Surface-based technologies and the settlement of *Mytilus galloprovincialis*

Thesis submitted by

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Statement on the Contribution of Others

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Abstract

The mussel *Mytilus galloprovincialis* is a common aquaculture species, and also a major fouling organism that has negative economic impacts. There are no standard assay conditions for this important species and therefore the initial study of this thesis (Chapter 2) quantified the effect of key factors on the settlement of pediveligers and plantigrades to provide a standardised and reproducible assay method. Density dependent settlement did not occur for either pediveligers or plantigrades. Settlement increased in drop assays in a 12 h light:12 h dark cycle, while bottom shade had no effect of any magnitude. In addition, settlement was significantly enhanced by storing pediveligers for between 4 and 24 days at 4ºC. Overall, these data provide the template to optimise and standardise static laboratory settlement assays for mussels in order to develop materials that either enhance settlement for the aquaculture industry, or deter settlement for antifouling applications. Furthermore, simple mechanisms such as storage at 4ºC can enhance settlement beyond current methods used in aquaculture hatcheries.

In the third chapter of this thesis, key properties affecting the settlement and adhesion of marine invertebrate larvae were investigated. Surface wettability and microtopography can either enhance or deter larval settlement of many sessile marine organisms. To determine the effect of these surface properties on the settlement of pediveligers of *M. galloprovincialis* (Chapter 3), polymers spanning a range of wettability and microtextured polydimethylsiloxane (PDMS) were used. Furthermore, the adhesion strength of settled pediveligers on microtextured PDMS surfaces was quantified using a flow chamber. Settlement was enhanced at the hydrophilic end of the wettability spectrum, where mean settlement on nylon reached 33.5 ± 13.1%. In contrast, mean settlement on the most hydrophobic polymer, PDMS, was 4.2 ± 3.2%. Microtopography had a much stronger effect than wettability, where 400 µm textured PDMS enhanced settlement above 90%. Settlement preferences were also positively correlated to adhesion strength at flow rates of 4 knots, with all initially settled pediveligers on smooth PDMS detaching, while 79.9 ± 5.7 % of pediveligers remained on PDMS with a 400 µm texture.

This process was further developed in the subsequent chapter (Chapter 4). The global mussel aquaculture industry uses specialised spat catching and nursery culture ropes made of multi-filamentous synthetic and natural fibres to optimise settlement and retention of mussels on ropes for on-growing. However, the settlement ecology and preferences of mussels are poorly understood and only sparse information exists in a commercial context. This study quantified the settlement preferences of pediveligers and plantigrades of *M. galloprovincialis* for

increasingly complex surfaces and site-specific settlement locations on and within ropes at an industrial scale using optical microscopy and X-ray micro-computed tomography (µCT). *M. galloprovincialis* has clear settlement preferences for increasingly complex materials and high selectivity of settlement sites relative to the size of individuals from the pediveliger stage through to the plantigrade stage. Pediveligers of *M. galloprovincialis* initially settle inside ropes and move outwards as they increase in size. In contrast, smaller individuals that have not grown move deeper inside of the ropes over time. This study demonstrated that µCT is an excellent non-destructive technique for mapping attachment sites of individuals as early as 1 day post settlement. Furthermore it quantified the numbers of settled individuals on and within ropes providing for the development of technologies to optimise aquaculture practices.

Finally, in the last chapter of this thesis (Chapter 5), surface technologies to deter the settlement of pediveligers and plantigrades of *M. galloprovincialis* as fouling organism were investigated. Fouling-release materials minimise the adhesion of biofouling and are currently used as an antifouling strategy with non-target effects. Technologies to control the settlement and adhesion of the important fouling organism *M. galloprovincialis* on fouling-release materials were developed by incorporating the nanofillers titanium dioxide (TiO_2) and carbon nanotubes (CNTs) in PDMS matrices. The incorporation of TiO_2 prevented larval settlement when photoactivated with UV light, even at the lowest concentration of the nanofiller (3.75 wt%). Notably, there was 100% mortality of pediveligers exposed to photoactivated TiO_2. However, plantigrades initially settled to photoactivated TiO_2, but their adhesion strength was significantly reduced on these surfaces in comparison to blank PDMS. In addition, plantigrades had high mortality after 6 h. In contrast to the enhanced antifouling and fouling-release properties of PDMS filled with TiO_2, the incorporation of CNTs into PDMS had no effect on the settlement and adhesion of *M. galloprovincialis*.

In conclusion, this study provides an optimised and standardised static laboratory settlement assay for mussels. Subsequently, surface-based technologies to control the settlement of *M. galloprovincialis* were investigated to develop materials that either enhance settlement and retention for the aquaculture industry, or prevent settlement thereby reducing biofouling. Microtopography and complexity of surfaces are both settlement cues for pediveligers, with complexity playing a key role for settlement and selection of attachment sites. These results highlight the efficacy of multi-filament ropes used in the mussel aquaculture industry as these complex materials allow pediveligers to settle at protected sites within the ropes and migrate to the exterior of the rope post-metamorphosis as individuals increase in size. They also provide a new and fundamental understanding of the settlement process to optimise the retention of hatchery reared mussel larvae to support a sustainable mussel aquaculture industry. Finally, in an attempt to reduce the negative economic impacts of mussels as a fouling organism by

improving antifouling and fouling-release properties against *M. galloprovincialis*, TiO_2 was incorporated into coatings and was highly effective in preventing larval settlement, causing high rates of mortality and weakening the adhesion strength of any settled organism. In contrast, the incorporation of CNTs as nanofiller was ineffective. While TiO_2 has limitation in its application, it can provide an alternative non-biocidal antifouling strategy in the photic zone.

In summary, the outcomes of this thesis provide a novel and innovative contribution to understanding the mechanisms driving mussel settlement preferences and technologies to control mussel settlement and growth.

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General Introduction

1.1 Mussels and their economic impact

To meet the increasing demand of high quality seafood for human consumption, the global production of farmed fish and shellfish is increasing (Naylor et al. 2000; McKindsey et al. 2011). The mollusc aquaculture industry comprised a quarter of global aquaculture production in 2008, with the cultivation of mussels worth more than 1.2 billion USD (FAO 2010). The genus *Mytilus* with two key species, *Mytilus galloprovincialis* and *M. edulis*, is the major contributor to mussel production (Gosling 2003; Stevens et al. 2008).

Both species occur on rocky shores of intertidal to subtidal zones with a wide distribution from the subtropics to the Arctic (Gosling 2003; Wonham 2004) and can hybridise where they geographically overlap, with hybrids being fertile (Gosling 1992). The distinction of *M. galloprovincialis*, *M. edulis*, and their hybrids based on morphologic characters alone is unreliable and genetic analysis is required (Wonham 2004), causing taxonomic confusion (McDonald et al. 1991). The shells of *Mytilus* species have a pronounced triangular shape and their meat has a high content of protein and micronutrients (Karakoltsidis et al. 1995). Consequently, mussels are a healthy and high demand food resource, with the mussel aquaculture industry growing continuously (Gosling 2003).

The success of the mussel aquaculture industry relies greatly upon high settlement rates and retention of mussel spat on ropes for on-growing (Hickman 1992). Specialised spat catching and nursery culture ropes, made of multi-filamentous synthetic and natural fibres, are used in the mussel aquaculture industry to optimise the settlement and retention of mussels (Cáceres-Martínez et al. 1994; Walter and Liebezeit 2003; Filgueira et al. 2007; Brenner and Buck 2010; Hayden and Woods 2011). Mussel seed is either collected from the wild using spat catching ropes, or alternatively from drift macroalgae and natural mussel beds. In addition, mussel seed is also produced in closed life-cycle hatchery culture. After collection, or hatchery production, the mussel seed is settled onto nursery culture ropes and to ensure attachment and retention, they are covered with a cotton mesh socking that degrades over time (Gosling 2003; Carton et

al. 2007; Hayden and Woods 2011; McKindsey et al. 2011). Despite these efforts, losses of mussels during the grow-out phase typically exceed 50% (Carton et al. 2007; Hayden and Woods 2011) and the causes are poorly understood. However, it is assumed that these are based on multiple factors, including stressors during seeding (Carton et al. 2007), predation (Hickman 1992), biofouling (Figueras 1990; Hickman 1992), disease (Gosling 2003), parasites (Figueras et al. 1991), secondary migration (Buchanan and Babcock 1997; Hayden and Woods 2011), self-thinning (Lachance-Bernard et al. 2010), and the dropping-off of heavy mussel aggregations due to their weight (Figueras et al. 1991; Sukhotin and Kulakowski 1992). Optimal seeded culture ropes are therefore critical for the retention and optimal growth of mussels (Figueras 1990).

In order to control the density of mussels on culture ropes, mussels are mechanically stripped and re-seeded onto new ropes at a lower density (Hickman 1992; Hayden and Woods 2011). This standard practice, termed thinning, is done at least once during the grow-out phase and is similar to the initial seeding practise, where mussels are placed on ropes into a degradable socking (Figueras 1990). Thinning is not exclusive for the cultivation of *Mytilus* and also commonly applied in *Perna* culture (Carton et al. 2007; Hayden and Woods 2011; McKindsey et al. 2011).

A biomass of approximately 10 kg of mussels per metre is the upper limit for ensuring rapid growth and good retention of mussels (Figueras 1990; Sukhotin and Kulakowski 1992). When the load on the ropes exceeds this critical value, growth decreases and the risk of heavy mussel aggregations dropping off due excessive weight is increased (Figueras 1990). Therefore, the mussel aquaculture industry relies on an understanding of fundamental drivers for larval selection of preferred settlement sites to ensure supply of mussel spat, in conjunction with management of density on culture ropes to achieve optimal spat retention.

In contrast to the aquaculture industry where mussel settlement and retention is critical, other marine industries consider mussels to be a significant and costly fouling organism that incurs high management and control costs. Mussels are highly successful fouling organisms (Briand 2009) because of their wide physiological tolerances, ability to withstand air exposure, high fecundity, and their ability to settle on a wide variety of substrates in high densities (Gosling 2003). The blue mussel, *M. galloprovincialis*, is a particularly critical fouling species with high recruitment rates and the ability to outcompete other species (Erlandsson et al. 2006).

As biofoulers, mussels have negative economic impacts on marine industries, including cooling water conduits of vessels (Lee and Chown 2007), desalination plants and power generation

cooling systems (Henderson 2010), fin fish aquaculture (Braithwaite and McEvoy 2005; de Nys and Guenther 2009; Fitridge et al. 2012), petroleum extraction (Page et al. 2010) and shipping (Townsin 2003). The shipping industry is affected by the fouling of internal spaces, internal sea chests and water circulation systems, and external equipment and hulls, which leads to increased drag and fuel requirements (Townsin 2003). The unrestricted water flow onboard vessels through a sea chest for engine cooling, fire fighting purposes, and ballast is critical for ongoing and efficient operation (Coutts et al. 2003; Coutts and Dodgshun 2007). Major operational problems arise when this water flow is restricted due to biofouling and engine cooling fails causing marine engines to wear prematurely (ASA 2007). Furthermore, due to their wide environmental tolerances mussels can be easily translocated to non-indigenous habitats and are considered a major bioinvader (Lee and Chown 2007).

In order to prevent biofouling, biocidal antifouling coatings are commonly used (Finnie and Williams 2010), but with impacts on marine environments (ten Hallers-Tjabbes and Walmsley 2010). Well known impacts include the accumulation of biocides in the water column, sediments, and marine organisms (Thomas et al. 2002; Konstantinou and Albanis 2004; Gammon et al. 2009; Turner et al. 2009). Therefore, the development of environmentally acceptable technologies for the prevention of biofouling is important (Callow and Callow 2011). Larval settlement is an important initial step for the establishment and growth of biofouling and understanding the factors affecting settlement is therefore fundamental to managing and minimising biofouling by mussels.

1.2 Settlement and adhesion of the mussel *Mytilus galloprovincialis*

Larvae of the genus *Mytilus* initially settle as pediveligers (Figure 1.1a) and once successfully settled and metamorphosed, the post-larvae (Figure 1.1b) are termed plantigrades (Bayne 1976), spat, seed or juveniles (Alfaro et al. 2004). In contrast to many sessile invertebrate larvae, mussel settlement is not permanent and individuals of *Mytilus* can detach and resettle in an alternative habitat (Bayne 1964a; Gosling 2003). Pediveligers initially settle on filamentous substrata (‘primary’ settlement; McGrath et al. 1988; Cáceres-Martínez et al. 1994), including branching algae (Bayne 1964a) and hydroids (Genzano et al. 2003). This initial settlement is followed by metamorphosis and a period of growth, thereafter juvenile mussels undergo pelagic migration to settle into adult mussel beds (‘secondary’ settlement; McGrath et al. 1988; Cáceres-Martínez et al. 1994; Dobretsov and Wahl 2001; Gosling 2003).

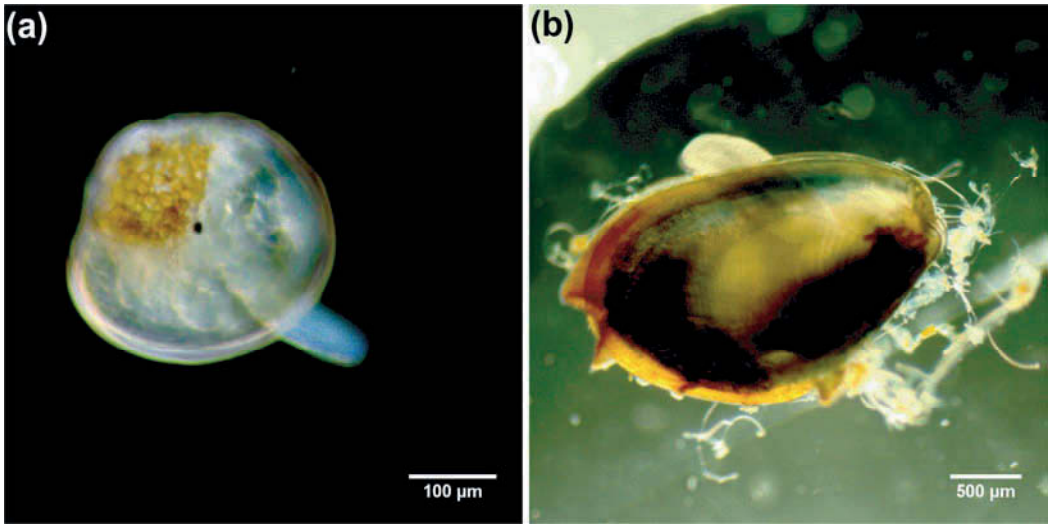

Figure 1.1: (**a**) Pediveliger and (**b**) plantigrade of *M. galloprovincialis*.

These dense aggregations are highly productive assemblages and also offer protection against predation and wave action (Seed and Suchanek 1992). Detachment and re-attachment may occur numerous times before plantigrades recruit into mussel beds (Buchanan and Babcock 1997) and direct settlement on adult mussel beds also occurs (McGrath et al. 1988; Cáceres-Martínez et al. 1993). It remains unclear which factors trigger primary or secondary settlement (Carton et al. 2007), but it is hypothesized that this is a strategy to avoid negative intraspecific competition with adults (Bayne 1964a).

The target species of this thesis, *M. galloprovincialis*, initially settles as a pediveliger larva, with a width and length of approximately 190 and 260 µm, respectively. The main morphological features of pediveliger larvae are eyespots, a large velum used for swimming and feeding, and a foot (Bayne 1976). The foot, a complex muscular and glandular organ with cilia, is used for crawling and byssus secretion (Gosling 2003; Pernet et al. 2003). Pediveligers actively explore the substratum by crawling (Waite 1987; Gosling 2003; Pernet et al. 2003) and are capable of discriminating between different substrata (Gosling 2003).

Pediveligers are selective in their preference of settlement sites (Petersen 1984; Cáceres-Martínez et al. 1994; Filgueira et al. 2007; Brenner and Buck 2010) and if the substratum is unsuitable, they withdraw their foot and swim off (Bayne 1976). Pediveligers can delay metamorphosis for several weeks (Bayne 1965) and repeat the exploratory pattern of swimming and crawling behaviour until a suitable settlement site is found (Gosling 2003). If a suitable

substrate is found, the foot is extended through the two partly opened shells (Tamarin et al. 1976) and the tip of the foot is positioned with its distal depression on the surface of interest (Tamarin et al. 1976; Waite 1987; Silverman and Roberto 2010). The foot tip mediates scrubbing of the surface by muscular contractions and movements within the foot (Waite 1987; Silverman and Roberto 2010). The foot muscles then push the depression firmly against the surface and the water is squeezed out (Waite 1987). As a result, a water-tight and air-tight seal is formed (Silverman and Roberto 2010). Adhesive proteins are secreted into the distal depression from specialised glands to deposit a byssal plaque and thread onto the substrate (Silverman and Roberto 2010). Once attached to the surface, the foot is retracted and multiple threads can be produced by repeating the previously described procedure.

The byssal adhesive is composed of a proteinaceous mixture (Lin et al. 2007) and its composition differs between species (Silverman and Roberto 2010), the age of the mussel (Petrone et al. 2008), and the region of the byssus (Silverman and Roberto 2007; Gilbert and Sone 2010). The quality of the adhesive joint depends strongly on surface characteristics and the interaction of the adhesive with the surface (Wiegemann 2005). The ability of an adhesive to spread is linked to the surface properties of the adherend surface (wettability) which in turn affects the quality of the bond (Waite 1987; Wiegemann 2005)

1.3 Surface-based technologies to control the settlement of *Mytilus galloprovincialis*

The settlement of *M. galloprovincialis* is controlled by a diversity of factors. In general, the settlement of marine invertebrate larvae is cued by biological, chemical and physical properties associated with surfaces (Rittschof et al. 1998; Clare and Aldred 2009), including the presence of conspecifics (Elbourne et al. 2008), surface chemistry (Krishnan et al. 2006), surface wettability (Aldred et al. 2006), surface charge (Petrone et al. 2011), elastic modulus (Brady Jr 2001; Chaudhury et al. 2005), topography (Bers and Wahl 2004; Scardino et al. 2006; Scardino and de Nys 2011) and colour (Kobak 2001; Swain et al. 2006). Manipulating these surface-based properties is an obvious avenue to develop materials that either enhance settlement and retention for the aquaculture industry, or prevent settlement thereby reducing biofouling. Surface modification approaches are considered to be the most promising environmentally sustainable antifouling alternative to toxic biocides (Vladkova 2010; Webster and Chisholm 2010). Given the economic importance of the mussel *M. galloprovincialis*, there is an imperative to understand key surface parameters affecting settlement and adhesion. In this section, a range of surface-based technologies are introduced, with wettability, topography and

the embedment of nanofillers into polymeric matrices being most critically reviewed as they are focus of subsequent chapters of this thesis.

1.3.1 Wettability

Understanding the role of wettability is fundamental to manipulating surfaces with the aim of selectively promoting or deterring the settlement of mussels. Surface wettability is the tendency of a liquid to spread on a solid substrate and is characterised by the angle between the solid surface and the liquid, termed 'contact angle' (Jung and Bhushan 2009). The contact angle θ defines hydrophilic ($\theta < 90°$) and hydrophobic surfaces ($\theta > 90°$). For example, glass ($\theta \approx 25°$; Altankov et al. 1996), epoxy ($\theta \approx 54°$; Occhiello et al. 1991) and nylon ($\theta \approx 67°$; Gotoh and Kikuchi 2005) are hydrophilic, while polydimethylsiloxane (PDMS; $\theta \approx 97°$; Aldred et al. 2010a) and Teflon ($\theta \approx 112°$; Guiseppi-Elie et al. 1986) are hydrophobic surfaces. Hydrophilic surfaces have good wetting characteristics and high surface energies, whereas hydrophobic surfaces have poor wettabilities, low surface energies, and non-stick characteristics (Genzer and Efimenko 2006). Surface energy is also a measure of wettability; the higher the surface energy, the more wettable a surface. Extremes exist for both categories with superhydrophilic ($\theta < 5°$) and nearly non-wettable superhydrophobic surfaces ($\theta > 150°$).

In the case of mussels, the adhesive has been shown to spread further on self-assembled monolayers (SAMs) with lower water contact angles (Aldred et al. 2006) and therefore wettability provides a mechanism to manipulate adhesion strength. A generalized and non-linear relationship between the relative adhesion strength of fouling organisms and the surface energy is characterised by the Baier-curve (Baier 2006). The main feature of this curve is minimised adhesion in a narrow area of surface energy (20-30 mN m^{-1}), while adhesion increases for all lower and higher surface energy values (Brady and Singer 2000; Baier 2006; Pereni et al. 2006). However, the strength of adhesion is also affected by the time of contact, the forces applied to remove the fouling organism, and the bioadhesive, which differ among organisms (Baier 2006).

Sessile organisms rely on bio-molecules made of multi-protein complexes as adhesives to fix themselves to the substratum (Kamino 2008). This biological adhesive is therefore critical for both larval and adult attachment. For example, the mussel *M. edulis* has a positive correlation between adhesive plaque spreading and increased wettability (Aldred et al. 2006). Similarly, the pad diameter of successfully attached spores of the marine alga *Ulva linza* (Callow et al. 2005)

also increase with increasing wettability of the surface (hydrophilic). A further approach to control the settlement of marine organisms focuses on the combination of both hydrophilic and hydrophobic characteristics into one surface (Krishnan et al. 2008). These amphiphilic surfaces reduce the adhesion strength of cells of the diatom *Navicula* (Krishnan et al. 2006) and sporelings of the green algae *Ulva* (Gudipati et al. 2005).

Once a surface is submerged, the initial contact angle of the surface changes over time due to the adsorption of molecules and the colonisation of unicellular organisms forming a biofilm (Maki and Mitchell 2002). Therefore, the effect of wettability is short-term and initially different contact angles of materials converge after a submersion period of a few days (Dahlström et al. 2004). The bacterial biofilm community and density are not influenced by the initial surface wettability and consequently organisms relying on biofilm settlement cues are not affected by initial wettability (Huggett et al. 2009).

A range of marine organisms show very species-specific settlement responses to wettability, however, any effect of wettability may be lost with biofilm formation over time. For example, the barnacle *Balanus improvisus* (O'Connor and Richardson 1994; Dahlström et al. 2004), the ascidian *Ascidia nigra* (Gerhart et al. 1992), and the bryozoan *Bugula neritina* (Gerhart et al. 1992) prefer to settle on hydrophobic surfaces, while the mussel *M. galloprovincialis* prefers hydrophilic surfaces (Yamamoto et al. 1997). Similarly, *M. edulis* attaches more rapidly to hydrophilic than to hydrophobic surfaces (Aldred et al. 2006). Importantly, despite the fact that mussels are commonly used in laboratory assays (Briand 2009), studies on the direct effect of wettability on mussel settlement are sparse and are limited to plantigrades (Young and Crisp 1982; Yamamoto et al. 1997; Nishida 2003; Aldred et al. 2006).

For plantigrades, Aldred et al. (2006) found a positive correlation between adhesive plaque spreading and increased wettability, whereas Young and Crisp (1982) found that the plaque area was smaller on hydrophilic than on hydrophobic surfaces. The contrary findings may be due to the diversity of materials used by Young and Crisp (1982) (slate, glass, paraffin wax, polytetrafluoroethylene) with different surface properties other than wettability, such as elastic modulus, polarity, and surface topography, also affecting the settlement of mussels. There is a clear need to better understand the effects of wettability on *M. galloprovincialis*, and in particular for pediveligers, which have not been investigated. Pediveligers are the critical initial settlement step in the supply of seed-stock for the mussel aquaculture industry, and also the initial step for the establishment of mussels as fouling organisms.

1.3.2 Microtopography

A further direct application to manipulate mussel settlement and retention is surface topography. The concept of selective settlement based on the ability to attach to textured surfaces has been demonstrated for a range of marine organisms, including the barnacle species *B. improvisus* (Berntsson et al. 2000) and *B. amphitrite* (Schumacher et al. 2007a; Aldred et al. 2010b; Chaw et al. 2011), the serpluid tube worm *Hydroides elegans* (Scardino et al. 2008), the bryozoan *B. neritina* (Scardino et al. 2008), and the green alga *U. linza* (Callow et al. 2002; Schumacher et al. 2007a). For these species, settlement success is correlated to the number of attachment points between the settling organisms and the settlement surface (Callow et al. 2002; Scardino et al. 2006; Schumacher et al. 2007a; Scardino et al. 2008).

As a result, topographic surface features can either enhance (Chaw et al. 2011) or reduce the settlement (Schumacher et al. 2007b) of sessile marine organisms. In general, surface microtopographies slightly smaller than the size of settling organisms result in a reduced contact area available for the adhesion and attachment of settling organisms (Berntsson et al. 2000; Callow et al. 2002; Scardino et al. 2006, 2008). As a consequence, the number of attachment points are reduced (Scardino et al. 2006) and the settling organism requires more energy to settle (Callow et al. 2002). In contrast, microtopographies slightly larger than the size of settling organisms induce settlement by providing an optimal number of attachment points and thus, a more secure site for adhesion (Callow et al. 2002; Scardino et al. 2008).

