need to be conducted in a flow-through system with feeding throughout the assay period.

However, confounding effects of incorporating microalgae need to be initially addressed and controlled. Results from analysing physicochemical properties of substrates with appropriate biofilms and feeding spat would enhance attraction of spat and improve the efficiency of the catchment. An ideal substrate also would ensure that juvenile mussels stay on the ropes and not migrate to a suitable secondary site. Furthermore, settlement deterring bacteria, such as *Pseudoalteromonas* sp. AMGP1 would need to be controlled in areas dedicated for mussel aquaculture to ensure that juvenile mussels retain on ropes.

Finally, the effect of settlement-inducing bacteria as a source of larval nutrition was investigated in this study. A new approach for labelling with heavy metals, such as strontium and ytterbium (Yb) were achieved in this study and unlike strontium; Yb was a more suitable marker due to its absence in seawater. Furthermore, this study is the first to have used Yb-labelled bacteria for feeding mussel larvae and juveniles. Results from this study indicated that both mussel larvae and spat are bacterivores. Bacterivory of mussel larvae and juveniles may also indicate that this feeding mode occurs in the sea throughout the year. In addition to food and source of energy, these bacteria may act as potential probionts to improve digestion of larval feed. It is likely that larvae and juveniles consume mixed cultures of both bacteria and microalgae. However, it is not clear if larvae selectively choose microalgae over bacteria. The present study was an exploratory work to investigate the effect of targeted settlement-inducing bacteria on larval nutrition. It is possible that other species of bacteria may be better suited as diets for mussel larvae and juveniles. Further work is essential in this area to determine whether bacteria can be used as alternative sources of larval nutrition. Information on the exact preference of

larval diet would minimise the current costs involved in culturing microalgae to support growth of mussel larvae and juveniles.

In summary, it is clear that mono-species bacterial biofilms have the ability to shape a habitat by either inducing or deterring settlement of mussel larvae. Since the settlement-inducing bacterial strains have no toxic effects on larvae, chemicals produced by these bacteria may be suitable for coating ropes in a commercial mussel hatchery to increase mussel settlement and spat production. Since metabolites from bacteria are natural, larvae might interact with such chemicals more often in the sea and hence these chemicals would be more ecologically appropriate. This thesis covered a wide range of topics for the settlement and nutrition of mussel larvae and juveniles and provides techniques and direction for future investigation. In addition, suggestions for future work are detailed in the following section to guide further research on this commercially renowned mussel species.

10.1 **Future directions**

- The structural identity of larval settlement inductive and toxic molecules from mono-species bacteria can be investigated to understand the exact chemistry of the molecules present in the biofilm exudates and the mechanism of chemical induction can be investigated through omics-based (proteomics, genomics and transcriptomics) approaches.

- With the knowledge about the chemical nature of the bacterial toxins, assay kits can be developed to analyse their presence. This may help in early detection of bacterial toxins that cause larval mortality in hatchery settings.

- Large-scale production of and continual supply of mono-species bacterial biofilms need to be optimised for any large-scale larval settlement and nutrition studies. This research would have potential commercial benefits. Continuous culture techniques for bacteria are already well developed and understood from both microbiological and engineering perspectives.

- Biofilm-mediated antifouling is an area for addressing biofouling issues in New Zealand waters. In addition, antifouling-metabolites can be used to control several indigenous and non-indigenous biofouling species of macro-algae and invertebrates.

- Bacterial biofilms or their metabolites need to be tested on farm ropes to encourage attachment and retention of mussel seeds. Field-based and large-scale experiments need to be conducted and environmental parameters need to be initially assessed.

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