This has been clearly demonstrated for zoospores of *U. linza*, with > 94% settlement in depressed regions representing energetically favourable sites (Long et al. 2010a). A number of surface topography features have been identified that affect the settlement and attachment of marine organisms, including feature width (Carman et al. 2006; Schumacher et al. 2007b; Long et al. 2010a, 2010b), height (Callow et al. 2002; Carman et al. 2006; Schumacher et al. 2007a; Long et al. 2010a) spacing (Callow et al. 2002; Carman et al. 2006; Schumacher et al. 2007a; Long et al. 2010a), design (Callow et al. 2002; Carman et al. 2006; Long et al. 2010b), geometry (Schumacher et al. 2007b; Carman et al. 2006), spatial arrangement (Schumacher et al. 2007a), roughness (Schumacher et al. 2007b), and the number of distinct features in the design (Long et al. 2010a). The final outcome of many of these studies is the development of a trademarked technology, Sharklet AF™, which is a bio-inspired surface design based on the scales of sharkskin and their riblets (Carman et al. 2006). This unique pattern contains rectangular ribs with varying length from 4-16 µm, which are arranged in adjacent diamond patterns (Carman et al. 2006) and is an excellent example of the translation of fundamental biology to an applied material to control settlement and growth.

Microtopography also affects the exploration behaviour of marine invertebrate larvae and may significantly perturb the exploration of settlement sites (Chaw et al. 2011). In contrast to these negative impacts, topographic surface features can also have a positive effect on the settlement on many marine organisms (Köhler et al. 1999). In general, textured surfaces provide an attractive surface for larval settlement with a high surface to volume ratio and crevices providing protection of hydrodynamic forces, drifting objects, and predation. As such, post-settlement mortalities may be reduced (Filgueira et al. 2007).

For mussels, topographic features play a key role in settlement choice and this effect is far greater than chemical cues (Gribben et al. 2011). Previous studies identified low mussel settlement on smooth surfaces (Eyster and Pechenik 1987; Petraitis 1990; Köhler et al. 1999), while topographic features enhanced their settlement (Köhler et al. 1999; Marsden and Lansky 2000; Alfaro and Jeffs 2002; Gribben et al. 2011). Furthermore, the absence of filamentous substrata prolongs the larval stage and delays metamorphosis (Lutz and Kennish 1992). Attachment of mussels is also more rapid on surfaces with topographic features (Gosling 2003), including branching algae (Alfaro and Jeffs 2002), shells (Petersen 1984), and hydroids (Alfaro and Jeffs 2002), with fine-branching substrata being preferred over medium- and coarse-branching materials (Alfaro and Jeffs 2002). Further, mussels actively select favourable sites and higher numbers of spat attach to node areas of substratum rather than to inter-node areas, regardless of branching (Alfaro and Jeffs 2002). In contrast, adult mussels settle on all types of firm substrates (Seed 1976) to form dense mussel beds (Gosling 2003). Microtopography therefore provides a potential mechanism to either enhance or deter the settlement of mussels as either pediveligers or plantigrades, but has not been defined to accurate size.

1.3.3 Other material properties and features

To date, surface based approaches are the most promising environmentally friendly antifouling alternative to biocides, which are commonly used in antifouling coatings (Finnie and Williams 2010; Vladkova 2010). In general, non-biocidal surface antifouling strategies are based on the prevention of initial attachment of fouling organisms and facilitating removal by reducing bioadhesion (Callow and Callow 2011). Research on surface-based technologies has mainly focussed on the manipulation of topographies and wettability, with both properties influencing the attachment and strength of fouling organisms to surfaces. Surface based approaches are versatile and include bioinspired engineered topographies, amphiphilic nanostructured coatings,

phase-segregating siloxane-polyurethane copolymers, superhydrophilic zwitterinonic polymers, inorganic-organic nanohybrids, nanocomposites, and superhydrophobic surfaces (Webster and Chisholm 2010; reviewed by Callow and Callow 2011). A number of these may be directly applicable to mussel settlement and the focus of this thesis is to incorporate carbon nanotubes (CNTs) and titanium dioxide (TiO_2) as nanofillers into a polydimethylsiloxane (PDMS) matrix to improve antifouling and fouling-release properties. These approaches have been selected due to the potential to provide a rapid translation from fundamental research to applied industrial materials.

1.3.3.1 Fouling-release coatings and CNTs

The concept of reducing the adhesion of biofouling using fouling-release coating systems has been commercially applied since 1987 (Townsin and Anderson 2009). Current fouling-release coatings are based on materials with low surface energies including silicone elastomers, fluoropolymers, flourosilicones and hybrids of siloxanes with other materials (Anderson et al. 2003; Webster and Chisholm 2010). As initial settlement is not deterred, hydrodynamic shear forces are required to remove poorly adhered organisms (Finnie and Williams 2010). To enhance the release characteristics of these coatings, CNTs have been incorporated into silicone elastomers as a nanofiller. The adhesion strength of *U. linza* and *B. amphitrite* is successfully reduced when PDMS matrices are filled with CNTs (Beigbeder et al. 2008, 2010). Furthermore, the incorporation of nanofillers provides mechanical reinforcement of generally weak silicone elastomers (Frogley et al. 2003; Paul et al. 2006; Tjong 2006; Ci et al. 2008). The use of CNTs as nanofiller in targeted surfaces is a future direction for fouling-release coating systems to improve mechanical, as well as release, properties.

1.3.3.2 Fouling-release coatings and TiO_2

Similar to CNTs, the incorporation of the photocatalyst TiO_2 also successfully improves the mechanical properties of silicone elastomers (Mirabedini et al. 2008). In addition, the incorporation of TiO_2 has the potential to improve antifouling properties against a broad range of fouling organisms due to its photocatalytic effect. Photocatalysis is a chemical oxidation process activated by light. When a photocatalytic surface is illuminated by light with sufficient energy (3.2 eV for TiO_2 with photoactivation taking place in the range 300-388 nm), a photoexcited electron in the valence band is promoted to the conduct band (Wang et al. 2007; Gaya and Abdullah 2008). This results in the formation of a positive hole (h^+) in the valence

band and an electron (e^-) in the conduct band, also termed an electron-hole pair (Fujishima et al. 2008; Thiruvenkatachari et al. 2008). As a result, highly reactive superoxides ($O_2•^-$), hydroperoxyl radicals (HOO•), and hydroxyl radicals (OH•), which oxidise a large variety of organic compounds, are formed (Fujishima et al. 2008; Gaya and Abdullah 2008).

The photoreactions are limited to the surface of the photoexcited photocatalyst (TiO_2), and therefore only molecules that are in direct contact with the catalyst surface undergo photocatalytic degradation (Gaya and Abdullah 2008). As the photokilling zone is restricted to the near-surface and reactions with non-target organisms are unlikely, the incorporation of TiO_2 in fouling-release coatings is an obvious avenue to prevent biofouling. However, there has been no effort to deconstruct the effect of TiO_2 on the settlement of key fouling organisms other than bacteria, where materials were successful in eliminating *Escheria coli* from surfaces (Ho et al. 2003). This is an obvious avenue to further improve the effectiveness of fouling-release coating systems to control initial settlement.

1.4 Aims and chapter summary

The aims of this study are to quantify the settlement ecology and preferences of *M. galloprovincialis*. The first step in the complex process of manipulating surfaces to control settlement and recruitment is to determine the settlement preferences of both pediveligers and plantigrades to the surface properties of wettability, topography, and the incorporation of CNTs and TiO_2 as nanofillers into PDMS matrices. Expected outcomes of this study will be the definition of the underlying principles required for the development of innovative surface-based technologies for the aquaculture industry to enhance settlement and retention, and antifouling technologies to prevent the settlement and adhesion of *M. galloprovincialis*.

In Chapter 2, as a critical first step, standardised and optimised methods for static laboratory based settlement assays of *M. galloprovincialis* are established for the development of surface based antifouling and aquaculture technologies. The effects of the key factors of density (conspecifics), assay type, light, bottom shade (colour), and storage of larvae on the settlement of pediveligers and plantigrades are quantified.

In Chapter 3, the effects of wettability and microtopography on the settlement of pediveligers of *M. galloprovincialis* are quantified. Commercially available polymers that encompass surface wettabilities from hydrophilic to hydrophobic are used, as well as microtextured PDMS

surfaces with groove widths ranging from 10 to 1000 μm. Furthermore, the adhesion strength of settled pediveligers on micro-textured PDMS surfaces is quantified.

In Chapter 4, the settlement of *M. galloprovincialis* is determined at an industrial scale by mapping the settlement locations of individuals on culture ropes from a commercial mussel hatchery. Settlement sites within the rope are quantified from the initial pediveliger stage through to the final plantigrade stage. Finally, active selection of favourable substrata with increasing complexity is also quantified prior to and post metamorphosis.

Based on the positive effect of surface topography (Chapter 3) and complexity (Chapter 4) on the settlement of *M. galloprovincialis*, the effectiveness of innovative antifouling technologies to prevent the settlement of pediveligers and plantigrades of *M. galloprovincialis* are explored in Chapter 5. The efficacy of CNTs and the photocatalyst TiO_2 as nanofillers incorporated into PDMS surfaces are quantified. The settlement of *M. galloprovincialis* on all surfaces, and the strength of adhesion are quantified.

The results of this study are synthesized and discussed in Chapter 6. This Chapter also addresses future research directions for the application and development of surface technologies for the aquaculture industry to enhance settlement, and antifouling technologies to prevent the settlement of mussels.

Chapter 2

Optimising settlement assays of pediveligers and plantigrades of *Mytilus galloprovincialis* [1]

2.1 Introduction

Larval settlement is an essential step in closed life cycle aquaculture, and also the initial step for the establishment and growth of biofouling. Understanding the factors affecting larval settlement is therefore fundamental to facilitating sustainable aquaculture industries and managing biofouling impacts. This is particularly relevant for mussels, which are a common aquaculture species (Gosling 2003), but are also a problematic fouling organism (Briand 2009). The mussel aquaculture industry is worth 1.2 billion USD (FAO 2010), with the genus *Mytilus* being the major contributor to production (Gosling 2003; Stevens et al. 2008). The success of this industry relies greatly upon high settlement rates and retention of mussel spat on ropes for on-growing. Specialised ropes have been developed to optimise retention (Filgueira et al. 2007), but losses typically exceed 50% (Carton et al. 2007). In contrast, mussels are also a major fouling organism with negative economic impacts for marine industries, including cooling water conduits of vessels, desalination plants and power generation cooling systems (Holmes 1970; Henderson 2010), fin fish aquaculture (Braithwaite and McEvoy 2005; de Nys and Guenther 2009), petroleum extraction (Page et al. 2010) and shipping (Townsin 2003).

Given the economic importance of mussel settlement, there is an imperative to understand the mechanisms that influence the settlement and recruitment of mussel larvae. Mussel larvae settle initially as pediveligers (Bayne 1976) and are selective in their preference of settlement sites (Petersen 1984; Caceres-Martinez et al. 1994a; Filgueira et al. 2007; Brenner and Buck 2010). Once pediveligers settle and metamorphose, the post-larvae are termed plantigrades (Bayne 1976), spat, seed or juveniles (Alfaro et al. 2004). In contrast to many sessile invertebrate larvae, mussel settlement is not permanent and plantigrades of many species can detach and resettle in an alternative habitat (Bayne 1964a; Buchanan and Babcock 1997; Gosling 2003).

[1] Chapter 2 is adapted from Carl C, Poole AJ, Vucko MJ, Williams MR, Whalan S, de Nys R. 2011. Optimising settlement assays of pediveligers and plantigrades of *Mytilus galloprovincialis*. Biofouling 27:859-868.

Determining the settlement preferences of both pediveligers and plantigrades is the first step in the complex process of manipulating surfaces to affect settlement and recruitment. The key to developing surface-based antifouling and aquaculture technologies is the establishment of reliable, reproducible laboratory assays for settlement (Elbourne et al. 2008). Laboratory based assays are used commonly to quantify the settlement behaviours of both pediveliger larvae (Eyster and Pechenik 1987; Bao et al. 2007a; Yang et al. 2011) and plantigrades of *Mytilus* species (Cáceres-Martínez et al. 1994; Aldred et al. 2006) as larvae of this genus are relatively easy to culture, and settle quickly (Briand 2009). However, previous laboratory studies have been hindered by low settlement rates of pediveligers, with rates of less than 10% settlement in the absence of settlement cues (Eyster and Pechenik 1987; Dobretsov and Qian 2003) and with a complete lack of settlement in numerous studies (Satuito et al. 1995; Bao et al. 2007a; Yang et al. 2008). Although there is a clear need to optimise settlement assays, surprisingly, there are no established protocols for standardised assays of *Mytilus* species. Furthermore, comparisons across studies are difficult without standardised methods, due to the variability of parameters used in assays (Briand 2009). The fundamental parameters for settlement assays are (1) the number of test organisms used in assays as density dependent conspecific interactions may occur, (2) the type of assay conducted, (3) the light regime, (4) the effect of surface colour on settlement when testing a range of surface materials, and (5) the effect of storage, which extends the period of available larvae for assays.

Establishing the effect of conspecifics (density) on settlement is a critical first step in the development of a reliable assay method (Elbourne et al. 2008). Settlement to conspecifics is a common phenomenon in marine invertebrate larvae (Burke 1986) and can significantly affect larval settlement assays and corresponding interpretations (Head et al. 2003). Despite the gregarious distribution of adult mussels in the wild (Okamura 1986), little is known about conspecific effects on pediveligers and plantigrades of mussels in laboratory assays. The settlement and the attachment strength of the adult zebra mussel *Dreissena polymorpha* was positively correlated to their density in laboratory studies (Kobak 2001, 2006). However, there are no studies that specifically test the effect of density of conspecifics on settlement for *Mytilus* species.

A further factor affecting settlement is the type of assay deployed. The most common type used in laboratory settlement studies is the volume assay, where a dish is filled with a specific volume of water containing the test organism (Head et al. 2003; Scardino et al. 2009; Yang et al. 2011). In this case, larvae are provided with more than one surface choice. For example, complications arise if larvae settle on the walls of the dish rather than the bottom, where the test surface is placed. To avoid this problem, drop assays have been used in antifouling studies with

barnacle cypris larvae (Schumacher et al. 2007a; Aldred et al. 2010a, 2010b). For drop assays, a number of test organisms are introduced into a drop with a specific volume onto the test surface, providing only one surface choice for settlement. This assay method needs to be considered in laboratory settlement assays for *Mytilus* species.

Light also influences the settlement and metamorphosis of marine invertebrate larvae (Crisp 1974; Ettinger-Epstein et al. 2008). Pediveligers of *Mytilus edulis* are negatively phototactic as they approach settlement (Bayne 1964b). Similarly, large mussels attach in higher numbers in the dark (Kobak 2001) producing more byssal threads (Davis and Moreno 1995) and consequently, higher attachment strength (Kobak 2006). Previous laboratory-based settlement assays with mussels have been conducted under light conditions of 12 h light:12 h dark (Petersen 1984; Pechenik et al. 1990; Cáceres-Martínez et al. 1994), complete darkness (Alfaro et al. 2006; Bao et al. 2007a) and full light (Eyster and Pechenik 1987; Aldred et al. 2006). To improve comparability of studies quantifying settlement, it is important to unify protocols and establish conditions that optimise settlement. Surface colour also contributes to settlement of invertebrate larvae (Kobak 2001; Swain et al. 2006), however, there are also examples where surface colour has no effect on larval settlement (Hodson et al. 2000; Guenther et al. 2009). Given there are no studies on the effect of surface colour on the settlement of *Mytilus*, it is important to determine a preference, or lack thereof, within an optimised assay.

Finally, larval competency is another important consideration in optimising laboratory assays, as invertebrate larvae can be difficult to obtain due to seasonal availability. This is also the case for larvae of *Mytilus* species with a limited window of availability (Bayne 1976). However, this limitation can be avoided by refrigerating larvae to extend their duration, a commonly used method for *Balanus amphitrite* cyprids (Elbourne et al. 2008; Murosaki et al. 2009). Refrigerated straight-hinge larvae of the mussel *M. galloprovincialis* can survive for up to three months with no effect on survival, growth, settlement behaviour and metamorphosis (Satuito et al. 2005). The effect of refrigerating competent pediveligers remains mostly unknown and it is important to close this knowledge gap.

To optimise laboratory-based settlement assays, specifically for the development of surface-based technologies to either enhance or reduce settlement of pediveligers and plantigrades of *M. galloprovincialis*, the effects of five key factors on the settlement were quantified. These are (1) density (conspecifics), (2) assay type, (3) light, (4) bottom shade (colour), and (5) storage of larvae.

2.2 Material and methods

2.2.1 Culture of pediveligers

Larvae of the mussel *M. galloprovincialis* were supplied by the Victorian Shellfish Hatchery in Queenscliff, Victoria, Australia. Spawning and larvae production are described in detail in Pettersen et al. (2010). The larvae were shipped as veligers (19 days after fertilisation), within 24 h, to James Cook University, Townsville, Queensland, Australia. Veligers were then transferred into aerated (0.2 µm filtered air; Midisart 2000 filters) 3 L Erlenmeyer flasks containing autoclaved filtered seawater (FSW) at a density of 2 veligers mL^{-1}. All FSW in culture and settlement assays was filtered to 0.2 µm and UV sterilised. The larvae were maintained at 18˚C under constant light, and fed *Chaetoceros muelleri* (CS-176), *Pavlova lutheri* (CS-182), and *Isochrysis galbana* (clone T.ISO, CS-177) at a target cell density of 100,000 cells mL^{-1} *I. galbana* equivalents (33,300 cells mL^{-1} *I. galbana*; 33,300 cells mL^{-1} *P. lutheri*; 25,000 cells mL^{-1} *C. muelleri*). The larvae were fed daily and the water was changed every other day. Larvae were maintained until the pediveliger stage was reached and competent to settle. Pediveligers were then pooled and used in settlement assays. All pediveligers used in an experiment were from a single batch.

2.2.2 Culture of plantigrades

Plantigrades (9 weeks after fertilisation) were also supplied by the Victorian Shellfish Hatchery and shipped, within 24 h, to James Cook University, Townsville. Plantigrades were transferred into aerated 3 L Erlenmeyer flasks with autoclaved FSW at a density of 2 plantigrades mL^{-1}. The plantigrades were maintained at 18˚C with 12 h light:12 h dark cycle and were fed 50,000 cells mL^{-1} of *I. galbana*, *P. lutheri*, and *C. muelleri* in equal parts twice daily. Food was maintained in excess. The water was changed every other day. The plantigrades were reared for 2 weeks and then used in settlement assays.

2.2.3 Laboratory experiments

2.2.3.1 Conspecific effects

2.2.3.1.1 Pediveligers. The first step in establishing a standardised laboratory assays is the determination of the effects of density of conspecifics as a factor in settlement. The increase in larval density within the small volume of a drop may affect a response to conspecifics that differs from that within the larger volume assay. Therefore, drop and volume assays were performed using 0.8 and 5 mL of FSW, respectively, at densities of 1, 2, 5, 10, 25, and 50 pediveligers per dish (n = 10) (Greiner bio-one 657185). The settlement assays were conducted in a temperature controlled culture cabinet (Sanyo MLR-351) at 18°C in a 12 h light (5.25 W m^{-2}):12 h dark cycle to maximise settlement (see Section 2.3.2). The settlement of the pediveligers was determined after 48 h. Pediveligers were considered to be settled when they attached to the surface with byssal threads.

2.2.3.1.2 Plantigrades. To determine density-dependent effects of plantigrades, a volume assay was conducted at densities of 1, 2, 5, 10, 25, and 50 plantigrades (n = 10) per dish (Greiner bio-one 657185) with 5 mL of FSW. Only the volume assay was conducted due to the larger size of the plantigrades (> 1.2 mm shell length), which prevented using higher densities in the small volume of a drop. The assay was conducted in a temperature controlled culture cabinet (Sanyo MLR-351) at 18°C in the light (5.25 W m^{-2}) (set to a 12 h light:12 h dark cycle). The settlement of the plantigrades was recorded after 6 h. Plantigrades were considered to be settled when they produced byssal threads to attach to the surface.

2.2.3.2 The effect of assay type, light, and bottom shade on the settlement

To formally test the effects of assay type, in conjunction with light and bottom shade, on the settlement of pediveligers of *M. galloprovincialis*, a factorial design was employed (n = 5). Drop and volume assays were conducted with 0.8 and 10 mL FSW, respectively. Laboratory settlement assays were conducted with approximately 20 pediveligers in each Petri dish (55 mm diameter, Techno Plas S5514UV10) in three temperature controlled culture cabinets (Sanyo MLR-351) at a constant 18°C over 48 h. A total of 30 Petri dishes were used for each assay type and were then split into three groups each containing 10 dishes. Each group was assigned to one light treatment performed in a separate culture cabinet with controlled conditions among treatments. The three tested light treatments over 48 h were (1) a 12 h light:12 h dark cycle, (2)

48 h light, and (3) 48 h dark. The light intensity was a constant 5.25 W m^{-2}. To differentiate between the effect of light and a preference for bottom shade, five clear Petri dishes were placed on white craft paper and the remaining five dishes on black craft paper (Canson, White 143 and Black 142) for each light treatment.

Only pediveligers were tested for the effects of assay type, light, and bottom shade as the settlement time of plantigrades is shorter than any of the light cycles used for pediveligers. Complete settlement of plantigrades occurred within 6 h (see Section 2.3.1).

2.2.3.3 Storage of larvae

To determine the effect of refrigeration on the settlement of pediveligers, competent pediveligers were refrigerated for 32 days at 4°C in the dark. Settlement assays were conducted with larvae refrigerated for 0, 4, 8, 12, 16, 24 and 32 days. As a control, drop assays were conducted on day 0 with pediveligers before refrigeration (n = 9). The remaining pediveligers, from a cohort spawned together, were placed at a density of 5 pediveligers mL^{-1} with 40 mL autoclaved FSW each, into 18 tubes (Corning 430829). At each sampling point, three randomly selected tubes were removed from the refrigerator to minimise container effects, and the refrigerated pediveligers were immediately used in assays. Drop assays (n = 9) were conducted in a temperature controlled culture cabinet (Sanyo MLR-351) at 18°C in a 12 h light (5.25 W m^{-2}):12 h dark cycle to maximise settlement (see Section 2.3.2). Approximately 20 pediveligers were pipetted with 800 µL FSW in a drop assay on a Petri dish (Techno Plas S5514UV10) and the number of settled pediveligers was determined after 48 h. To minimise stress and avoid repeated sampling, remaining pediveligers not used in assays were discarded. Pediveligers were inactive at 4°C and did not settle within the storage tubes.

2.2.4 Statistical analysis

All data were analysed by analysis of variance (ANOVA, SPSS Version 19). The assumptions of normality and homogeneity of variance were examined using Q-Q plots of residuals and plots of standardised residuals versus means, respectively (Quinn and Keough 2002). Data were arcsine square-root transformed where required. Post hoc comparisons (Tukey's HSD; α = 0.05) were performed as appropriate.

For ANOVAs testing the effect of density on the settlement of pediveligers in both the drop and volume assays, and settlement of plantigrades in volume assays, the single and two individual treatments were excluded from the formal analysis as transformations did not satisfy the assumption of homogeneity of variance. To test the effects of assay type (drop and volume assay), light treatments (12 h light:12 h dark, light, and dark), and bottom shade (black and white) on the settlement of pediveligers, three-factor ANOVA was used with assay type, light, and bottom shade as fixed factors. To assess differences in the settlement of pediveligers when stored at 4°C, ANOVA was performed with the period of storage as a fixed factor. Results are reported as mean ± 1 standard error (SE).

2.3 Results

2.3.1 Conspecific effects

2.3.1.1 Pediveligers

Pediveligers of *M. galloprovincialis* responded negatively to the presence of conspecifics with settlement decreasing significantly with increasing density in both the drop (one-factor ANOVA: $F_{(3,36)} = 6.31$, $p = 0.002$; Figure 2.1a) and volume assays ($F_{(3,36)} = 5.58$, $p = 0.003$; Figure 2.1b). In the drop assay, settlement ranged from 10.8 ± 3.4% to 50 ± 9.1%, and was generally higher than in the volume assay where settlement ranged from 3.0 ± 0.7% to 30 ± 11.1%. For both assay types the settlement of pediveligers was significantly lower with 50 larvae, than with 5 and 10 larvae (Figure 2.1a,b). The threshold, where settlement fell below 20%, was reached in the drop assay at a higher density (> 25 larvae) than in the volume assay (> 10 larvae).

2.3.1.2 Plantigrades

There was no effect of density on the settlement of plantigrades of *M. galloprovincialis* and no significant difference in settlement among densities (one-factor ANOVA: $F_{(3,36)} = 0.16$, $p = 0.922$). Settlement within 6 h ranged from 85.0 ± 7.7% to 100.0 ± 0.0% (Figure 2.2).

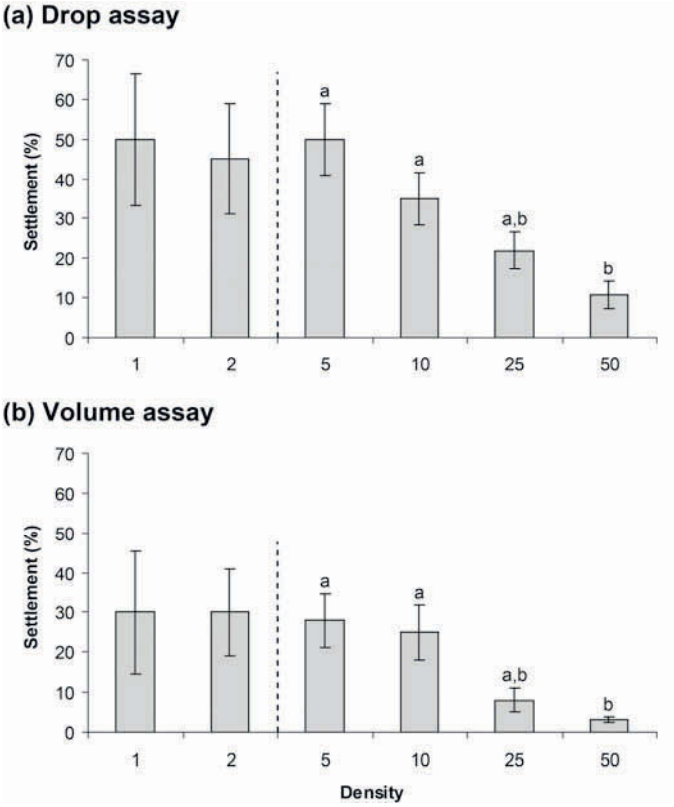

Figure 2.1: Settlement (%) of pediveligers of *M. galloprovincialis* after 48 h at different densities in (**a**) drop and (**b**) volume assays. Means ± SE are shown (n = 10). Superscript letters indicate significant differences (Tukey's HSD; α = 0.05). Statistical analyses were only preformed for densities ≥ 5 larvae per dish (to the right of the dotted line).

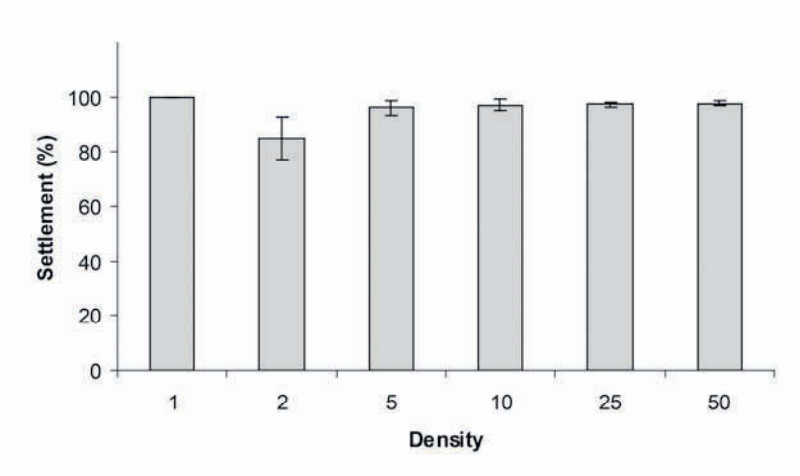

Figure 2.2: Settlement (%) of plantigrades of *M. galloprovincialis* after 6 h at different densities in volume assays. Means ± SE are shown (n = 10).

2.3.2 The effect of assay type, light, and bottom shade on the settlement

There was a complex interactive effect of assay type, light and bottom shade on settlement of pediveligers (p = 0.022; Table 2.1; Figure 2.3a,b). This effect was primarily driven by higher settlement on black bottom shades in comparison to white bottom shades in the drop assay in the light treatment (12.3 ± 2.8% on black and 6.2 ± 0.8% on white bottom shades) in contrast to lower settlement in the volume assay in the 12 h light:12 h dark regime (11.4 ± 1.8% on black and 19.9 ± 1.4% on white bottom shades). In all other combinations of treatments (4 out of 6) there was no differentiation between bottom shade (Figure 2.3a,b).

Table 2.1: Three-factor ANOVA of the effect of assay type (drop and volume assay), light (12 h light:12 h dark cycle, 48 h dark, and 48 h light), and bottom shade (black and white) on the settlement of pediveligers of *M. galloprovincialis*.

Source	***df***	**Mean**	***F***	***p***
Assay type	1	0.17	17.22	< 0.001
Light	2	0.13	13.92	< 0.001
Bottom shade	1	0.00	0.01	0.936
Assay type × Light	2	0.01	1.12	0.335
Assay type x Bottom shade	1	0.06	6.70	0.013
Light × Bottom shade	2	0.01	1.12	0.335
Assay type × Light × Bottom shade	2	0.04	4.12	0.022
Error	48	0.01		

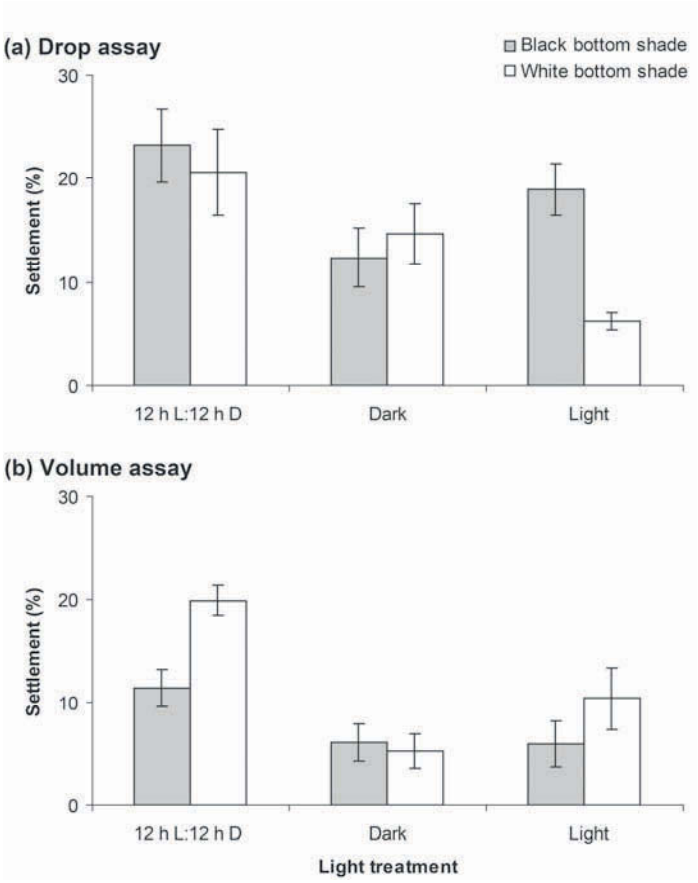

Figure 2.3: Settlement (%) of pediveligers of *M. galloprovincialis* in (**a**) drop and (**b**) volume assays under different light conditions (12 h light:12 h dark over 48 h, 48 h dark, and 48 h light) and bottom shades (black and white) after 48 h. Means ± SE are shown (n = 5).

The treatment combination with the highest mean settlement of pediveligers was the 12 h light:12 h dark regime in the drop assay (23.2 ± 3.5% on black and 20.6 ± 4.1% on white bottom shades, Figure 2.3a). More generally, settlement tended to be higher in the drop assay than the volume assay, with a mean overall settlement of 16.0 ± 1.5% for the drop assay, compared to 9.8 ± 1.2% for the volume assay. The only combination of treatments with higher numbers of pediveligers settled in the volume assay, compared to the drop assay, was with light on the white bottom (10.4 ± 3.0% in volume and 6.2 ± 0.8% in drop assay). Notably, settlement only occurred on the bottom of the dishes.

2.3.3 Storage of larvae

Pediveligers settled in higher numbers when stored at 4°C over time (Figure 2.4), and the period of storage had a significant effect on settlement (one-factor ANOVA: $F_{(6,56)} = 44.00$, $p < 0.001$). The settlement of refrigerated pediveligers increased approximately 10 fold from day 0 (7.1 ± 1.7%) to days 4 (80.4 ± 9.4%) to 12 (84.8 ± 9.2%). Subsequently, the settlement of pediveligers stored for more than 12 days decreased consistently over time, with lower settlement after 16 days (61.9 ± 6.2%), and 24 days (50.1 ± 7.3%). The lowest settlement was observed in refrigerated pediveligers stored for 32 days (11.8 ± 4.7%) with no significant difference between the settlement of pediveligers stored for 32 days and the control (day 0).

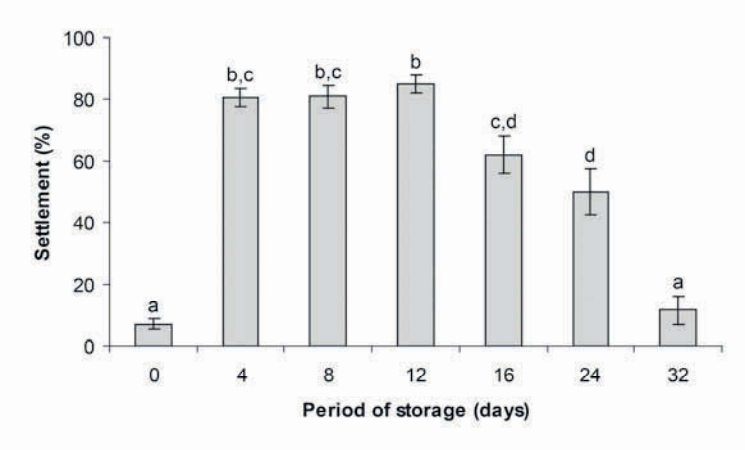

Figure 2.4: Settlement (%) of pediveligers of *M. galloprovincialis* stored at 4ºC over 32 days. Settlement was determined after 48 h. Means ± SE are shown (n = 9). Superscript letters indicate significant differences (Tukey's HSD; $\alpha = 0.05$).

2.4 Discussion

This study provides a foundation for understanding factors affecting the settlement of pediveligers and plantigrades of *M. galloprovincialis* in laboratory studies, and establishes standardised and optimised methods for static settlement assays. Density dependent conspecific effects, assay type, light regime, and storage of pediveligers contribute to the settlement of *M. galloprovincialis*. Further, the efficient manipulation of these parameters is a key in

developing reliable and reproducible laboratory assays for the development of surface based technologies to either enhance settlement for the aquaculture industry, or to reduce biofouling.

The results of this study demonstrate that conspecific density had no effect on the settlement of plantigrades of *M. galloprovincialis*. In contrast, settlement of pediveligers was significantly reduced at the highest tested density in both drop and volume assays. This is surprising, as pediveligers occur in high densities in the wild (King at al. 1990; Alfaro and Jeffs 2003) and is in contrast to the positive effect of larval density on the settlement of many other sessile invertebrate larvae, including barnacles (Head et al. 2003, 2004; Elbourne et al. 2008), and sponges (Ettinger-Epstein et al. 2008). The densities at which conspecific effects occur can be highly variable among organisms, ranging from less than one barnacle cypris larvae mL^{-1} (Head et al. 2004) to 2.5 sponge larvae mL^{-1} (Ettinger-Epstein et al. 2008). Chemical cues associated with conspecific larvae and adults can increase larval settlement (Clare and Matsumura 2000; Toonen and Pawlik 2001). In this study, conspecifics had a negative effect on the settlement of pediveligers, which could be a technique to avoid intraspecific competition (Hills and Thomason 1996). The threshold level, where settlement of pediveligers dropped below 20%, occurred at higher densities in the drop assay (> 25 larvae) than in the volume assay (> 10 larvae). This outcome was unexpected given the smaller volume and surface area available in the drop assay, leading to enrichment of any chemical cues, and space limitations. Laboratory assays with *M. galloprovincialis*, and possibly other *Mytilus* species, are therefore enhanced if performed with 10 to 20 pediveligers for drop assays, and 5 to 10 pediveligers for volume assays. At these densities, pediveliger settlement is optimised (~ 28%) and variability is reduced. The consistently high settlement (up to 100%) of plantigrades within 6 h, regardless of densities, shows conspecific cues do not influence settlement and supports a method whereby higher densities of plantigrades can be used. Ideally, less than 20 individuals should be used in assays, as plantigrades have a propensity to clump and settle on each other, complicating analyses and interpretations.

Settlement of pediveligers of *M. galloprovincialis* was consistently higher in drop assays than in volume assays. Aldred et al. (2010a) conducted settlement assays with cyprids of the barnacle *B. amphitrite*, where settlement in drop assays was higher than in volume assays, with a reduced error. A further advantage of the drop assay is that only one surface choice is given and thus, the test organism cannot settle on the walls of the dish. Settlement on walls is problematic because it is impossible to differentiate between the actual effect of the test surface, and the preference to settle on the wall over the test surface when a choice is given. In spite of these disadvantages, the volume assay has the benefit of being less sensitive to movement and assays can be conducted under moving water conditions (Eyster and Pechenik 1987; Cáceres-Martínez

et al. 1994). Although drop assays are only feasible for static studies, they are the assay of choice for laboratory settlement assays with pediveligers of *M. galloprovincialis*. Notably, settlement was increased using the drop assay, in comparison to previous studies, where settlement was less than 10% (Dobretsov and Qian 2003), or did not occur (Satuito et al. 1995; Bao et al. 2007a; Yang et al. 2008), in the absence of settlement cues. An optimised static assay system with high levels of settlement provides a reproducible tool for the comparative screening and development of technologies, chemical or physical, to enhance or deter settlement. This provides the most rapid and simple tool to move effective surfaces to more complex testing under flow regimes, or field studies to address complex highly variable interactions on settlement and recruitment (Tamburri et al. 1996; Pernet et al. 2003).

A further factor having a significant effect on the settlement of pediveligers in assays is light exposure. Increased settlement of pediveligers occurred when assays were at an optimal 12 h light:12 h dark cycle. Standard photoperiods are commonly used in assays for a range of test organisms and are species-specific. For example, laboratory studies using the tube worm *Hydroides elegans* are conducted using a 15 h light:9 h dark cycle (Bryan et al. 1997, 1998), whereas settlement assays with other invertebrate larvae, such as the sponge *Luffariella variabilis* (Ettinger-Epstein et al. 2008), the barnacle *B. amphitrite* (Kawahara et al. 1999; Aldred et al. 2010a, 2010b) and the bryozoan *Bugula neritina* (Dahms et al. 2007; Scardino et al. 2008) are commonly conducted in the dark due to negative effects of light on their settlement and light sensitive larvae. The influence associated with bottom shade is minor for *M. galloprovincialis*. In this study, only black and white bottom shades were tested as a baseline, at the extremes of the colour spectrum. Any effect of black and white is overshadowed by light and assay type, and while colour clearly influences settlement of different organisms (Swain et al. 2006; Robson et al. 2009; Satheesh and Wesley 2010; Shine et al. 2010) there is no consistent effect of shade affecting the settlement of *M. galloprovincialis*.

One of the major findings of this study is that the storage of competent pediveligers of *M. galloprovincialis* at 4°C significantly increases settlement. This is in contrast to findings of Satuito et al. (2005), where straight hinged veligers of *M. galloprovincialis* were refrigerated for 1- 3 months with no effect on survival, growth, settlement behaviour and metamorphosis. This may be due to the use of different stages of ontogeny (veliger vs. pediveliger stage) and the period of refrigeration (30-90 days vs. 4-32 days), or a combination thereof. Settlement of refrigerated pediveligers was enhanced by more than 70% when refrigerated between 4 and 12 days. The high number of settled pediveligers after refrigeration provides an alternative to chemical settlement inducers (Dobretsov and Qian 2003; Yang et al. 2008) and extends the period of available larvae for assays. However, settlement of refrigerated pediveligers stored for

more than 12 days decreased continuously over time, raising the question of whether delayed settlement contributes to depletion of energy reserves needed for settlement and metamorphosis (Pechenik et al. 1998) and a decrease of selectivity (Marshall et al. 2003), as identified with the 'desperate larvae hypothesis' (Marshall and Keough 2003). Accordingly, refrigerated pediveligers need to be used with caution in settlement assays as the effect on the settlement behaviour, such as time of exploration prior to settlement, remains unclear. However, the use of relevant controls to compare surfaces may be suitable in extending periods where assays can be conducted. As static laboratory studies are, in principle, a preliminary method to select the best surface to either deter or enhance settlement prior to testing surfaces under flow, or in field trials, a comparative use of refrigerated larvae with high settlement may prove to be a useful tool in assessing extremes of deterrence for antifouling purposes. The use of refrigerated pediveligers is also a promising approach to increase the efficiency of the mussel aquaculture industry by enhancing settlement and retention on ropes.

In conclusion, a baseline method was established for reproducible and effective laboratory settlement assays of *M. galloprovincialis*. Settlement of pediveligers was optimised with a several fold increase compared to previous studies without the use of chemical cues. This was achieved with low densities of pediveligers ($\leq$ 25 individuals dish^{-1}) in drop assays with a photoperiod of 12 h light:12 h dark. Settlement above 80% occurred when pediveligers were refrigerated, which may have application in laboratory antifouling assays assessing extremes of deterrence, or industrial application to enhance settlement and retention of spat in the aquaculture or in laboratory antifouling assays.

Chapter 3

Enhancing the settlement and attachment strength of pediveligers of *Mytilus galloprovincialis* by changing surface wettability and microtopography [2]

3.1 Introduction

Settlement of marine invertebrate larvae is cued by biological, chemical and physical properties associated with surfaces (Rittschof et al. 1998; Clare and Aldred 2009), with surface wettability (Aldred et al. 2006) and topography (Bers and Wahl 2004; Scardino et al. 2006; Scardino and de Nys 2011) being key features. Marine invertebrate larvae show remarkable variability in their settlement choices across the range of surface wettabilities (Genzer and Efimenko 2006). Surfaces with a low wettability (hydrophobic) are preferred by the barnacle *Balanus improvisus* (O'Connor and Richardson 1994; Dahlström et al. 2004), the ascidian *Ascidia nigra* (Gerhart et al. 1992), and the bryozoan *Bugula neritina* (Gerhart et al. 1992). In contrast, the barnacle *B. amphitrite* prefers surfaces with a high wettability (hydrophilic) (Gerhart et al. 1992; O'Connor and Richardson 1994; Hung et al. 2008; Aldred et al. 2010a). However, more recently Petrone et al. (2011) observed no effect of surface energy on the settlement of *B. amphitrite*, with surface charge influencing cyprid settlement. Understanding the species-specific larval response to surface properties, including wettability, will facilitate the manipulation of surfaces to selectivity deter or promote settlement of targeted marine invertebrate larvae.

Similarly, surface topography also influences settlement of marine sessile organisms and can either enhance (Chaw et al. 2011; Gribben et al. 2011) or reduce (Berntsson et al. 2000) larval settlement. Furthermore, there is a correlation between settlement success and the number of attachment points between larvae (Scardino et al. 2006, 2008), and algal spores (Callow et al.

[2] Chapter 3 is adapted from Carl C, Poole AJ, Sexton BA, Glenn FL, Vucko MJ, Williams MR, Whalan S, de Nys R. 2012. Enhancing the settlement and attachment strength of pediveligers of *Mytilus galloprovincialis* by changing surface wettability and microtopography. Biofouling 28:175-186.

2002), and the settlement surface. As such, the attachment of marine organisms is linked to surface topography features of width, spacing, feature height (Schumacher et al. 2007a), geometry, spatial arrangement, roughness (Schumacher et al. 2007b), and the number of distinct surface features in the design (Long et al. 2010a). In general, surface microtopographies slightly smaller than the size of larvae/spores result in a reduced contact area available for the adhesion and attachment of settling organisms (Callow et al. 2002; Scardino et al. 2006, 2008). In contrast, microtopographies slightly larger than the size of larvae/spores induce settlement by providing a more secure site for adhesion, and refuge from hydrodynamic forces (Callow et al. 2002; Scardino et al. 2008).

Both surface wettability and topography therefore have direct and demonstrated applications to manipulate larval settlement. The mussel aquaculture industry relies on successful settlement and retention of mussel spat on ropes for on-growing (Hickman 1992). Mussel larvae (pediveligers) actively explore the substratum with a foot prior to attachment and are capable of discriminating between different substrata (Gosling 2003). The mussel *Mytilus edulis* attaches more rapidly to hydrophilic surfaces than to hydrophobic surfaces and there is a positive correlation between adhesive plaque spreading and increased wettability (Aldred et al. 2006). In contrast, Young and Crisp (1982) showed that the plaque area was smaller on hydrophilic than on hydrophobic surfaces. The contrary findings may be due to the diversity of materials used by Young and Crisp (1982) (slate, glass, paraffin wax, polytetrafluoroethylene) with different surface properties other than wettability, e.g. elastic modulus, polarity, and surface topography. The latter also plays a key role in the settlement of larvae of *Perna canaliculus*, where they attach more readily on surfaces with topographic features (Gribben et al. 2011), including branching algae and hydroids (Buchanan and Babcock 1997; Alfaro and Jeffs 2002).

Specialised spat catching and nursery ropes are currently used in the mussel aquaculture industry to optimise the settlement and retention of mussels (Filgueira et al. 2007; Hayden and Woods 2011). Ropes are commonly made of nylon, polyethylene terephthalate, or polypropylene (Cáceres-Martínez et al. 1994; Walter and Liebezeit 2003; Filgueira et al. 2007; Brenner and Buck 2010) and these polymers differ in their wetting characteristics, which affect the settlement choice of mussels. Furthermore, the structure of ropes play a key role for larval settlement and retention of mussels (Filgueira et al. 2007; Brenner and Buck 2010), including the thickness of rope filaments and surface area (Walter and Liebezeit 2003; Filgueira et al. 2007). Surface topography is a potential mechanism to enhance larval settlement, and if successful to manipulate the density and retention of mussels to minimise losses. These are important issues for the global mussel aquaculture industry, and the commercially important species *M. galloprovincialis* (Gosling 2003; Dias et al. 2009). However, there has been no effort

to deconstruct the effects of the surface properties of materials used for mussel settlement, to deliver the fundamental drivers for larval selection of preferred surfaces for settlement and attachment.

Manipulating the surface properties of wettability and surface topography is an avenue to develop materials that optimise the settlement and attachment of spat in the mussel aquaculture industry. The aim of this Chapter is to determine the effect of wettability on the settlement of pediveligers of *M. galloprovincialis* by using commercially available polymers that encompass surface wettabilities ranging from hydrophilic to hydrophobic. Further, settlement preferences and attachment strength of pediveligers on microtextured polydimethylsiloxane (PDMS) surfaces with groove widths ranging from 10 to 1000 μm were determined to facilitate the development of surface based technologies to enhance settlement and retention for the mussel industry.

3.2 Materials and methods

3.2.1 Culture of pediveligers

Larvae of the mussel *M. galloprovincialis* were supplied by the Victorian Shellfish Hatchery in Queenscliff, Victoria, Australia. Larvae were maintained (see Chapter 2, Section 2.2.1) until the pediveliger stage was reached and competent to settle (approximate width of 190 μm and length of 260 μm). Pediveligers were then pooled and used in settlement assays.

3.2.2 Laboratory experiments

3.2.2.1 Polymers with different wettabilities

3.2.2.1.1 Surfaces and characterisation. Polymers ranging from hydrophilic to hydrophobic were used to determine the effect of wettability on the settlement of pediveligers of *M. galloprovincialis*. The ten surfaces tested were epoxy (R180 Epoxy Resin mixed 5:1 with H180 Standard Hardener; Nuplex Industries), nylon 6 (Quadrant EPP), polyethylene terephthalate (PET; Bayer Material Science), polyurethane (PU; Colex International Limited, which was hot pressed in a 4-tonne hydraulic press at 155°C for 5 min to flatten the material), polycarbonate (PC; Bayer Material Science), polystyrene (PS; Polystrom), polypropylene (PP;

Roechling), high-density polyethylene (HDPE; Simona), polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning), and polytetrafluoroethylene (PTFE; Fluoro Pacific).

3.2.2.1.2 Contact angle. Surface wettability is the tendency of a liquid to spread on a solid substrate and is characterised by the angle between the solid surface and the liquid (contact angle). Contact angles were measured with a goniometer (First Ten Ångstroms, FTÅ200, USA) using the sessile drop method. Polymeric test surfaces were placed onto a platform underneath a syringe and measurements were obtained using Milli-Q water at different locations on each surface (n = 10). A drop of 2 µL water was formed on the needle tip of a syringe and then applied on the polymer surfaces by raising the platform supporting the polymer until it made contact with the drop. The platform was then lowered until the drop detached from the needle tip. Images of the drop were captured within 1-2 s until the contact angle was stable. Images were analysed using the software FTÅ32 (First Ten Ångstroms). Due to the hydrophilic nature of epoxy and nylon 6, the contact angles were constantly decreasing and stable contact angles could not be recorded. Therefore, contact angles for these two materials were obtained from Occhiello et al. (1991) and Gotoh and Kikuchi (2005).

3.2.2.1.3 Settlement assay. Polymers were cut into squares (30 × 30 mm), washed with a detergent solution (Decon 90; Decon Laboratories Ltd.), rinsed with distilled water, and air dried before they were used in assays. They were placed individually in covered Petri dishes (Techno Plas 10603700) to prevent evaporation. Blank Petri dishes were used as a control. Approximately 20 pediveligers were pipetted with 800 µL filtered seawater (FSW; 0.2 µm filtered and UV sterilised) in a drop assay on each test surface. The assay was conducted in a temperature controlled culture cabinet (Sanyo MLR-351) at 18°C in a 12 h light (5.25 W m^{-2}):12 h dark cycle. The settlement of pediveligers was determined after 48 h. Pediveligers were considered settled when they attached to the surface with byssal threads. This experiment was conducted with a total of three larval batches from three independent spawning events, with replicates of each polymer type ranging between 6 and 12 depending on larval batch.

3.2.2.2 Microtextured PDMS

To determine the effect of microtopography on settlement and attachment strength of *M. galloprovincialis*, surface topographies with groove widths ranging from 10 to 1000 µm in

PDMS (Sylgard 184; Dow Corning) were used. Microtextured PDMS surfaces had a linear grating of approximately square wave profile and a mark-space ratio of 1 (groove width = land area width). Due to widely differing grating sizes and depth variations, different photo- and electron-beam polymer resist substrates and photolithography techniques were used in surface production (see Gonzalez-Macia et al. 2010 and Seisyan 2011 for review of these methods). The master surfaces for casting PDMS copies also varied as described below.

3.2.2.2.1 Spin on photo-resists – maP-1200 series. Grating samples with 10 and 20 µm groove widths were produced using two different positive maP-1200 series photo-resists. The resists were spun onto four inch quartz/chrome plates and baked on a hotplate (at 100°C) for 90-300 s (depending on resist thickness). Micropatterning was conducted via a contact mask UV exposure (ABM flood source, similar to a mask aligner) through high resolution chromium masks and developed in one part AK 400 developer and 4 parts de-ionized water. The resist thickness was adjusted to match the grating width to obtain an approximately 1:1 aspect ratio.

3.2.2.2.2 Nyloprint ST-43 and ST-92. To produce samples with 40, 60 and 80 µm groove widths, a completely new method using a negative pre-clad photo resist on a steel backing plate called Ciba ST-43 is described. This alcohol-wash photopolymer plate is a negative pre-clad photo resist (200 µm thick) on a metal backing sheet. For larger grating samples (groove width of 100-1000 µm) a similar substrate (Ciba ST-92) was used with a thicker resist (600-650 µm). After exposing the plates to collimated UV light through a high definition printed transparency mask, they were developed in an 80:20 ethanol/water mixture with brushing and agitation. The ST-43 and ST-92 samples were then passed through a 3 kW UV unit to harden developed areas and baked at 80°C for 30 min. These substrates could then be used for casting PDMS.

3.2.2.2.3 Nickel electroforms for casting PDMS. The electron-beam lithography and spun UV resist plates were sputtered with approximately 100 nm of nickel (200 W for 7 min) in a physical vapour deposition unit and electroplated at 8.0 A for 7 h in a nickel sulphamate bath (pH 4; temperature 45°C) (Sexton and Marnock 2000). Casting copies of the nickel masters (250 µm thick) were then produced by electroplating and separating. Shims (approximately 200 µm thick) and the Nyloprint masters were not copied because the surface was durable enough for direct casting.

3.2.2.2.4 PMDS preparation and casting. Dow Corning Sylgard 184 PDMS was used to produce all microstructured test surfaces. PDMS was prepared using a 10:1 ratio of base elastomer to curing agent. The two components were mixed thoroughly and de-gassed in a

vacuum desiccator to remove bubbles. Grating surfaces were prepared by spraying a thin layer of release agent onto the nickel shim or Nyloprint master prior to casting. The Nyloprint samples required a 30 min bake at 75°C after this process step. A stainless steel square mold was fixed to the grating surface with removable tape to form a well and the PDMS was poured onto the surface and cured at 75°C for 30 min to generate 75 × 75 mm (10 – 80 µm) and 125 × 125 mm samples (100 – 1000 µm) with a thickness of approximately 8 mm. 20 to 30 copies could be generated from one master. For the 800 and 1000 µm groove width gratings, depth was limited to 600 µm (the maximum depth of the Nyloprint resist).

3.2.2.2.5 Settlement assay. To determine the effect of microtopography on settlement and attachment strength of *M. galloprovincialis*, square-wave profile textured PDMS surfaces with feature widths of 10, 20, 40, 60, 80, 100, 200, 250, 300, 400, 600, 800, and 1000 µm were used. Smooth PDMS and blank Petri dishes (Techno Plas 10603700) were used as controls. There were six replicates for each surface. Circles (35 mm diameter) were cut from the PDMS surfaces using a hole punch and the PDMS samples (84 in total) were then randomly placed in polyvinylchloride (PVC) plates. Each PVC plate (28 in total) had three machined holes, where the PDMS samples fitted precisely. To secure the surfaces to the plate, the PDMS samples were fixed with cloth tape (Scotch® Colored Duct Tape 330BLU-DC-NA) from the bottom. This minimises handling of the surfaces between quantitative settlement assays (larval counts) and subsequent adhesion strength measurements in the flow chamber.

To remove compounds not cross-linked into the PDMS matrices, the surfaces were washed with a detergent solution (Decon 90), washed thoroughly with water, rinsed with distilled water, and air dried before they were used in the assay. Approximately 20 pediveligers were pipetted with 800 µl FSW in a drop assay on each test surface (n = 6) and covered individually with a Petri dish bottom (IWAKI 1000-053) to prevent evaporation. The assay was conducted in a temperature controlled culture cabinet (Sanyo MLR-351) at 18°C in a 12 h light (5.25 W m^{-2}):12 h dark cycle. The settlement of pediveligers was determined after 48 h and individuals were considered to be settled when they produced byssal threads to attach to the surface. The number and the area of byssal threads were not measured. All pediveligers used were from a single batch.

3.2.2.2.6 Adhesion strength. To quantify the adhesion strength of settled pediveligers of *M. galloprovincialis*, a flow chamber was used (Figure 3.1). The flow chamber consists of an open raceway, 130 mm in height, 1400 mm in length and 150 mm in width. The water is pumped into the raceway (Onga® Hi-flo 415) and dispersed through a PVC fitting (diameter of

25 mm) filled with 23 small pipes (180 × 5 mm) orientated in the direction of the flow to ensure a homogeneous, laminar-like flow based on Enríquez and Rodríguez-Román (2006). The homogeneity of the generated water flow was assessed using dye, which diluted equally without the formation of eddies. The flow rate was varied using stop-valves between the pump and before the raceway. Flow speeds were quantified using the time taken for a floating particle to move 1 m within the raceway. The volumetric flow (flow rate) for each flow speed was calculated using the time required to fill a fixed volume (5 L). The adhesion strength of settled pediveligers of *M. galloprovincialis* on textured PDMS surfaces was determined by quantifying settled numbers before and after the exposure to flow speeds of 0.5, 1, 2, and 4 knots, equivalent to 0.3, 0.5, 1.0, and 2.1 m s^{-1}. The flow rate ranged from 13.9 (0.5 knots) to 111.2 L min^{-1} (4 knots). The PVC plates holding the PDMS test surfaces were placed in the flow chamber with grooves parallel to the flow (one plate at a time) and secured in the raceway using customised fittings. A small PVC ramp was placed in front of the plate holding the test surfaces to ensure an even flow over the plate. The test surfaces were exposed to each speed for 2 min after which the number of remaining mussels was counted.

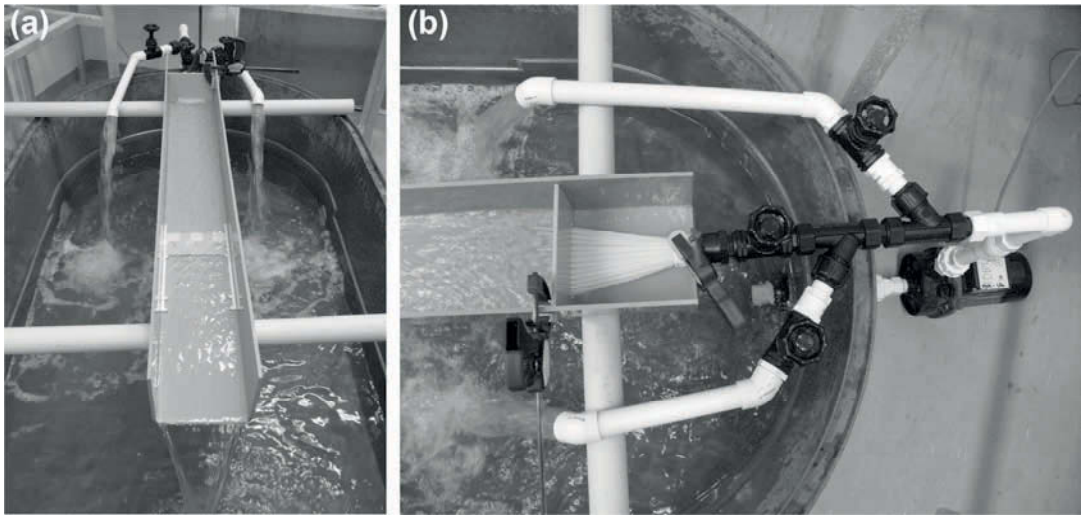

Figure 3.1: Flow chamber set up. (**a**) Front view of the raceway with a height of 130 mm, length of 1400 mm and a width of 150 mm. The samples were placed in a PVC frame, which was then placed in the raceway and secured using customised fittings. (**b**) The water is pumped into the raceway and dispersed through a PVC fitting filled with 23 small pipes orientated in the direction of the flow to ensure a homogeneous, laminar-like flow (Image courtesy of M.J. Vucko, JCU).

3.2.2.3 PDMS aspect ratios

3.2.2.3.1 Preparation of PDMS samples with different aspect ratios using Laminar 5038 resist. The varying aspect ratio plates were produced using Laminar 5038 resist film, which has a thickness of 35 µm. Three layers of resist were laminated onto a polished stainless steel plate at 113°C to give an overall final thickness of 105 µm. The resist samples were exposed for 35 s through a high definition photo-mask using a collimated UV light source (ABM), development of the samples were carried out in 10 g L^{-1} Na_2CO_3 with slight agitation, at ambient temperature.

Electroforming of the shim and the PDMS casting of the samples was carried out in the same manner as previously described in this Chapter (see Section 3.2.2.2).

3.2.2.3.2 Settlement assays and adhesion strength. A range of microtopographies that enhance settlement of *M. galloprovincialis* were identified in the previous experiment and used to determine the effect of aspect ratio of textured surfaces on the settlement of pediveligers. These included PDMS samples with square wave profiles comprising aspect ratios (width:depth features) of 1:1, 1:2, 2:1, and 2:2. The test surfaces had feature widths of 200 and 400 µm and therefore, the width:depth features of the test surfaces were 200:200 (1:1), 200:400 (1:2), 400:200 (2:1), and 400:400 µm (2:2). Smooth PDMS and blank Petri dishes (Techno Plas 10603700) were used as controls.

The experimental setup, bioassay procedure, and adhesion strength measurements followed the methods described in the previous experiment (see Section 3.2.2.2).

3.2.3 Statistical analysis

To test for the effect of larval batch on polymers with different wettabilities, a two factor PERMANOVA was used with wettability as a fixed factor and larval batch as a random factor using PRIMER 6 (v. 6.1.13) and PERMANOVA+ (v. 1.0.3) (Clarke and Gorley 2006). For the PERMANOVA, the Bray-Curtis dissimilarity measure was used and *p*-values were calculated using unrestricted permutation of untransformed raw data with 9999 random permutations.

All remaining data were analysed by either univariate or repeated measures analysis of variance (ANOVA; SPSS Version 19). The assumptions of normality and homogeneity of variance were examined using Q-Q plots of residuals and Levene's test, respectively (Quinn and Keough 2002). Data were converted to proportions and arcsine square-root transformed where required and not stated otherwise. Post hoc comparisons were performed to establish significant differences (Tukey's Honestly Significant Difference test, Tukey's HSD; $\alpha = 0.05$).

One-factor ANOVA was used to separately test the effect of the surface characteristics of microtexture and aspect ratio on the settlement of pediveligers of *M. galloprovincialis*. Microtexture and aspect ratio were treated as fixed factors. To determine differences in the number of remaining mussels with increasing flow rates, repeated measures ANOVA was used with 'flow' as the within-subject variable and 'texture' and 'aspect ratio' as between-subject factors. Variance-covariance sphericity of the data used in repeated measures analyses were estimated using the Huynh-Feldt correction. Correlations between the number of settled and remaining pediveligers after being exposed to increasing water flow were assessed using Pearson's product-moment correlations for each flow rate. Results are reported as mean ± 1 standard error (SE).

3.3 Results

3.3.1 Polymers with different wettabilities

The settlement of pediveligers of *M. galloprovincialis* on polymers showed a similar trend among batches and the highest number of pediveligers consistently settled on nylon, regardless of batch. The highest number of pediveligers settled on the two polymers with the highest wettability (lowest hydrophobicity), with 20.5 ± 5.0% (mean of three batches ± 1 SE) and 33.5 ± 13.1% settlement on epoxy and nylon, respectively (Figure 3.2). The lowest number of pediveligers settled on PDMS (4.2 ± 3.2%), representing the least wettable (most hydrophobic) polymer in the tested range. However, there was no consistent trend between settlement and wettability. There was a significant interactive effect of 'larval batch' and 'wettability' of tested polymers on settlement, preventing the analysis of the main effects (Table 3.1). This interaction term was primarily driven by differences in the overall settlement between batches.

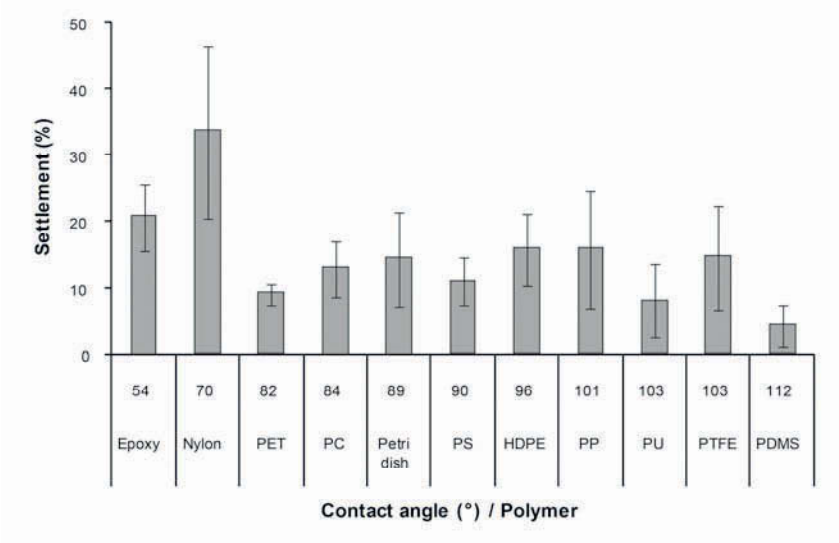

Figure 3.2: Mean settlement (± 1 SE) of pediveligers of *M. galloprovincialis* on polymers ranging from hydrophilic to hydrophobic (n = 3).

Table 3.1: Two-factor PERMANOVA analysis on Bray-Curtis distances for differences in the settlement of different larval batches of pediveligers (random factor) on polymers ranging from hydrophilic to hydrophobic (fixed factor).

Source	*df*	Mean Square	*F*	*p*
Wettability	10	8485	1.81	0.070
Batch	2	21296	11.43	< 0.001
Wettability × Batch	20	4802	2.58	< 0.001
Residual	246	1863		

3.3.2 Microtextured PDMS

3.3.2.1 Settlement assays

Microtopographies significantly enhanced the settlement of pediveligers of *M. galloprovincialis* (one-factor ANOVA: $F_{(14,75)} = 38.68$, $p < 0.001$; Figure 3.3). Settlement on smooth controls and textures of 10 and 20 µm were significantly lower than settlement on textures ≥ 40 µm, with the exception of textures ranging from 100 to 250 µm (Tukey's HSD; $\alpha = 0.05$). Over 65% of pediveligers settled on textures sized between 40 to 80 µm and 300 to 1000 µm, whereas the settlement on textures between 100 and 250 µm were below 45%. The lowest settlement

occurred on the 10 µm texture (8.4 ± 2.8%), while the highest number of pediveligers settled on the 400 µm texture (91.6 ± 1.7%) within 48 h.

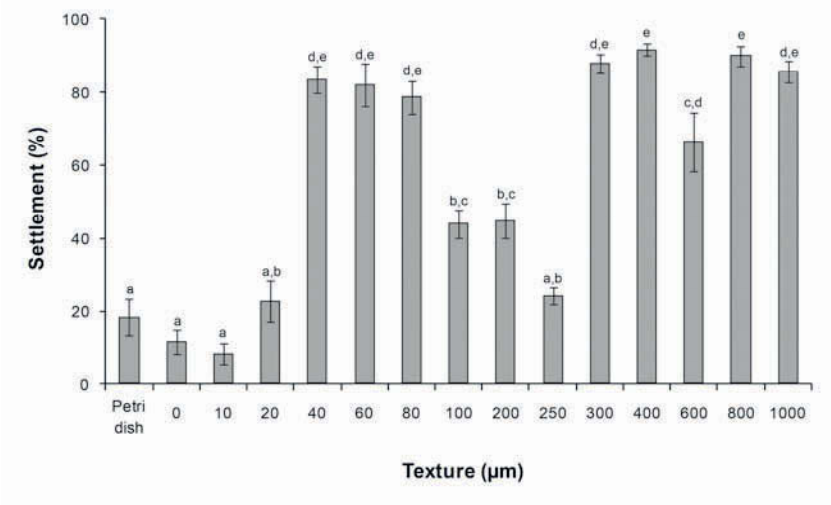

Figure 3.3: Mean settlement (± 1 SE) of pediveligers of *M. galloprovincialis* on textured PDMS ranging from 0-1000 µm (n = 6). Superscript letters indicate significant differences (Tukey's HSD; $\alpha = 0.05$).

3.3.2.2 Adhesion strength

The detachment (release) of pediveligers increased with increasing flow rates (Figure 3.4a-d). At the lowest tested flow rate of 0.5 knots, the percentage of remaining pediveligers ranged between 62.5 ± 11.6% (10 µm) and 94.4 ± 5.6 % (250 µm) (Figure 3.4a). The number of detached pediveligers increased at flow rates of 1 and 2 knots with the percentage of remaining pediveligers ranging between 23.0 ± 9.3% (smooth PDMS) and 81.7 ± 5.9 % (400 µm) at 1 knot (Figure 3.4b) and 4.0 ± 3.7% (smooth PDMS) and 79.9 ± 5.7% (400 µm) at 2 knots (Figure 3.4c). The highest level of detachment occurred on exposure to 4 knots, with no pediveligers remaining on smooth PDMS. In contrast, 79.9 ± 5.7 % of pediveligers remained on the 400 µm texture (Figure 3.4d).

Although there was a significant difference in the number of remaining pediveligers between microtextures and flow (Table 3.2a), there was also a significant interaction term between 'flow rate' and 'texture' ($F = 6.26$, $p < 0.001$). This interaction was mainly driven by differences in adhesion strength on different microtextures with increasing flow rates.

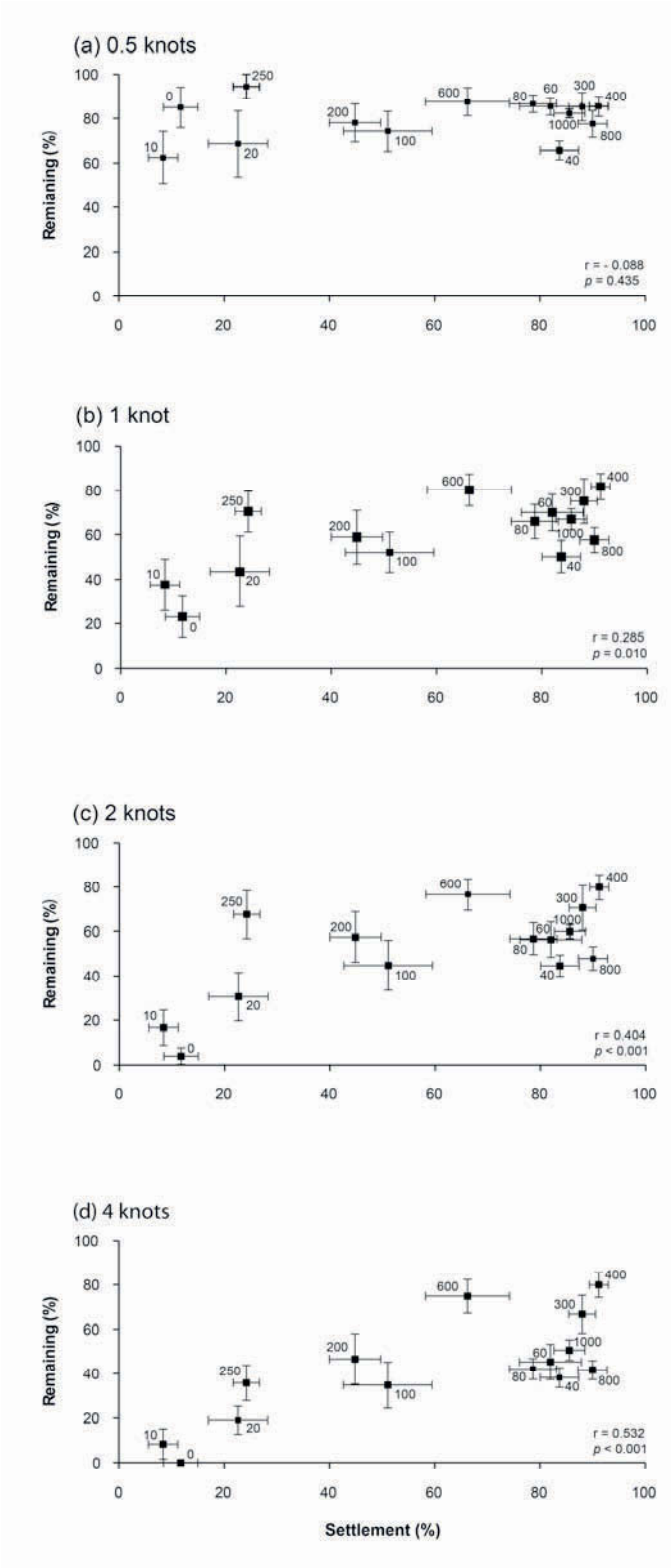

Figure 3.4: The correlation between initial settlement (%) and remaining pediveligers of *M. galloprovincialis* (%) after being exposed to (**a**) 0.5 knots, (**b**) 1 knot, (**c**) 2 knots, and (**d**) 4 knots. Means ± 1 SE are shown (n = 6).

Table 3.2: Repeated measures ANOVA of remaining mussels on textured PDMS exposed to flow rates of 0.5, 1, 2, and 4 knots on (**a**) microtextured PDMS and (**b**) PDMS with different aspect ratios.

Source	*df*	Mean Square	*F*	*p*
(a) Microtextured PDMS				
*Test of within-subjects effects**				
Flow	3	3.83	207.53	< 0.001
Flow × Texture	39	0.12	6.26	< 0.001
Error (Flow)	201	0.02		
Test of between-subjects effects				
Texture	13	1.06	4.21	< 0.001
Error	67	0.25		
(b) PDMS aspect ratios				
*Test of within-subjects effects**				
Flow	2.48	1.46	27.30	< 0.001
Flow × Aspect ratio	9.92	0.14	2.66	0.010
Error (Flow)	57.06	0.05		
Test of between-subjects effects				
Aspect ratio	4	1.31	5.46	0.003
Error	23	0.24		

*Results are based on the Huynh-Feldt correction

3.3.2.3 Correlation of settled and remaining pediveligers

Overall, higher numbers of pediveligers remained on textured surfaces with high initial larval settlement (Figure 3.4a-d). There was no significant correlation between the lower flow rate of 0.5 knots and initial settlement (Pearson's product-moment correlation: r = -0.088, p = 0.435). However, when flow increased above 1 knot the number of remaining pediveligers was positively correlated to settlement (1 knot, r = 0.285, p = 0.010). This association became stronger with increasing flow rates and was strongest at the highest tested flow rate (4 knots, r = 0.532, p < 0.001).

3.3.3 PDMS aspect ratios

3.3.3.1 Settlement assay

Aspect ratio of narrow features had a significant effect on the settlement of pediveligers (one-factor ANOVA: $F_{(5, 30)} = 63.11$, $p < 0.001$; Figure 3.5). Settlement was lowest on controls with 3.0 ± 1.0% and 18.0 ± 2.5% on smooth PDMS and Petri dishes, respectively. Settlement increased with increasing texture for both width and depth from 43.3 ± 6.9% (1:1) to 92.3 ± 2.6% (2:2). Settlement was significantly lower on the square wave profile with a 1:1 aspect ratio (43.3 ± 6.9%), than on all other textured surfaces (Tukey's HSD; $\alpha = 0.05$). Notably all textures with a 400 µm feature had high settlement, regardless of orientation.

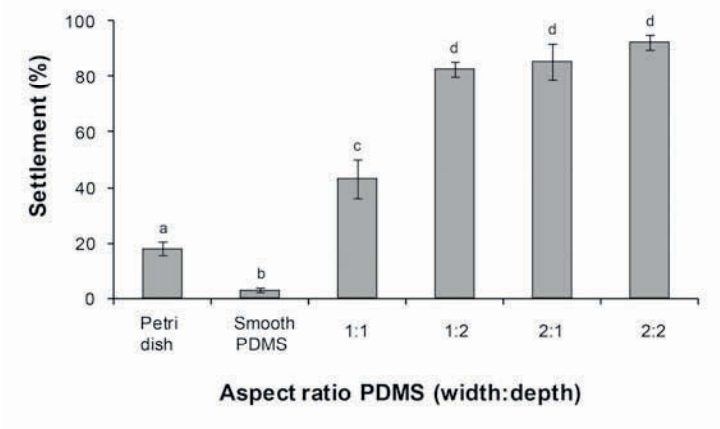

Figure 3.5: Settlement (%) of pediveligers of *M. galloprovincialis* on textured surfaces with different aspect ratios (widths:depths), ranging from 1:1 (200:200 µm) to 2:2 (400:400 µm). Means ± 1 SE are shown ($n = 6$). Superscript letters indicate signififcant differences (Tukey's HDS; $\alpha = 0.05$).

3.3.3.2 Adhesion strength

The number of remaining pediveligers decreased with increasing velocities (Figure 3.6a-d). Aspect ratio (repeated measures ANOVA: $F = 5.46$, $p = 0.003$; Table 3.2b) and flow ($F = 27.30$, $p < 0.001$) significantly affected the number of remaining mussels, with a significant interaction among these two factors ($F = 2.66$, $p = 0.010$). There were no remaining pediveligers on smooth PDMS after being exposed to 4 knots. For the textured surfaces, the numbers of remaining pediveligers were similar among aspect ratios and ranged between 59.2 ± 6.6% (2:2) and 71.5 ± 5.4% (1:2).

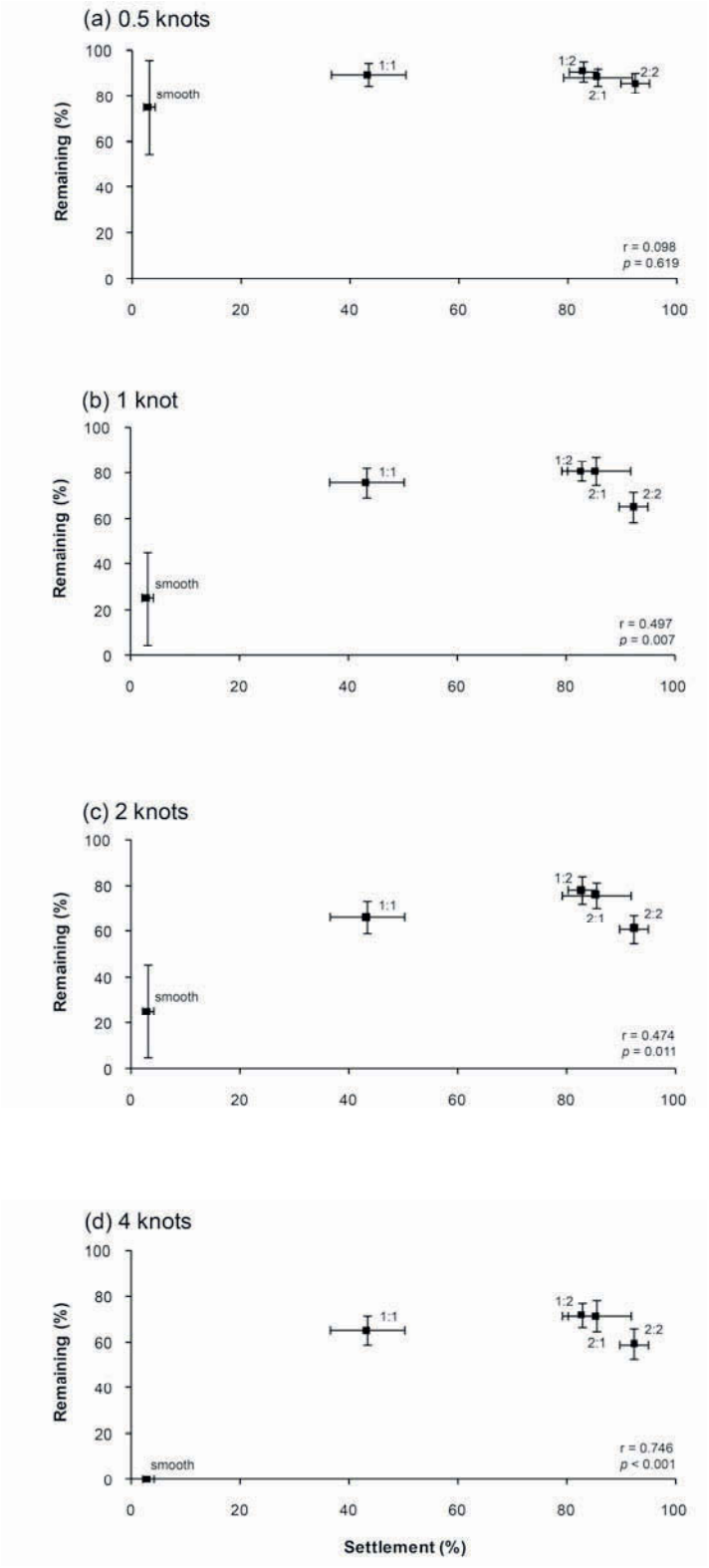

Figure 3.6: The correlation between initial settlement (%) on textured surfaces with different width:depth features after 48 h and remaining pediveligers of *M. galloprovincialis* (%) after being exposed to (**a**) 0.5 knots, (**b**) 1 knot, (**c**) 2 knots, and (**d**) 4 knots. Means ± 1 SE are shown (n = 6).

3.3.3.3 Correlation of settled and remaining pediveligers

Generally, topographic features enhanced settlement and retention (Figure 3.6a-d). The correlation for the lowest tested velocity was not significant (Pearson's product-moment correlation: r = 0.098, p = 0.619) and the number of remaining pediveligers was significantly correlated to settlement for flow rates $\geq$ 1 knot, as in the assay utilising scale of microtopography. The association between retention and initial settlement became stronger with increasing flow rates and was strongest at 4 knots (r = 0.746, $p < 0.001$).

3.4 Discussion

This study demonstrates the importance of wettability and microtextured surfaces to the settlement of pediveligers of *M. galloprovincialis*. While wettability has a negligible effect on settlement, the presence of topographic structures plays a key role. This is clearly seen where PDMS without topography has the lowest settlement among all tested polymers, but has settlement of over 90% with the addition of topography (400 µm). Overall, the effect of texture overshadows any effect of wettability on the settlement of pediveligers of *M. galloprovincialis.*

Settlement of pediveligers was higher on polymers with hydrophilic characteristics. The highest settlement occurred on the most hydrophilic polymers (epoxy and nylon) and was more than five times the settlement on the most hydrophobic polymer (PDMS). Mussels attach to surfaces using proteinaceous bioadhesives (Lin et al. 2007) and the quality of the adhesive joint depends strongly on surface characteristics and the interaction of the adhesive with the surface (Wiegemann 2005). The ability of an adhesive to spread is linked to the wettability of the adherend surface which in turn affects the quality of the bond (Waite 1987; Wiegemann 2005). Aldred et al. (2006) found a positive correlation between increased surface wettability and the capacity of the adhesive (plaque) to spread for the mussel *M. edulis*. Furthermore, mussel byssal production decreased with increasing surface wettability, and therefore fewer byssus threads are required to establish a strong attachment, which results in energetic advantages; larval attachment was consistent on surfaces with different wettabilities, irrespective of the number of byssus threads used to attach (Aldred et al. 2006). This is in contrast to findings of the present study, where settlement of pediveligers differed between wettabilities, and this may be due to different stages of ontogeny in the two studies (plantigrades *vs.* 15 mm spat). The composition of adhesive secretions differs between different aged mussels, with a higher production of

polysaccharides at the young larval stage, while proteinaceous secretions becoming more dominant with increasing age (Petrone et al. 2008).

Overall, settlement was low (< 35%) on all polymers. This is starkly contrasted with the strong effect of microtopography on PDMS, where there was more than 90% settlement. This is consistent with other studies identifying low mussel settlement on smooth surfaces (Eyster and Pechenik 1987; Petraitis 1990; Köhler et al. 1999), and topographic features increasing settlement (Köhler et al. 1999; Alfaro and Jeffs 2002; Gribben et al. 2011). Mussel larvae actively explore the substratum with a foot by crawling (Waite 1983; Gosling 2003; Pernet et al. 2003) and make an active settlement choice of where to settle (Köhler et al. 1999; Alfaro and Jeffs 2002). The results of this study demonstrate that flat surfaces without topographic features are not selected resulting in low numbers of settled pediveligers. Furthermore, settlement on microtextured surfaces with profiles smaller than 40 µm (10 and 20 µm) was low. The diameter of the foot of pediveligers is approximately 40 µm and it is proposed that textures smaller than the diameter of the foot at this stage of ontogeny are detected as an unattractive surface for settlement. The mussel scrubs the surface with its foot prior to attachment (Waite 1987) and smaller topographic features may therefore inhibit settlement.

There was a very strong effect of texture ≥ 40 µm, with an increase of settlement to 80% compared to smooth controls. In general, textured surfaces provide an attractive surface for settlement with its high surface to volume ratio and crevices providing protection of hydrodynamic forces and predation. Decreased settlement to topographies ranging from 100 to 250 µm is in accordance with attachment point theory (Callow et al. 2002; Scardino et al. 2006, 2008). Pediveligers have a width and length of approximately 190 and 260 µm, respectively. Therefore, topographies within this size range restrict the fit between larvae and surface feature (Figure 3.7) and also impede the contact area required for optimal adhesion and attachment (Scardino et al. 2008). A similar negative effect of surface topographies smaller than larvae occurs for the settlement of the barnacle *B. improvisus* (Andersson et al. 1999; Berntsson et al. 2000) and *B. amphitrite* (Schumacher et al. 2007a; Aldred et al. 2010a), the serpluid tube worm *Hydroides elegans* (Scardino et al. 2008), and the bryozoan *B. neritina* (Scardino et al. 2008). In contrast, microtopographies slightly larger than larvae improve settlement (Scardino et al. 2006). Consequently, the 400 µm textured surface had the highest settlement of pediveligers of *M. galloprovincialis*, providing optimal attachment and a more secure settlement site under flow. Accordingly, the highest number of settled pediveligers remained on the 400 µm texture after being exposed to 4 knots. While Schumacher et al. (2007a) found an isolated effect of feature depth on the settlement of the barnacle *B. amphitrite*, there was no distinct effect of either feature depth or feature width on the settlement of *M. galloprovincialis* in this study

(aspect ratio). When either of the topographical dimensions were 400 µm, there was no significant difference between test surfaces and pediveligers fitted into the grooves with more than 80% settling within 48 h.

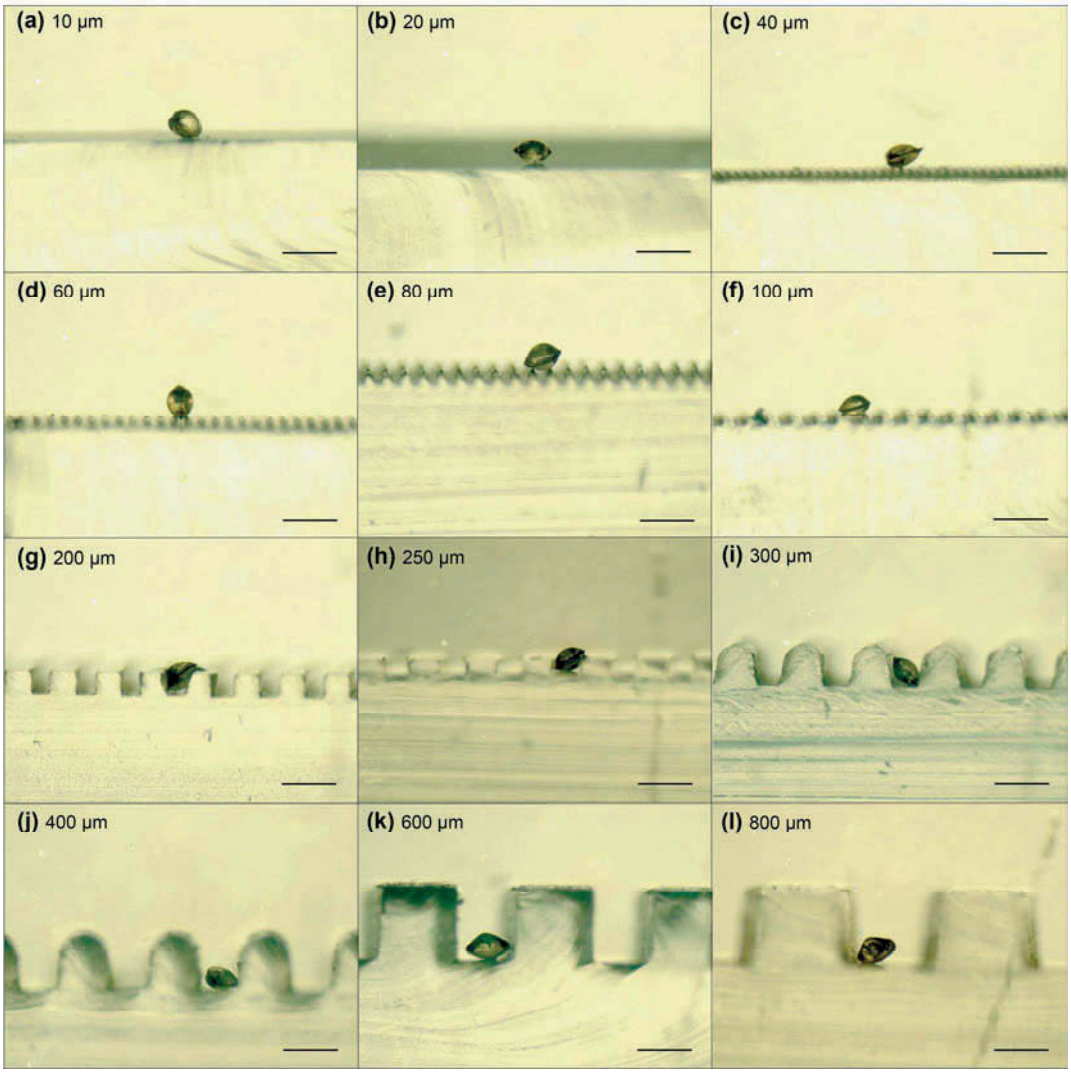

Figure 3.7: Cross-sections of the textured PDMS surfaces used in settlement assays with pediveligers of the mussel *M. galloprovincialis* on (**a**) 10 µm, (**b**) 20 µm, (**c**) 40 µm, (**d**) 60 µm, (**e**) 80 µm, (**f**) 100 µm, (**g**) 200 µm, (**h**) 250 µm, (**i**) 300 µm, (**j**) 400 µm, (**k**) 600 µm, and (**l**) 800 µm textured surfaces. Scale bars = 500 µm.

The concept of selective settlement based on the ability to attach (Scardino et al. 2008), does not explain the high settlement and low adhesion strength on surfaces with topographies between 40 and 80 µm. The numbers of remaining pediveligers on these topographies were

similar to those on topographies with low settlement (100 to 250 µm) after being exposed to the highest velocity. While the effects of surface topography on the settlement of marine organisms are well documented (Petronis et al. 2000; Callow et al. 2002; Scardino et al. 2006, 2008; Aldred et al. 2010a; Long et al. 2010a, 2010b), the quantification of attachment strength (Becker 1993; Finlay et al. 2002, 2008) and the correlation thereof with settlement preferences is less common (Aldred et al. 2010a). Furthermore, direct measurement of mussel adhesion is rare and limited to plantigrades and adults (Aldred et al. 2006; Burkett et al. 2009; Babarro and Reiriz 2010; Brenner and Buck 2010). This is the first study to demonstrate the positive correlation of settlement preferences and attachment strength of pediveligers of *M. galloprovincialis*. This association was the strongest for the highest tested flow rate (4 knots), while there was no difference in the number of remaining pediveligers for the lowest flow rate (0.5 knots). Applied water forces may need to be even higher when testing for differences in the attachment strength among other textured materials, given PDMS is a fouling-release material. Combining microtopography on preferred hydrophilic surfaces such as nylon may provide enhanced settlement and even stronger attachment strength. However, when using microtextured materials other than PDMS there may be unpredictable non-linear interactions between the response to feature size/aspect ratio and polymeric material due to differences in surface properties other than wettability. Consequently, the effect of feature sizes may not translate to all surface materials and feature sizes.

In conclusion, texture is a key factor in the active selection of settlement sites of pediveligers of *M. galloprovincialis* with a far greater effect than wettability. Textured surfaces with 400 µm grooves have the highest settlement and attachment strength. Furthermore, settlement preferences are positively correlated to the number of remaining pediveligers (attachment strength) after being exposed to a flow rate of 4 knots. These results provide the underlying principles to develop improved materials for the enhanced settlement and attachment of mussel larvae and provide an opportunity to regulate density and retention of mussels for the aquaculture industry.

Chapter 4

Where to settle – settlement preferences and site-specific locations of *Mytilus galloprovincialis* [3]

4.1 Introduction

The genus *Mytilus* with two key species, *Mytilus galloprovincialis* and *M. edulis,* is the major contributor to the mussel aquaculture industry (Gosling 2003; Stevens et al. 2008), and is worth more than 1.2 billion USD per annum (FAO 2010). Mussel seed for this industry is either collected from the wild using spat catching ropes, or alternatively collected from drift macroalgae and natural mussel beds. In addition, mussel seed is produced in closed life-cycle hatchery culture. The success of the global mussel aquaculture industry relies heavily on high settlement rates and retention of mussel spat on ropes for on-growing (Hickman 1992). To optimise these critical factors, specialised spat catching and culture ropes made of multi-filament synthetic and natural fibres are used (Cáceres-Martínez et al. 1994; Walter and Liebezeit 2003; Filgueira et al. 2007; Brenner and Buck 2010; Hayden and Woods 2011). However, little is known about the fundamental drivers for larval selection of preferred settlement sites for attachment on materials. Understanding these drivers is vital to develop and implement mechanisms to optimise and manage the settlement of mussels, and ensure a sustainable practice in the mussel aquaculture industry.

In contrast to many sessile invertebrate larvae, mussel settlement is not permanent and individuals of *Mytilus* can detach and resettle in an alternative habitat (Bayne 1964a; Gosling 2003). Larvae of *M. galloprovincialis* initially settle as pediveligers and actively explore the substratum by crawling with a foot (Waite 1987; Gosling 2003; Pernet et al. 2003), which is a complex muscular and glandular organ with cilia, also used for byssus secretion (Gosling 2003; Pernet et al. 2003). Pediveligers are capable of discriminating between different substrata (Gosling 2003) and are selective in their preference of settlement sites (Petersen 1984; Cáceres-Martínez et al. 1994; Filgueira et al. 2007; Brenner and Buck 2010). If the substratum is

[3] Chapter 4 is currently under review as is at the journal PLoS One.

unsuitable, they can withdraw their foot and swim off (Bayne 1976). In these circumstances metamorphosis can be delayed for several weeks (Bayne 1965) and pediveligers repeat the exploratory pattern of swimming and crawling behaviour until a suitable settlement site is found (Gosling 2003). If a suitable substratum is found, an adhesive plaque and byssal thread is deposited onto the surface (Silverman and Roberto 2010). Following metamorphosis, post-larvae are termed plantigrades (Bayne 1976), spat, seed or juveniles (Alfaro et al. 2004). Anecdotal observations at mussel hatcheries suggest that pediveligers initially settle inside the complex structure of multi-filament ropes, supporting an influence of topography on initial settlement preference in large-scale commercial production.

In laboratory based studies, topographic features have a demonstrated role in settlement choice of pediveligers and this effect is far greater than chemical cues (Gribben et al. 2011) and wettability (see Chapter 3, Section 3.31 and 3.3.2). Textured surfaces provide an attractive surface for larval settlement with a high surface area to volume ratio, and crevices provide protection from hydrodynamic forces, drifting objects, and predation. This reduces post-settlement mortality (Filgueira et al. 2007). In general, mussel settlement is lower on smooth surfaces, with topographic features strongly enhancing settlement (Köhler et al. 1999; Gribben et al. 2011; see also Chapter 3, Section 3.3.2.1). Attachment is also more rapid on surfaces with topographic features (Gosling 2003), including branching algae (Alfaro and Jeffs 2002), shells (Petersen 1984), and hydroids (Alfaro and Jeffs 2002), with fine-branching substrates being preferred over medium- and coarse-branching materials (Alfaro and Jeffs 2002). Furthermore, mussels actively select favourable sites and higher numbers of spat attach to node areas of substrata than to inter-node areas regardless of the branching of substrata (Alfaro and Jeffs 2002), while the absence of filamentous substrata prolongs the larval stage and delays metamorphosis (Lutz and Kennish 1992). More specifically, preferences can be defined to accurate sizes, with a topographic width 400 µm being a preferred initial settlement site for pediveligers of *M. galloprovincialis* (see Chapter 3, Section 3.3.2.1). Importantly, any changes in preference as pediveligers grow remain undefined.

In contrast to pediveligers, adult mussels settle on all types of firm substrata (Seed 1976) to form dense mussel beds (Gosling 2003), suggesting that the effect of topography as a settlement cue becomes less important for adult mussels. However, there has been no effort to deconstruct the direct effect of microtopography on the settlement of mussels beyond the metamorphosis of pediveligers.

Using the combination of manipulative studies and quantitative measurements, the aim of this Chapter is to determine settlement preferences and locations of *M. galloprovincialis* under

commercial hatchery operating conditions. Firstly, settlement preferences of pediveligers and plantigrades are quantified for surfaces with increasing complexity. Secondly, in an applied approach, the site-specific settlement location of pediveligers on and within culture ropes from a commercial mussel hatchery is quantified and mapped for 16 days post initial settlement using optical microscopy and X-ray micro-computed tomography. This Chapter thereby contributes to the fundamental understanding of key drivers for larval settlement selection of *M. galloprovincialis*, from the pediveliger through to the plantigrade stage, within an applied commercial hatchery operation.

4.2 Materials and methods

4.2.1 Culture of pediveligers and on-growing

Approximately 100 adult mussels of *M. galloprovincialis* were collected as broodstock (F2) from Clifton Springs Aquaculture Fisheries Reserve, Victoria, Australia, and transported to the Victorian Shellfish Hatchery in Queenscliff, Victoria, Australia. Detailed spawning procedure and larvae production procedures are provided in Pettersen et al. (2010).

Larvae were reared in 9 aerated flow-through tanks, each holding 400 L filtered seawater (FSW; 1 µm and UV sterilised) with an initial flow rate of 1.7 L min^{-1}. The flow rate was increased to 2.5 L min^{-1} after 5 days post fertilisation, and to 3.3 L min^{-1} after 8 days post fertilisation. The larvae were maintained at a density of 25-40 larvae mL^{-1} at 16˚C under constant light. Larvae were fed *Chaetoceros calcitrans* (CS-178), *Pavlova lutheri* (CS-182), and *Isochrysis galbana* (clone T.ISO, CS-177) daily in equal parts at a target background residual cell density of 50,000 cells mL^{-1}. After 12 days post fertilisation, *C. muelleri* (CS-176) was added to the diet (33% *I. galbana*, 33% *P. lutheri*, 11% *C.calcitrans*, 22% *C. muelleri*). The water in all tanks was changed every other day.

The pediveliger stage was reached at 22 days post fertilisation and larvae were competent to settle. In the conditions used for settlement and on-growing under hatchery production, reproduced in assays below, pediveligers of several larval rearing tanks were combined until the required number of approximately 19,300,000 larvae was achieved for transferring these into one of six nursery tanks at a density of approximately 4.8 pediveligers mL^{-1} in a closed culture system. Each nursery tank held approximately 4000 L FSW and 750 m of spat catching ropes (150 ropes, each 5 m) as a settlement surface. The outdoor nursery tanks were aerated and covered with a shade cloth to reduce the growth of filamentous algae and to minimise water

temperature fluctuations. The water was completely renewed every second day. Mussels were fed daily at a target cell density of 50,000 cells mL^{-1} of *C. muelleri* (50%), *P. lutheri* (25%), and *I. galbana* (25%).

4.2.2 Laboratory settlement assays

To quantify the effects of surface complexity on the settlement of *M. galloprovincialis*, three settlement surfaces with increasing complexity were tested. These were smooth polypropylene rods (Gehr GmbH, Germany), textured polypropylene rods with a square wave profile of 400 µm (Gehr GmbH, Germany), and multi-filament braided polypropylene nursery ropes (Whittam ropes, Australia) used by local mussel farmers in Port Phillip Bay, Victoria, Australia. Polypropylene rods were textured by cutting a 400 µm wide and 400 µm deep thread into the rod, with a spacing of 400 µm between the thread. This feature size (400 µm) is highly effective in enhancing the settlement rate of pediveligers of *M. galloprovincialis* (see Chapter 3, Section 3.3.2). Each test surface (rods and rope) was cut to a length of 100 mm. The rods and rope had a diameter of approximately 20 and 12 mm, respectively. To suspend the test surfaces in water, a hole was drilled through the top end of each polypropylene rod and fishing line inserted. Similarly, fishing line was inserted into the top end of each braided rope for suspension. To ensure full suspension of each test surface in the settlement assays, a stainless steel weight (~19 g) was glued (Silkaflex-PRO, Silka) to the bottom end of each test surface.

Mussels were collected for no choice and choice settlement assays at 22 (pediveliger stage), 30, and 38 days post fertilisation (plantigrade stage) from the Victorian Shellfish Hatchery. Pediveligers were collected from a larval rearing tank approximately 1 h prior to the transfer to nursery tanks at 22 days post fertilisation. Plantigrades were collected off a rope from a designated outdoor nursery tank at 30 and 38 days post fertilisation.

4.2.2.1 No choice assays

In no choice settlement assays using *M. galloprovincialis*, one each of the three settlement surfaces (n = 10) were suspended individually in glass beakers filled with 900 mL FSW. Each test surface was suspended from a small rod using fishing line. The rod was placed horizontally on the top of the beaker, with the test surface immersed vertically in the water. The water was aerated using glass pipettes to ensure an even suspension of mussels in the water column.

Approximately 100 individual mussels (pediveligers or plantigrades) were placed in each glass beaker and maintained in a temperature controlled room at 17°C in a 12 h light:12 h dark cycle. The settlement of individuals on all surfaces including the beaker was measured after 48 h. Test surfaces were dipped three times in the water column to remove unattached mussels. Subsequently, these and other mussels suspended in the water column were captured on a 100 µm sieve and counted. Mussels attached to smooth and textured polypropylene rods, and glass beakers, were counted using a dissecting microscope (Olympus SZX7). To count the number of mussels attached to the ropes, these samples were teased apart and individually rinsed with FSW. The detached mussels were retained with a sieve (100 µm) and subsequently counted.

4.2.2.2 Choice assays

In choice settlement assays using *M. galloprovincialis*, the three surfaces were randomly attached to a rod and suspended in a glass beaker (n = 10) as previously described. Each beaker was filled with 900 mL FSW and aerated using glass pipettes. Approximately 100 individual pediveligers or plantigrades were added to each beaker and maintained in the same room in a 12 h light:12 h dark cycle. After 48 h, the number of unattached mussels and individuals suspended in the water column, as well as mussels settled onto each surface including the beaker was recorded (see Section 4.2.2.1).

4.2.3 Site-specific settlement onto ropes under hatchery conditions

4.2.3.1 Rope samples in small-scale nursery tanks

To determine the site-specific settlement onto and within culture ropes under commercial hatchery operating conditions, a total of 468 test rope pieces (Whittam ropes, Australia) were immersed in three independent outdoor tanks at the Victorian Shellfish Hatchery in Queenscliff. Each tank held 156 rope pieces and was a small-scale of the commercially used nursery tanks at the hatchery described previously. Each test rope piece had a length of 150 mm and was tied to the bottom and the top of the tank using fishing line so that all rope samples were immersed vertically in the water at a depth of approximetaly 130 mm below the surface. Each small tank was filled with 130 L FSW and approximately 20,000 pediveligers (22 days post fertilisation) were added to each tank. The feeding regime, density of larvae and rope per unit volume were equal to hatchery standards for the commercial tanks (see Section 4.2.1). The water was

completely renewed in each tank after 24 h and therefore all non-settled larvae were also removed from the tanks after 24 h. Sets of ropes (n = 3) were collected from each tank one day after settlement (23 days post fertilisation) and subsequently on every fourth day for 16 days (39 days post fertilisation). Within each rope, from each set, a section of 100 mm within the centre of the rope was marked using cable ties and the number of settled mussels within the marked section counted using a dissecting microscope (Olympus SZX7). Subsequently, mussels were removed from the outside of the rope using tweezers, preserved in 70 % ethanol and images were taken for size measurements (Image J). The assayed ropes were then discarded.

4.2.3.2 Rope samples in individual containers

To quantify the movement of mussels from the inside of the rope outwards as individuals grow larger, a total of 15 rope pieces were collected from each small-scale nursery tank (3 tanks; 45 rope pieces in total) after one day post settlement (23 days post fertilisation). The ropes were then placed individually into independent labelled containers, each holding 1500 mL of FSW. Notably, ropes in individual containers were not subject to longer term larval supply and provide a contrast to rope samples maintained in small-scale nursery tanks as movement between ropes was excluded. This confirms that all subsequent mussels are a product of the initial settlement of pediveligers at 23 days post fertilisation. The water in each container was gently aerated and the containers were maintained at 17°C with 12 h light:12 h dark cycle. The feeding density was maintained at 50,000 cells mL^{-1} as described previously for nursery tanks (see Section 4.2.1). The water and holding containers were changed every other day. To determine the initial settlement and subsequent movement of mussels on the ropes over time, one set of ropes (n = 3) from each small-scale tank was removed and assayed on 1, 5, 9, 13, and 17 days post settlement by quantifying the number of settled mussels on the outside of the rope within each marked 100 mm section as previously described. All mussels were removed using tweezers, counted and preserved in 70% ethanol for size measurements. To minimise stress and avoid repeated sampling, all assayed ropes were discarded.

4.2.3.3 Imaging settlement locations with X-ray micro-computed tomography

In addition, the specific settlement locations and the depth of settlement within the rope were qualitatively determined using X-ray micro-computed tomography (µCT). One rope piece was collected from each small-scale nursery tank (n = 3) on 1, 5, 9, 13, and 17 days post settlement

(23, 27, 31, 35, and 39 days post fertilisation). Immediately on collection, all ropes were placed in 70% ethanol for 30 min and subsequently vacuum packed in plastic wrap (Sunbeam VAC 660) to minimise dislocation of mussels during transportation. For X-ray µCT imaging, the samples were transported from the hatchery in Queenscliff to the µCT facility located at the Australian National University, Canberra. The in-house designed X–ray µCT device is built on a 3 m parallel optical rail and consists of a X-ray source (X-Tek RTF-UF225), rotation stage (Newport RV120PP) and X-ray camera (Roper PI-SCX100:2048) (see Sakellariou et al. 2004 for details). The location of the rotation stage and the camera are adjustable on the rail, which results in changes of the magnification of the sample. The magnification is determined by the proximity of the sample to the X-ray source, compared with the distance between the camera and the source (Ribi et al. 2008). For this study, the sample distance was 800 mm, which resulted in a voxel size of approximately 39 µm. A 'voxel' is a cubic volume element, which represents a three-dimensional data point in the tomogram. The three-dimensional tomogram (Figure 4.1a) visualises the structure and variation of composition within the sample and was generated by collecting a series of two-dimensional radiographs, collectively called projection data (Sakellariou et al. 2004). In order to collect the projection data at different viewing angles, the sample was rotated through 360º. To minimise the scan time and to stabilise the samples, a total of three rope samples (one sample from each small-scale nursery tank collected at the same time point) were vertically placed side by side in a small plastic container fixed on the rotation stage for each scan. The rope samples were kept vacuum packed and individually labelled using aluminium foil stripes allowing identification in the tomogram. The X-ray source was run with 80 kV at 120 µA to optimise the contrast of the mussel shells in the rope samples. A total of 1440 projections were obtained per revolution and the scan time was 5 h for each scan. Volumes of 1024^3 voxels were generated and each rope sample was scanned approximately 35 mm in length. To generate a tomogram, the projection data were processed with a Feldkamp reconstruction algorithm (Feldkamp et al. 1984; Sakellariou et al. 2004). All data processing was carried out on a Compaq AlphaServer super-computer located at the Australian Partnership for Advanced Computing, Australia's national supercomputing facility.

The images from three-dimensional tomograms were visualised and analysed using the in-house developed program 'NCViewer' This program has the feature to visualise the tomogram slice by slice in each plane (x, y, z; Figure 4.1b-d). The size of each mussel was individually measured in each plane. To measure the settlement depth of mussels within the rope, the distance of each individual to the exterior of the rope was quantified in the z-plane (Figure 4.1d). Uniform distance measurements were obtained using the plastic wrap, in which each rope sample was vacuum-sealed as a reference. Mussels were clearly distinguishable in the tomograms by an oval shape with a hollow centre, which characterised the two shell valves

(Figure 4.1b-d). The contrast of the mussels increased with age as the X-ray density of the calcium carbonate of the shell increased with growth. To quantify settlement depth within ropes among mussel with different sizes, mussels were grouped into eight size classes based on their maximum shell length measured in any of the three planes (x, y, z): 250-349, 350-449, 450-549, 550-649, 650-749, 750-849, 850-949, and > 950 µm in shell length.

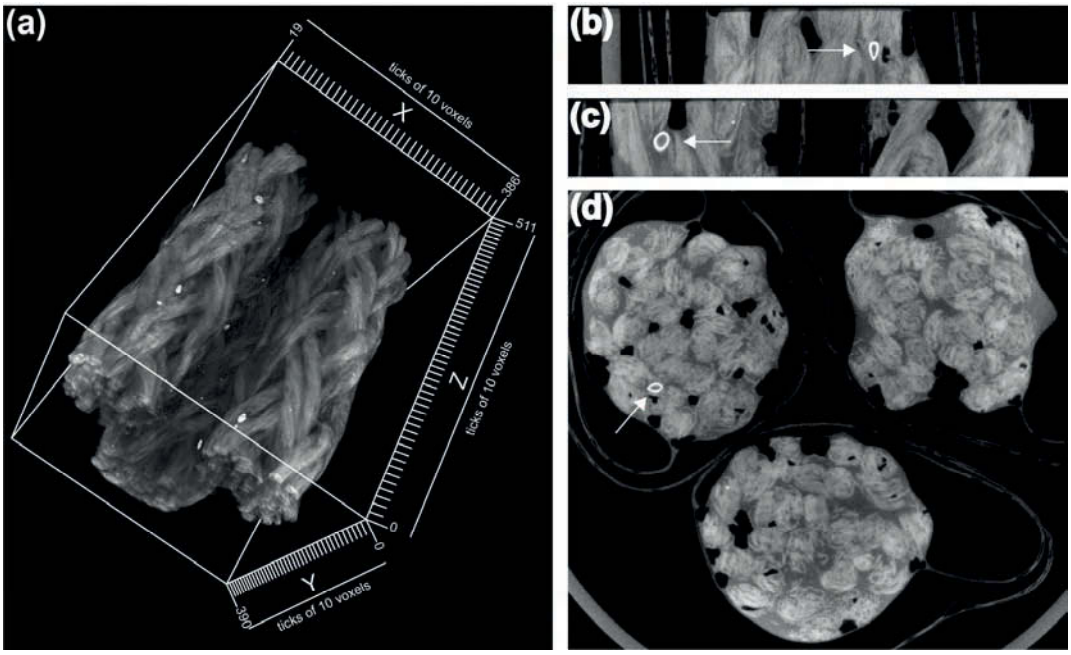

Figure 4.1: (**a**) Three-dimensional tomogram of rope samples collected 17 days post settlement with a reduced volume of 512^3 voxels for visualising purposes. Voxel size is 78 µm. (**b**) Site-specific settlement location of a mussel in x-plane, (**c**) y-plane, and (**d**) z-plane. Arrows indicate a mussel, which is clearly distinguishable by the oval shape with a hollow centre, which characterises the two shell valves. Tomograms with 1024^3 voxels were generated and used for analysis with a voxel size of approximately 39 µm.

4.2.4 Statistical analysis

Data were analysed by one- or two-factor permutational multivariate analysis of variance (PERMANOVA). The Bray-Curtis dissimilarity measure was used for all PERMANOVAs and *p*-values were calculated using unrestricted permutation of untransformed raw data and permutation of residuals under a reduced model with 9999 random permutations for one- and two-factor PERMANOVAs, respectively. If there was a significant difference, pair-wise *a*

posteriori comparisons were made among the significant groups using the Bray-Curtis similarity measure (α = 0.05). Statistical analyses were performed using PRIMER 6 (v. 6.1.13) and PERMANOVA+ (v. 1.0.3) (Clarke and Gorley 2006). Data are reported as mean ± 1 standard error (SE).

The effect of surface complexity on the settlement of *M. galloprovincialis* in no choice and choice assays were considered fixed factors. Beaker was included as a random factor for choice assays as test surfaces in one beaker were not independent. There was no interaction term (surface complexity × beaker) as surfaces were not replicated in each beaker. To test for differences in settlement of mussels on the outside of ropes under hatchery conditions over time (in small-scale nursery tanks and individual containers), time was treated as a fixed factor and tank was included as a random factor. There was no interaction term. To assess differences in the depth of settlement of mussels within ropes using µCT, PERMANOVAs were performed for each sampling point (days post settlement) with size class as a fixed factor and tank as a random factor. There was no interaction term.

4.3 Results

4.3.1 Laboratory settlement assays

4.3.1.1 No choice assays

For all no choice assays, increasing complexity of the test surfaces significantly enhanced the settlement of *M. galloprovincialis*, regardless of age (Figure 4.2a-c). Settlement was consistently the highest on rope samples, the most complex surface tested.

For no choice assays with pediveligers (22 days post fertilisation), settlement significantly differed among all tested surfaces (one-factor PERMANOVA: $F_{(9,27)}$ = 26.23, p < 0.001; Figure 4.2a). The lowest number of pediveligers settled on smooth polypropylene (0.8 ± 0.2%) and settlement increased with increasing surface complexity to more than 50% on rope. Consequently, less than 44% of pediveligers were unattached and suspended in the water column after 48 h in no choice assays with rope, while more than 88% of pediveligers were in the water column in assays with smooth and textured polypropylene. Overall, the number of individuals settled on beakers ranged from 0.7 ± 0.3% to 6.0 ± 1.5% pediveligers.

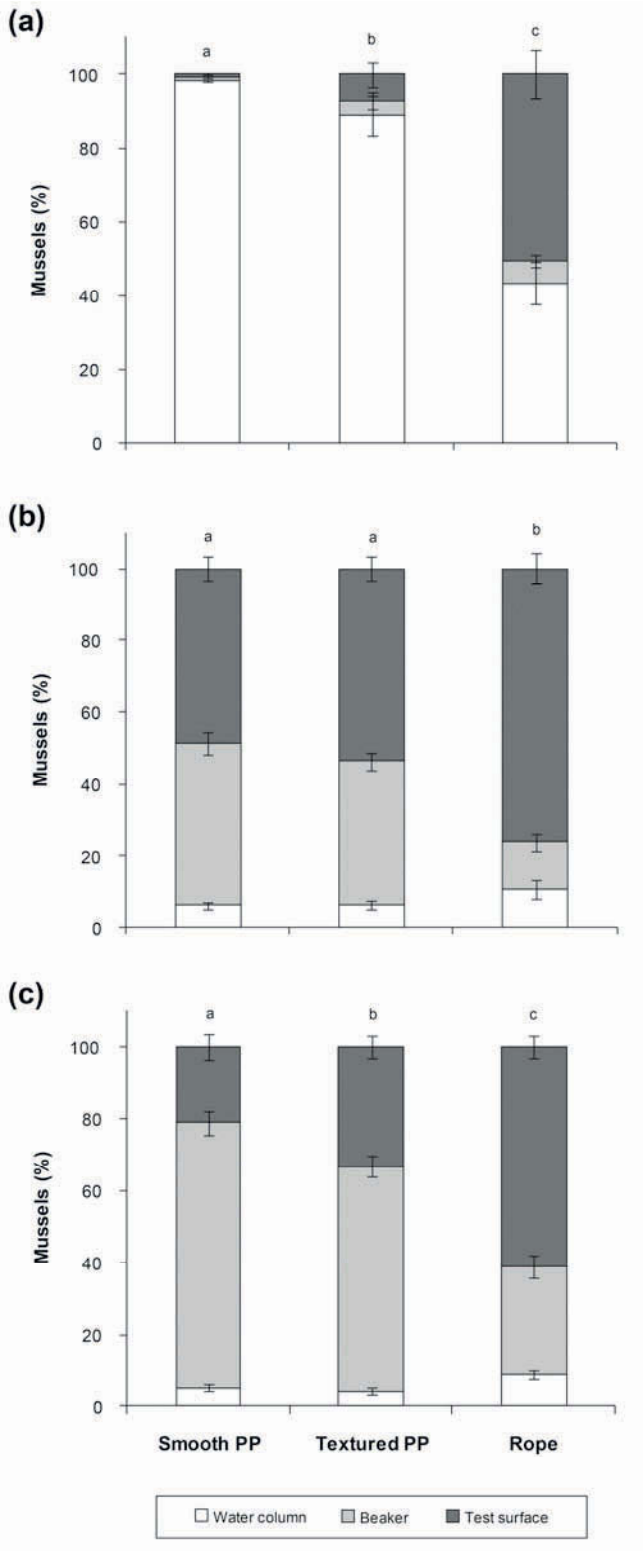

Figure 4.2: Number of mussels (%) settled on test surfaces (smooth polypropylene (PP), textured PP, rope) and glass beaker and suspended in the water column after 48 h in no choice assays. Settlement assays were conducted with mussels (**a**) 22 days, (**b**) 30 days, and (**c**) 38 days post fertilisation. Means ± SE are shown (n = 10). Superscript letters indicate significant differences between test surfaces (pair-wise *a posterior* test; $\alpha = 0.05$).

Similarly, surface complexity significantly enhanced the settlement of 30 day old plantigrades ($F_{(9,27)}$ = 12.00, p < 0.001; Figure 4.2b). Significantly fewer plantigrades settled on smooth (48.6 ± 3.4%) and textured polypropylene (53.6 ± 3.2%) than on rope samples (76.0 ± 4.3). The number of mussels suspended in the water column also decreased with increasing age and ranged from 6.2 ± 1.0% to 10.7 ± 2.6% for 30 day old plantigrades. The lowest settlement of plantigrades on beakers occurred in assays with rope samples (13.4 ± 2.4), while the highest number of plantigrades settled on beakers in assays with smooth polypropylene (45.2 ± 3.3%).

Choice assays conducted with 38 day old plantigrades were consistent with assays conducted 22 and 30 days post fertilisation, with a significant difference in the settlement of individuals between all surfaces ($F_{(9,27)}$ = 16.57, p < 0.001; Figure 4.2c). Plantigrades had significantly lower settlement on smooth polypropylene (21.1 ± 3.5%) than on textured polypropylene (33.3 ± 3.2%) or rope (60.9 ± 2.9%). Overall, the number of unattached plantigrades was below 9% in all assays and the settlement of mussels on beakers was higher than on the test surfaces with the exception of rope. Between 62.4 ± 2.8% and 73.9 ± 3.3% plantigrades settled on beakers in assays with textured and smooth polypropylene, respectively. In contrast, 30.1 ± 3.1% of plantigrades settled on beakers in assays with rope.

4.3.1.2 Choice assays

For all choice assays, significantly higher numbers of mussels, regardless of age, settled on rope samples, whereas there was no significant difference between smooth and textured polypropylene (Figure 4.3a-c). Broadly speaking, the choice assay provided similar results to the no choice assay with higher settlement on material with higher surface complexity.

For choice assays with pediveligers (22 days post fertilisation), surface complexity significantly enhanced the settlement of *M. galloprovincialis* (one-factor PERMANOVA: $F_{(2,18)}$ = 61.40, p < 0.001; Figure 4.3a). Less than 1% of pediveligers settled on smooth or textured polypropylene, while 48.1 ± 8.2% pediveligers settled on the rope. Approximately 50% of pediveligers were suspended in the water column after 48 h, whereas only 2.4 ± 0.4% pediveligers settled on the beaker.

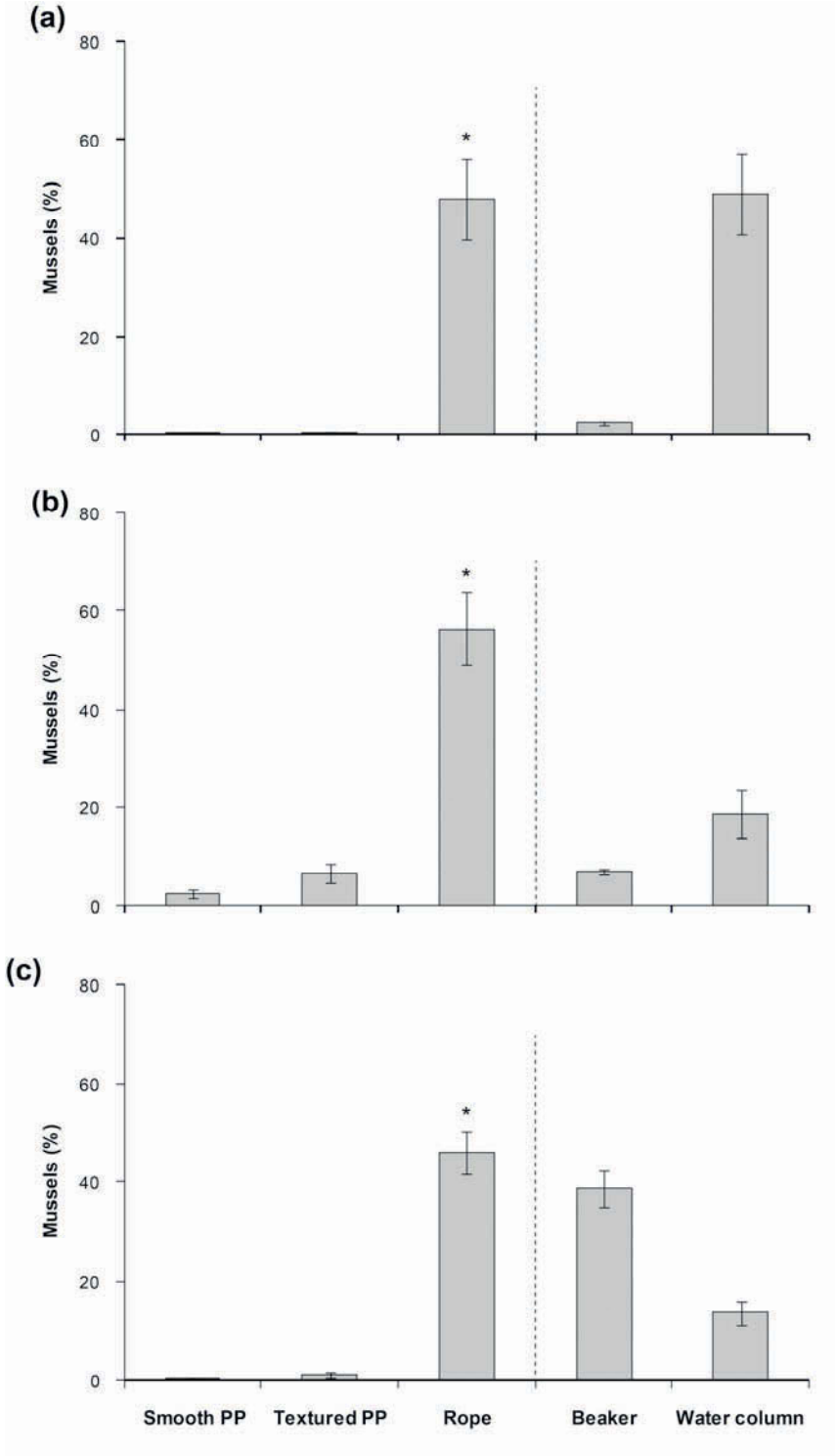

Figure 4.3: Settlement choice of mussels (%) on test surfaces (smooth polypropylene (PP), textured PP, rope) and glass beaker and suspended in the water column after 48 h in choice assays. Settlement assays were conducted with mussels (**a**) 22 days, (**b**) 30 days, and (**c**) 38 days post fertilisation. Means ± SE are shown (n = 10). Statistical analyses were only performed for test surfaces (to the left of the dotted line). Asterisk indicates significant differences between test surfaces (pair-wise *a posterior* test; α = 0.05).

In a similar manner to settlement preferences of pediveligers, surface complexity significantly influenced the settlement choice of 30 day old plantigrades ($F_{(2,18)}$ = 17.90, p < 0.001; Figure 4.3b). The settlement of plantigrades was low on smooth (2.4 ± 0.9%) and textured polypropylene (6.8 ± 2.0%), while the majority of plantigrades settled on the rope (56.4 ± 7.4%). More mussels were suspended in the water column (18.7 ± 4.8%) than attached to the glass beaker (7.0 ± 0.4%).

Finally, surface complexity had a consistent and positive effect on the settlement choice of older plantigrades (38 days post fertilisation) ($F_{(2,18)}$ = 54.17, p < 0.001; Figure 4.3c). The lowest number of plantigrades settled on smooth polypropylene (0.4 ± 0.2%) and settlement increased with increasing complexity to 46.1 ± 4.3% on the rope. Overall, fewer mussels were suspended in the water column (13.6 ± 2.4%) in comparison to the number of mussels settled on the glass beaker (38.7 ± 3.5%).

4.3.2 Site-specific settlement onto ropes under hatchery conditions

4.3.2.1 Rope samples in small-scale nursery tanks

The number of settled mussels appearing on the outside of ropes that were maintained in continuous culture in small-scale outdoor nursery tanks increased significantly with time (two-factor PERMANOVA: $F_{(4,30)}$ = 17.45, p < 0.001; Figure 4.4, solid line). The increase in settlement was approximately eight fold from one day post settlement (2.2 ± 0.6 individuals) to 17 days post settlement (17.9 ± 3.0 individuals), with the largest increase between days 1 and 5 post settlement (9.6 ± 1.8 individuals). This effect was consistent between ropes excised from culture in outdoor small-scale nursery tanks (these data) and those assayed in individual containers (Figure 4.4, dashed line, see Section 4.3.2.2).

Size measurements of mussels one day post settlement (23 days post fertilisation) could not be performed as removal off the rope using tweezers was destructive for their soft shells. However, the average length of mussels collected on the settlement day (22 days post fertilisation) was measured as pediveligers suspended in the water column could be pipetted and their mean shell length was 302.8 ± 4.3 µm. After 5 days post settlement, mussels reached a mean length of 374.2 ± 8.1 µm and their length increased with age to 540.9 ± 12.7 µm, 723.4 ± 11.6 µm, and 989.8 ± 18.7 µm for 9, 13, and 17 days post settlement, respectively.

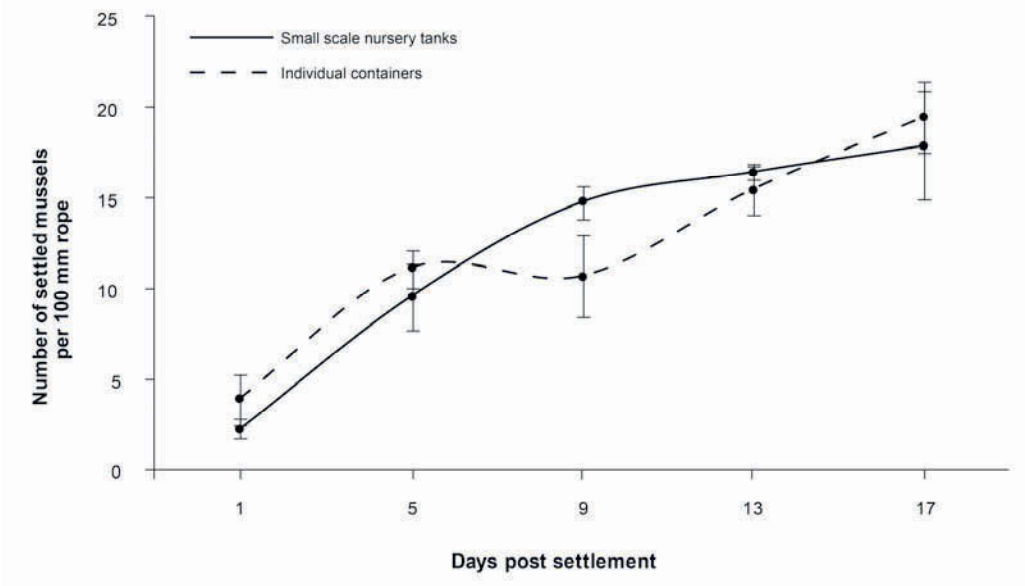

Figure 4.4: Mean number of settled mussels on the outside of 100 mm long rope samples maintained in small-scale nursery tanks (solid line) and individual containers (dashed line) over time (1 to 17 days post settlement, which correspond to 23 to 39 days post fertilisation). Means ± SE are shown (n = 3).

4.3.2.2 Rope samples in individual containers

In general, rope samples maintained individually in containers showed a similar result to settlement of mussels on ropes maintained in small-scale nursery tanks (Figure 4.4, solid line, see Section 4.3.2.1). Assays of ropes in individual containers further ensured no settlement on the ropes, confirming that all subsequent mussels were a product of the initial settlement of pediveligers at 23 days post fertilisation. It also ensured no migration between ropes occurred. The number of settled mussels on the outside of ropes increased with time (Figure 4.4, dashed line) and the time period had a significant effect on the settlement of individuals (two-factor PERMANOVA: $F_{(4,30)}$ = 7.03, p < 0.001). However, the origin (tank) of the rope sub-samples also had a significant effect on the settlement of *M. galloprovincialis* ($F_{(2,30)}$ = 2.47, p = 0.039). The lowest number of mussels settled on the outside of ropes one day post settlement (3.9 ± 1.4 individuals) and increased nearly three-fold within four days (5 days post settlement: 11.1 ± 1.1 individuals). After 9 days post settlement, the number of settled mussels on the outside of rope decreased slightly (10.7 ± 2.3 individuals). Subsequently, the settlement of mussels increased consistently over time, with higher settlement after 13 days (15.4 ± 1.4 individuals) and 17 days post settlement (19.4 ± 2.0 individuals).

The mean lengths of mussels maintained in individual containers were similar to individuals grown in small-scale nursery tanks, with 531.9 ± 14.9 µm, 714.0 ± 14.2 µm, and 946.0 ± 15.9 µm for 9, 13, and 17 days post settlement, respectively.

4.3.2.3 Imaging settlement locations with X-ray micro-computed tomography

Pediveligers of *M. galloprovincialis* initially settled inside of ropes and smaller size classes continued to move deeper inside the ropes over time (Table 4.1). Notably, there is a clear migration of mussels from within the interior to the exterior of the rope as they increase in size over time (Figure 4.5a-e). Furthermore, not all mussels grew rapidly and more than 30% of mussels ≤ 349 µm were still represented in samples 17 days post settlement.

At one day post settlement, all mussels ranged in size between 250-349 µm and settled inside of ropes with a mean distance to the exterior of 1308.5 ± 165.1 µm (Figure 4.5a; Table 4.1). Within the following 4 days, mussels moved deeper inside of ropes. Smaller mussels (250-349 µm) settled deeper within ropes (1949.3 ± 158.4 µm) than larger mussels (1715.2 ± 194.3 µm), although the difference was not significant (two-factor PERMANOVA: $F_{(1,84)} = 1.66$, $p = 0.288$). The majority of individuals (70%) had shell lengths ≥ 350 µm (Figure 4.5b, Table 4.1).

As the size of mussels increased 9 days post settlement (Figure 4.5c; Table 4.1), the settlement depth within ropes decreased for larger mussels (≥ 450 µm) by approximately half in comparison to smaller mussels (≤ 449 µm). Individuals ≤ 449 µm had a mean location of 1588.0 ± 226.1 µm from the exterior, while individuals in the size classes of 450-549 µm and 550-649 µm moved outwards with a mean location from the exterior of 710.3 ± 250.2 µm and 847.0 ± 82.2 µm, respectively. The largest mussels found after 9 days post settlement had shell length ≥ 650 µm and settled on the outside of the rope samples (0 µm distance). Statistical analysis revealed no significant effect of the size of mussels on their settlement sites at 9 days post settlement ($F_{(4,70)} = 3.41$, $p = 0.059$).

In contrast, the size of mussels significantly affected the depth of location of mussels within the rope at 13 days post settlement ($F_{(5,107)} = 4.31$, $p = 0.015$), as well as tank from which the rope samples were collected ($F_{(2,107)} = 3.31$, $p = 0.005$). As individuals grew larger, mussels moved further towards the exterior of ropes (Figure 4.5d; Table 4.1). Mussels of the largest size class (750-849 µm) were located significantly closer to the outside of the rope with a mean distance

to the exterior of 469.1 ± 261.2 µm, compared to smaller mussels in the size classes of 350-449 µm (t = 2.64, p = 0.008) and 450-549 µm (t = 2.45, p = 0.039), which were 1442.3 ± 90.9 µm and 1489.0 ± 149.3 µm from the exterior, respectively. Mussels ≤ 349 µm had the deepest location within ropes with mean distance of 2072.7 ± 245.7 µm from the exterior of ropes.

The largest variation in size classes was observed for rope samples 17 days post settlement (Figure 4.5e; Table 4.1). The site-specific settlement within ropes was significantly affected by the size of mussels ($F_{(7,47)}$ = 2.34, p = 0.041) and generally, the location of mussels within ropes (distance from exterior) decreased with increasing shell length. Small mussels (250-349 µm) settled approximately six-fold deeper within ropes than mussels ≥ 950 µm (t = 5.67, p = 0.016) at a depth of 2770.8 ± 108.5 µm from the exterior compared to 463.5 ± 146.9 µm. Overall, more than 30% of all mussels had shell lengths ≤ 349 µm at 17 days post settlement and doubled their distance to the exterior in comparison to their location one day post settlement.

Table 4.1: Mean distance (µm) of settlement locations of individual mussels to the exterior of ropes after 1, 5, 9, 13, and 17 days post settlement. Mussels are grouped in eight size classes (250-349, 350-449, 450-549, 550-649, 650-749, 750-849, 850-949, and > 950 µm in shell length) and the number of individual mussels in each size class is shown. Measurements are based on tomograms obtained using X-ray µCT of three individual rope pieces collected at each time point.

	1 day post settlement		**5 days post settlement**		**9 days post settlement**		**13 days post settlement**		**17 days post settlement**	
Mussel size class (µm)	Distance (µm) ± SE	№ of individuals	Distance (µm) ± SE	№ of individuals	Distance (µm) ± SE	№ of individuals	Distance (µm) ± SE	№ of individuals	Distance (µm) ± SE	№ of individuals
250-349	1308.5 ± 165.1	(39)	1949.3 ± 158.4	(27)	1588.0 ± 226.1	(13)	2072.7 ± 245.7	(35)	2770.8 ± 108.5	(22)
350-449			1715.2 ± 194.3	(63)	1797.6 ± 59.1	(22)	1442.3 ± 90.9	(18)	1983.0 ± 874.6	(8)
450-549					710.3 ± 250.2	(30)	1489.0 ± 149.3	(17)	1668.8 ± 821.6	(5)
550-649					847.0 ± 82.2	(16)	1648.9 ± 438.8	(22)	1747.7 ± 1467.3	(2)
650-749					0.0 ± 0.0	(2)	666.5 ± 347.2	(17)	1747.7 ± 1467.3	(2)
750-849							469.1 ± 261.2	(16)	1094.5 ± 579.2	(4)
850-949									754.6 ± 201.1	(12)
> 950									463.5 ± 146.9	(9)
Total number of individuals		(39)		(90)		(83)		(125)		(64)

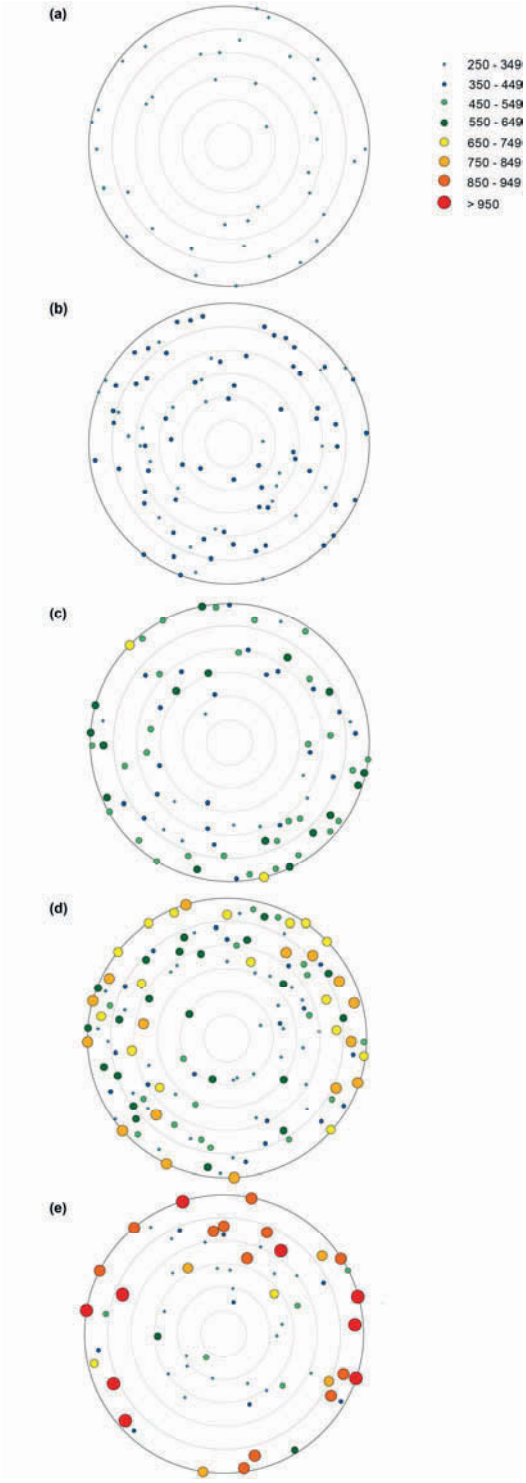

Figure 4.5: Schematic diagram to illustrate site specific settlement locations of individual mussels on and within ropes (**a**) 1 day, (**b**) 5 days, (**c**) 9 days, (**d**) 13 days, and (**e**) 17 days post settlement. The size classes of mussels (250-349, 350-449, 450-549, 550-649, 650-749, 750-849, 850-949, and > 950 µm in shell length) are indicated by different colours. The depth of settlement was identified using X-ray µCT and each schematic summarises the overall settlement on all three analysed ropes, therefore representing an overall rope length of approximately 105 mm.

4.4 Discussion

M. galloprovincialis has clear settlement preferences for more complex materials and high selectivity of settlement sites relative to the size of the individual, from the pediveliger stage through to the plantigrade stage (post metamorphosis). This is the first study to map the three-dimensional settlement preferences of mussels on culture rope and subsequently map their movement as individuals grow larger within complex materials, under commercial hatchery operating conditions. Mussels initially settle inside ropes and move outwards as they increase in size. In contrast, smaller individuals that have not grown and remain within the smallest size classes move deeper inside the ropes over time.

In general, mussels actively select favourable sites and substrata (Petersen 1984; Cáceres-Martínez et al. 1994; Alfaro and Jeffs 2002; Filgueira et al. 2007; Brenner and Buck 2010). The pediveliger larvae of mussels are highly selective in their preference of settlement surfaces as demonstrated in no choice assays. While approximately 50% of pediveligers settled on braided polypropylene rope within 48 h, less than 8% settled on textured polypropylene, and less than 1% on smooth polypropylene with no choice given between the test surfaces. Pediveligers of *Mytilus* have the ability to delay metamorphosis for several weeks (Bayne 1965), which facilitates high selectivity of settlement substratum. The absence of favourable substrata, such as fine-branching and filamentous materials (Buchanan and Babcock 1997; Alfaro and Jeffs 2002), prolongs the larval stage and delays metamorphosis (Lutz and Kennish 1992). This is reflected in the results of the no choice assay where the number of planktonic larvae (not attached) was high for textured and smooth polypropylene, with very low settlement on the latter. This implies that these two surfaces are unfavourable for settlement of plantigrades, although no alternative choice is given and topographic features were present (textured polypropylene). Topographic features are an important settlement cue for pediveligers (Gribben et al. 2011; see Chapter 3, Section 3.3.2). However, topography itself is not an exclusive key driver for larval settlement and the level of complexity of the surface plays a key role for settlement as demonstrated in this study.

When given a choice between the test surfaces, the settlement of *M. galloprovincialis* was always significantly higher on rope samples than on smooth and textured polypropylene, regardless of age. Pediveligers (22 days post fertilisation) showed the same preference for the most complex surface tested (rope) as plantigrades (30 and 38 days post fertilisation), which underlines the ability to differentiate between favourable and unfavourable surfaces (Bayne 1976) and to make an active settlement choice (Buchanan and Babcock 1997). There was no

change in surface preference with increasing size and ontogenetic stage. This may reflect the range of complexity and size within the rope structure, as opposed to a single texture feature.

In general, textured and complex surfaces provide an attractive substratum for settlement by offering an increased surface area and protection (Hodson et al. 2000; Filgueira et al. 2007; Koehl 2007), which in turn reduces post-settlement loss and mortality (Filgueira et al. 2007). This is especially important for pediveligers undergoing metamorphosis, as they do not feed and rely upon stored nutrients and energy while the adult gill/palp feeding mechanisms are developed (Bayne 1976). Imaging of specific settlement sites using µCT shows pediveligers initially settle inside ropes, which offers protection during this vulnerable stage. Settlement depth within the ropes increased to 5 days post settlement and decreased afterwards as mussels grew larger, showing an initial inwards, and subsequent outwards movement. This is in accordance with the observed time period needed for pediveligers to complete metamorphosis. In general, pediveligers of *Mytilus* metamorphose within 1 to 3 days after the first secretion of byssal threads depending on temperature (Bayne 1976). A sharp increase in food consumption was noticeable 4 days post settlement in this study, demonstrating that the majority of mussels completed metamorphosis within this time period and had begun active feeding. While the inside of ropes offers protection for pediveligers, the food availability and space may become limiting factors post-metamorphosis as the size of individuals increase. Consequently, mussels moved outwards as their shell lengths increased as quantified using optical microscopy and µCT imaging.

While optical microscopy can determine the number of settled mussels on the outside, µCT completes the picture and visualises each individual on the exterior and within ropes. As a result, µCT showed a high proportion of small mussels within the ropes over time, even at 17 days post settlement, which could not be determined using microscopy. In contrast to mussels with increasing shell length, mussels not growing and remaining within their size class moved deeper inside the rope over time. Mussels $\leq$ 349 µm doubled their penetration depth within 16 days, whereas growing mussels moved increasingly to the exterior. While µCT cannot determine whether visualised individuals are alive, the movement of the mean distance from the exterior towards the interior strongly supports that pediveligers that have not metamorphosed remained alive and mobile up to the completion of the study at 17 days post settlement.

In conclusion, more complex surfaces are preferred settlement sites with complexity being a key factor in the active selection of sites for settlement, metamorphosis and subsequent movement. Pediveligers and metamorphosed plantigrades preferentially settled onto ropes,

which represent the most complex tested surface. Furthermore, rope offers protection from hydrodynamic forces and predation. Small mussels initially settle within ropes (> 1000 µm deep) and move further inwards if they remain within the smallest size classes. In contrast, mussels with increasing size move rapidly towards the exterior. Overall, this Chapter identifies a key driver for settlement selection to support the development of new technologies to manage the settlement and retention of mussels for the aquaculture industry.

Chapter 5

Enhancing the efficacy of fouling-release coatings against fouling by *Mytilus galloprovincialis* using nanofillers [4]

5.1 Introduction

The negative economic impacts of biofouling are well documented (reviewed by Braithwaite and McEvoy 2005; Edyvean 2010; Flemming 2010) and affect all marine industries, including finfish aquaculture (de Nys and Guenther 2009; Fitridge et al. 2012), offshore and deep-sea structures used in the oil and gas industry (Apolinario and Coutinho 2009; Page et al. 2010), power stations (Henderson 2010), desalination plants (Henderson 2010), and shipping (Townsin 2003; Schultz et al. 2011). Mussels, in particular, are highly successful fouling organisms (Briand 2009) that incur high management and control costs (Page et al. 2010). Their success as foulers is due to their wide physiological tolerances, ability to withstand air exposure, high fecundity, and their ability to settle on a wide variety of substrata in high densities (Gosling 2003). The blue mussel *Mytilus galloprovincialis* is a particularly important fouling species with high recruitment rates and the ability to outcompete other species (Erlandsson et al. 2006).

Past and present antifouling technologies mostly rely on metal-based toxic coatings that kill the settling propagules of fouling organisms (Finnie and Williams 2010). However, the development of non-biocidal technologies is gaining increasing attention, with surface modification for fouling-release being the most promising antifouling alternative to toxic biocides (Vladkova 2010; Webster and Chisholm 2010). The concept of reducing the adhesion of biofouling using fouling-release coatings has been commercially applied since 1987 and has dramatically influenced the design of antifouling technologies (Townsin and Anderson 2009). Current fouling-release coatings are based on materials with low surface energies including silicone elastomers, fluoropolymers, flourosilicones and hybrids of siloxanes with other

[4] Chapter 5 is adapted from Carl C, Poole AJ, Vucko MJ, Williams MR, Whalan S, de Nys R. 2012. Enhancing the efficacy of fouling-release coatings against fouling by *Mytilus galloprovincialis* using nanofillers. Biofouling 28:1077-1091.

materials (Anderson et al. 2003; Webster and Chisholm 2010). As initial settlement is not prevented, hydrodynamic shear forces are required to remove attached organisms (Finnie and Williams 2010). Therefore, fouling-release coating systems are ineffective in environments with low shear forces, including slow moving or moored vessels, as well as aquaculture and oil and gas assets. There are many ways to improve these coatings and notably, a broad range of technologies can be incorporated to enhance fouling-release coatings (Scardino and de Nys 2011).

Polydimethylsiloxane (PDMS) is a model fouling-release material well suited for investigating surface modifications to either increase or decrease settlement and enhance the detachment (release) of fouling organisms (Carman et al. 2006; Schumacher et al. 2007a, 2007b; Long et al. 2010a; Martinelli et al. 2011). Surface microtopography features on PDMS matrices (Callow et al. 2002; Carman et al. 2006) have been shown to either enhance (see Chapter 3, Section 3.3.2) or reduce (Aldred et al. 2010a) the settlement and attachment strength of sessile organisms. In general, surface microtopographies slightly smaller than the size of the settling organism result in a reduced contact area available for adhesion and attachment (Berntsson et al. 2000; Callow et al. 2002; Scardino et al. 2006, 2008). As a consequence, the settling organism requires more energy to settle (Callow et al. 2002) and the number of attachment points is reduced (Scardino et al. 2006), resulting in reduced adhesion strength and enhanced fouling release properties (Aldred et al. 2010a; see Chapter 3, Section 3.3.2.2).

A further approach to enhance the release characteristics of these coatings is through incorporating nanofillers, which are fillers with at least one of its dimensions in the nanometer range, to reinforce polymers and manipulate mechanical properties (Sadhu and Bhowmick 2008). Titanium dioxide (TiO_2) is an interesting filler in that it has a photo-induced hydrophilic effect resulting in hydrophobic surfaces containing TiO_2 becoming hydrophilic under UV exposure (Fujishima et al. 2008). The most interesting aspect, however, is that TiO_2 is a photocatalyst that has the potential to improve antifouling properties against a broad range of fouling organisms due to its photocatalytic effect. TiO_2 is used to decompose organic compounds and its photocatalytic effect has been applied for the destruction of bacteria, viruses and cancer cells (Ho et al. 2003; Hajkova et al. 2007; Lee et al. 2010).

Notably, photoreactions are limited to the surface of the photoexcited photocatalyst (TiO_2) and therefore only molecules that are in direct contact with the catalyst surface undergo photocatalytic degradation (Gaya and Abdullah 2008). The photocatalytic effect is isolated to the surface due to a high reaction rate constant of the formed hydroxyl radicals (10^6 – 10^{11} $dm^3\,mol^{-1}\,s^{-1}$; Bhatkhande et al. 2002). This restricts the photo-killing zone to the near-

surface thereby potentially enhancing the antifouling attributes of non-biocidal fouling-release coatings whilst minimising effects on non-target organisms. However, there has been no effort to deconstruct the effect of TiO_2 on the settlement of key fouling organisms other than bacteria and diatoms, where materials were successful in eliminating organisms from surfaces (Ho et al. 2003; Kemmitt et al. 2012). Finally, the incorporation of TiO_2 also improves a wide range of mechanical properties of elastomers, including tensile strength, elastic modulus, hardness, abrasion resistance and energy to break (Mirabedini et al. 2008).

Carbon nanotubes (CNTs) are an alternative nanofiller to improve fouling-release properties. CNTs are cylindrical nanostructures formed by covalently bonded carbon atoms arranged in a hexagonal array (Thorstenson et al. 2001). The incorporation of CNTs affects the surface topography of the base material, as well as its wettability when immersed in water, and as a result influences the detachment of fouling organisms (Beigbeder et al. 2009). The fouling-release performance of coatings depends on the homogeneous dispersion of CNTs in the polymeric matrix and the interaction between both materials (Beigbeder et al. 2010). Furthermore, the concentration of CNTs is critical to the enhancement of fouling-release properties (Beigbeder et al. 2008; Beigbeder et al. 2010). Another important property of CNTs is the ability to mechanically reinforce silicone elastomers when dispersed in the polymeric matrix (Frogley et al. 2003; Paul et al. 2006; Tjong 2006; Ci et al. 2008).

The major objective of this Chapter was therefore to improve technologies to control the settlement of the important fouling organism *M. galloprovincialis* by incorporating the nanofillers TiO_2 and CNTs in PDMS, and also improve the efficacy of PDMS fouling-release coatings by minimising bio-adhesion. To determine the efficacy of nanofilled (TiO_2 and CNTs) PDMS, the settlement and adhesion strength of pediveligers and plantigrades of *M. galloprovincialis* was quantified. These two ontogenic stages represent primary and secondary settlers with different settlement preferences (Buchanan and Babcock 1997; Alfaro and Jeffs 2002). Firstly, PDMS matrices filled with TiO_2 in the range 3.75 – 15 weight percent (wt%), combined with microtexture with groove widths of 200, 300 and 600 µm, were assayed for effects on settlement and adhesion strength. Secondly, CNTs were incorporated into PDMS matrices in the range 0.2 – 6 wt% with the objective of reducing settlement and adhesion strength. In addition, the effect of these incorporated nanofillers on the surface and mechanical properties of the PDMS base material was quantified.

5.2 Material and Methods

5.2.1 Culture of pediveligers and plantigrades

Larvae and plantigrades of the mussel *M. galloprovincialis* were supplied by the Victorian Shellfish Hatchery in Queenscliff, Victoria, Australia. Spawning and larval production are described in detail in Pettersen et al. (2010). Veliger larvae (21 days after fertilisation) and plantigrades (57 days after fertilisation) were shipped within 14 h to James Cook University, Townsville, Queensland, Australia. Larvae and plantigrades were reared in a laboratory culture as described in detail in Chapter 2 (Section 2.2.1 and 2.2.2). Mussel larvae were maintained until the pediveliger stage was reached and competent to settle. Pediveligers were then pooled and used in settlement assays. Plantigrades were maintained for 9-15 days in the laboratory prior to use in settlement assays.

5.2.2 Laboratory experiments

5.2.2.1 TiO_2 in textured PDMS

5.2.2.1.1 Production of surfaces. PDMS (Sylgard 184; Dow Corning, USA) was used as a silicone elastomer to produce the micro-structured test surfaces with TiO_2 (Degussa TiO_2 P25) filler. PDMS was prepared using a 10:1 ratio of base elastomer (part A) to curing agent (part B). The nanofiller TiO_2 (a mixture of 79.5% anatase and 20.5% rutile) with an average primary particle size of 21 nm (specified by the manufacturer) was incorporated into the PDMS matrix at concentrations of 0 (blank control), 3.75, 7.5, 11.25 and 15 wt%. The two components were mixed thoroughly and degassed in a vacuum desiccator to remove bubbles. Casting of the blank and TiO_2-filled PDMS followed the production procedure described in Chapter 3 (Section 3.2.2.2.1). Smooth PDMS and microtextures with groove widths of 200, 300, and 600 µm were generated. Microtextured surfaces had a linear grating of approximately square wave profile and a mark-spacing ratio of 1 (groove width = land area width).

5.2.2.1.2 Characterisation of surfaces. To determine the effect of the nanofiller on surface topography, the smooth nanocomposites were coated with platinum-palladium (Pt-Pd; Cressington 208HR Sputter Coater, Cressington Scientific Instruments Ltd., UK) and images of the test surfaces were taken at random points using scanning electron microscopy (SEM; Hitachi S-4300 SE/N) at up to 3000× magnification. The distribution of TiO_2 particles on the

surfaces and throughout the PDMS matrices was examined using backscattered SEM. To quantify the effect of incorporated TiO_2 on the properties of PDMS, the hardness and contact angles of the smooth nanocomposites were measured. Hardness was quantified using a Shore Durometer (Durometer 1600 Shore OO; Shore Instrument & Mfg Co. Inc., USA) at random locations (n = 6). Contact angles θ were measured with a goniometer (First Ten Ångstroms, FTÅ200, USA) using the sessile drop method with a 2 µL Milli-Q water droplet described in Chapter 3 (Section 3.2.2.1.2). Contact angle measurements were carried out at different locations on each sample (n = 5) before and after the exposure to UV within 5 min after the removal from UV light. The test surfaces were placed in a QUV Accelerated Weathering Tester unit for 6 h (Q Panel Lab Products, USA) and a UV Irradiance Controller was used to control the UV intensity (UV A and B intensity: 1.5 mW cm^{-2}).

5.2.2.1.3 Settlement assays. To determine the effect of TiO_2 and microtopography on the settlement and attachment strength of pediveligers and plantigrades of *M. galloprovincialis*, TiO_2 was incorporated into PDMS as described above. The blank control and each of the TiO_2-filled PDMS materials were cast in a linear grating of square wave profile with features widths of 200, 300, and 600 µm and a smooth cast without any surface topography (n = 12 for pediveligers and plantigrades). Circles (25 mm diameter) were cut from the PDMS surfaces using a hole punch, washed with a detergent solution (Decon 90; Decon Laboratories Ltd.), rinsed with distilled water and air dried before use in assays. Circles were randomly placed in covered 6-well plates (IWAKI 3810-006) to prevent evaporation and for easier handling. For static drop settlement assays with pediveligers, approximately 20 individuals were pipetted in a 800 µL drop of filtered seawater (FSW; 0.2 µm filtered and UV sterilised) onto the test surfaces. Due to the larger size of plantigrades, only 5 individuals were used in each drop assay. To test for the effect of photocatalytic TiO_2, half of the replicates of each test surface (n = 6 for pediveligers and plantigrades) were placed in a temperature controlled culture cabinet (Sanyo MLR-351) at 18°C without light. The other half of the replicates of each test surface (n = 6 for pediveligers and plantigrades) were placed in a temperature controlled room at 18°C under a UV light bank (UV A and B intensity: 1.5 mW cm^{-2}, which equals less than ambient light in the tropics) in a 12 h light:12 h dark cycle. The light bank consisted of 12 Cosmedico 15508 180W RA plus Cosmolux lights (280-400 nm) and 11 Phillips Master TL5 HO 49W/865 lights (400-700 nm). To minimise temperature fluctuations, the 6-well plates holding the test surfaces were placed in a circulating water bath (18°C) and a fan was used to enhance air circulation under the UV lights. The settlement of pediveligers and plantigrades was determined after 48 h and 6 h, respectively. Pediveligers and plantigrades were considered settled when they attached to the surface with byssal threads.

5.2.2.1.4 Adhesion strength. To determine the adhesion strength of settled pediveligers and plantigrades of *M. galloprovincialis* on each surface, a flow chamber was used as described in Chapter 3 (Section 3.2.2.2.6). The test surfaces were carefully removed from the 6-well plates using forceps and then randomly placed in polyvinylchloride (PVC) plates. Each PVC plate had three machined holes side by side, where the test plugs fitted precisely. To secure the test surfaces to the plate, the test plugs were fixed with cloth tape (Scotch® Colored Duct Tape 330BLU-DC-NA) from the bottom. One PVC plate at a time was placed in the flow chamber with test surface grooves parallel to the flow and secured in the raceway using customised fittings. A small PVC ramp was placed in front of the plate holding the test surfaces to ensure an even flow over the samples. The test surfaces were exposed to 4 knots (equal to 2.06 m s^{-1}) for 2 min after which the number of remaining mussels was counted.

5.2.2.2 CNTs in PDMS

5.2.2.2.1 Production of surfaces. PDMS (Sylgard 184; Dow Corning, USA) was used as the silicone elastomer to produce the test surfaces and was prepared using a 10:1 ratio of base elastomer (part A) to curing agent (part B). Multi-walled CNTs (S-WNT-60100) with an outer diameter of 60-100 nm, length of 5-15 µm and a purity of > 98%, were purchased from Shenzhen Nanotech Port Co. Ltd (China). To mix the CNTs into the PDMS matrix at concentrations of 0.2, 0.5, 1, 2, 4, and 6 wt%, CNTs were dispersed in isopropyl alcohol and then placed in an ultrasonic system (Branson 3200 ultrasonic bath and Branson Sonifier B30; Branson Instruments, Danbury, USA) and sonicated for 5 h. Once the CNTs were fully dispersed, the base elastomer (part A) was added, mechanically mixed, and the isopropyl alcohol then allowed to evaporate under constant air flow with complete removal confirmed by constant weight measurements. After the isopropyl alcohol was evaporated, the curing agent (part B) was added and stirred in thoroughly. The mixture was de-gassed in a vacuum desiccator to remove bubbles before it was poured into stainless steel square moulds on a polypropylene surface.

5.2.2.2.2 Characterisation of surfaces. To determine the alignment of CNTs on the surface and their effect on surface topography, the nanocomposites were Pt-Pd coated and images were taken at random points using SEM (Hitachi S-4300 SE/N) up to 3000× magnification. The distribution and orientation of CNTs throughout PDMS matrices was examined by cutting cross-sections of the samples prior to SEM. To determine the effect of incorporated CNTs on

the properties of PDMS, the hardness and contact angles were quantified as described above (see Section 5.2.2.1.2).

5.2.2.2.3 Settlement assays. To quantify the effect of CNTs incorporated into PDMS on the settlement and attachment strength of pediveligers and plantigrades of *M. galloprovincialis*, CNTs were dispersed into a PDMS matrix as described. Blank PDMS was used as a control and circles (35 mm diameter) were cut from all test surfaces using a hole punch. There were six replicates for each surface for each ontogenetic stage and consequently 42 test surfaces in total for assays with pediveligers and plantigrades. Test surfaces were randomly placed in PVC plates as previously described. This minimised handling of the surfaces between settlement assays and subsequent adhesion strength measurements in the flow chamber. The surfaces were washed with a detergent solution (Decon 90), rinsed with distilled water, and air dried prior to use in assays.

Static drop settlement assays (n = 6 for pediveligers and plantigrades) were performed as previously described. Drop assays were covered individually to prevent evaporation (IWAKI 1000-053). Settlement assays were conducted in a temperature controlled culture cabinet (Sanyo MLR-351) at 18°C in a 12 h light:12 h dark cycle. The settlement of pediveligers and plantigrades was determined after 48 h and 6 h, respectively. Pediveligers and plantigrades were considered settled when they attached to the test surface with byssal threads.

5.2.2.2.4 Adhesion strength. The adhesion strength measurements followed the method described above (see Section 5.1.1.2).

5.2.3 Statistical analysis

Data were analysed by one- or two-factor permutational multivariate analysis of variance (PERMANOVA). The Bray-Curtis dissimilarity measure was used for all PERMANOVAs and *p*-values were calculated using unrestricted permutation of untransformed raw data with 9999 random permutations. If there was a significant difference, pair-wise *a posteriori* comparisons were made among the significant groups using the Bray-Curtis similarity measure (α = 0.05). To analyse the adhesion strength data, surfaces without initial settlement were excluded from PERMANOVAs. Statistical analyses were performed using PRIMER 6 (v. 6.1.13) and PERMANOVA+ (v. 1.0.3) (Clarke and Gorley 2006). Data are reported as mean ± 1 standard error (SE).

Correlations between the number of settled and remaining individuals after being exposed to a water flow of 4 knots were assessed using Pearson's product-moment correlation using SPSS (v. 19).

5.3 Results

5.3.1 TiO_2 in textured PDMS

5.3.1.1 Surface characterisation

The PDMS surfaces filled with TiO_2 had no surface topography and did not differ from the blank control surface at the micron scale. Backscattered SEM showed a homogeneous dispersion of the nanofiller on the surfaces of the nanocomposites (Figure 5.1a-e), however, TiO_2 formed small aggregates at the highest concentration of 15 wt% TiO_2 (Figure 5.1e). In contrast, micrographs of cross-sections of the nanocomposites showed large aggregates of the nanofiller throughout the matrices at all concentrations (Figure 5.1f-j).

The hardness of PDMS increased with the addition of increasing amounts of TiO_2 (Table 5.1). Consequently, the blank control was the softest surface with 84.1 ± 0.3 Durometer (Scale OO) and the hardness increased with the incorporation of TiO_2 up to 87.0 ± 0.3 Durometer (Scale OO) for the nanocomposite with the highest TiO_2 concentration (15 wt% TiO_2).

The incorporation of TiO_2 had an effect on the surface wettability (Table 5.1). The hydrophobicity of the TiO_2 filled matrices was lower than the blank PDMS control as shown by the contact angle measurements before the exposure to UV. While the contact angle of the blank PDMS remained unchanged after UV exposure, the hydrophobicity of the TiO_2-filled surfaces increased after exposure to UV light for 6 h. Notably, the TiO_2 matrices generated static electricity charges, which may cause errors in contact angle measurements. This charge effect was noted as the generated electrostatic field pulled the water droplet to the test surface more strongly than in the absence of charge (ASTM D5946-04), resulting in lower contact angles for statically charged surfaces. The surfaces did not show a static charge for contact angle measurements after 6 h of UV exposure.

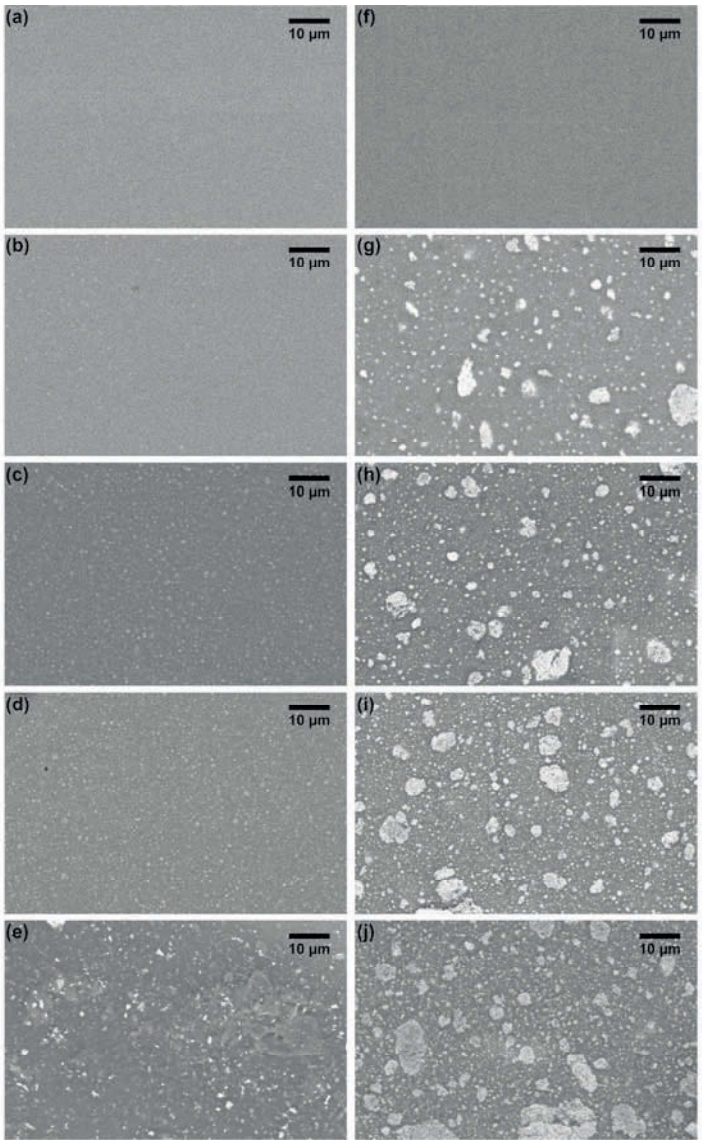

Figure 5.1: Backscattered SEM images of (**a-e**) the surface and (**f-j**) cross-sections through PDMS filled with various TiO_2 concentrations. (**a,f**) Blank PDMS control, (**b,g**) PDMS matrices filled with 3.75 wt% TiO_2, (**c,h**) 7.5 wt% TiO_2, (**d,i**) 11.25 TiO_2, and (**e,j**) 15 wt% TiO_2. Light coloured spots are TiO_2 particles.

Table 5.1: Hardness and water contact angles of nanocomposites filled with TiO_2.

Nanofiller TiO_2 (wt%)	Hardness (Durometer Scale OO) ± SE	Water contact angle θ (°) ± *SE*	
		No UV exposure	After 6 h UV exposure
0	84.1 ± 0.3	113.7 ± 0.3	113.8 ± 0.2
3.75	85.0 ± 0.3	104.8 ± 0.6	112.3 ± 0.4
7.5	85.1 ± 0.3	105.5 ± 0.7	111.9 ± 0.4
11.25	86.3 ± 0.2	104.8 ± 3.6	110.1 ± 0.6
15	87.0 ± 0.3	105.5 ± 3.1	110.1 ± 0.4

5.3.1.2 Settlement assay

The combination of TiO_2 and photoactivation (TiO_2 exposed to UV light) completely inhibited the settlement of all pediveligers of *M. galloprovincialis* at all tested concentrations of TiO_2 (Figure 5.2a). Notably, all pediveligers on surfaces filled with photoactivated TiO_2 were dead after 48 h. In contrast, pediveligers on blank surfaces were alive at the end of the experiment. Therefore, the strong and significant effect of TiO_2 (Table 5.2a) is based on its photoactivation, whereas UV light itself had no effect on survival of pediveligers. Consequently, settlement only occurred on control surfaces without TiO_2 in assays conducted under UV exposure. Among these, the lowest number of pediveligers settled on smooth PDMS (5.7 ± 1.5%) and increased with increasing size of texture from 200 µm (5.9 ± 3.4) to 600 µm (16.5 ± 6.4%); however, these increases were not significant (Table 5.2a).

In contrast, TiO_2 without photoactivation (assays conducted in the dark) had no effect on the settlement of pediveligers, while microtopography significantly enhanced the settlement of pediveligers (Figure 5.2b; Table 5.2b). Settlement on smooth PDMS surfaces was significantly lower with an overall mean settlement of 15.3 ± 0.6%, compared to 41.5 ± 1.2%, 51.9 ± 2.0%, and 47.4 ± 1.5% on 200, 300, and 600 µm textured surfaces, respectively. Settlement on 300 µm topographies was significantly higher than on other tested microtopographies (Table 5.2b). No mortalities of pediveligers were observed when settlement assays were conducted in the dark.

For settlement assays with plantigrades under UV exposure, neither microtexture nor the incorporation of TiO_2 had a significant effect on the settlement of plantigrades (Table 5.3a). More than 56% plantigrades settled on all surfaces under UV exposure, regardless of their microtopography and TiO_2 concentration (Figure 5.3a). The lowest number of plantigrades settled on smooth PDMS filled with 11.25 wt% TiO_2 (56.7 ± 6.2%), while the highest settlement occurred on surfaces with 300 µm texture without TiO_2 (90.0 ± 6.8%). Although plantigrades settled initially, all individuals were dead after 6 h on all TiO_2-filled surfaces when photoactivated under UV exposure. In contrast, all plantigrades were alive on blank surfaces exposed to UV confirming that UV itself had no effect on the survival of plantigrades.

When settlement assays were conducted in the dark, the overall settlement of plantigrades within 6 h was high on all surfaces ranging from 93.3 ± 4.2% to 100.0 ± 0.0% (Figure 5.3b). Similar to assays conducted under UV exposure, there was no effect of TiO_2 or microtexture on the settlement of plantigrades in the dark (Table 5.3b). Notably, no mortalities of plantigrades occurred for assays conducted in the dark.

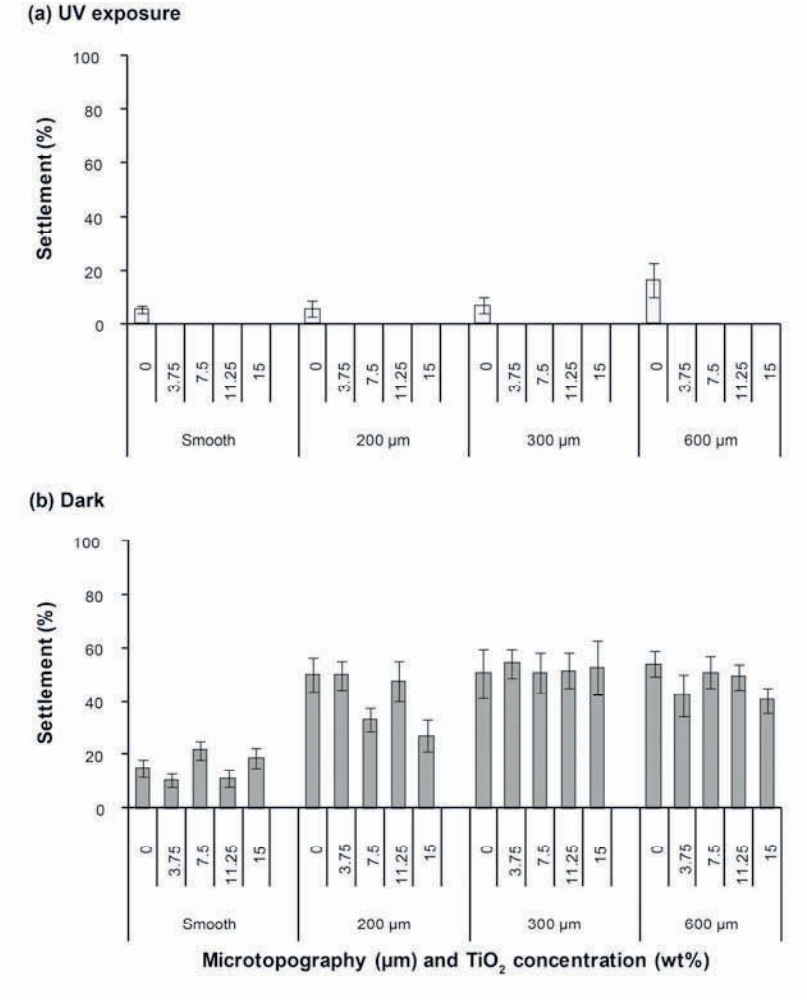

Figure 5.2: Settlement (%) of pediveligers of *M. galloprovincialis* on textured (200, 300, 600 µm) and smooth PDMS with different TiO_2 concentrations (0, 3.75, 7.5, 11.25, 15 wt%). Assays were conducted (**a**) under UV exposure and (**b**) in the dark. Means ± SE are shown (n = 6).

Table 5.2: PERMANOVA analysis on Bray-Curtis distances for differences in settlement of pediveligers (**a**) under UV exposure and (**b**) in the dark on surfaces with different microtextures (smooth, 200, 300, 600 µm) and TiO_2 concentrations (0, 3.75, 7.5, 11.25, 15 wt%).

Source	*df*	MS	*F*	*p*
(a) UV exposure				
Texture	3	434	0.81	0.510
TiO_2	4	22016	41.22	< 0.001
Texture × TiO_2	12	434	0.81	0.655
Residual	100	534		
Comparison *		*t*		*p*
0 – 3.75		6.42		< 0.001
0 – 7.5		6.42		< 0.001
0 – 11.25		6.42		< 0.001
0 – 15		6.42		< 0.001

Source	*df*	MS	*F*	*p*
(b) Dark				
Texture	3	13826	25.14	< 0.001
TiO_2	4	540	0.98	0.442
Texture × TiO_2	12	628	1.14	0.290
Residual	100	550		
Comparison**		*t*		*p*
0 - 200		5.05		< 0.001
0 – 300		6.27		< 0.001
0 – 600		6.50		< 0.001
200 – 300		1.94		0.046
200 – 600		1.72		0.073
300 - 600		0.83		0.420

* Pair-wise *a posterior* tests among TiO_2 concentrations (wt%).

** Pair-wise *a posterior* tests among microtextures (µm).

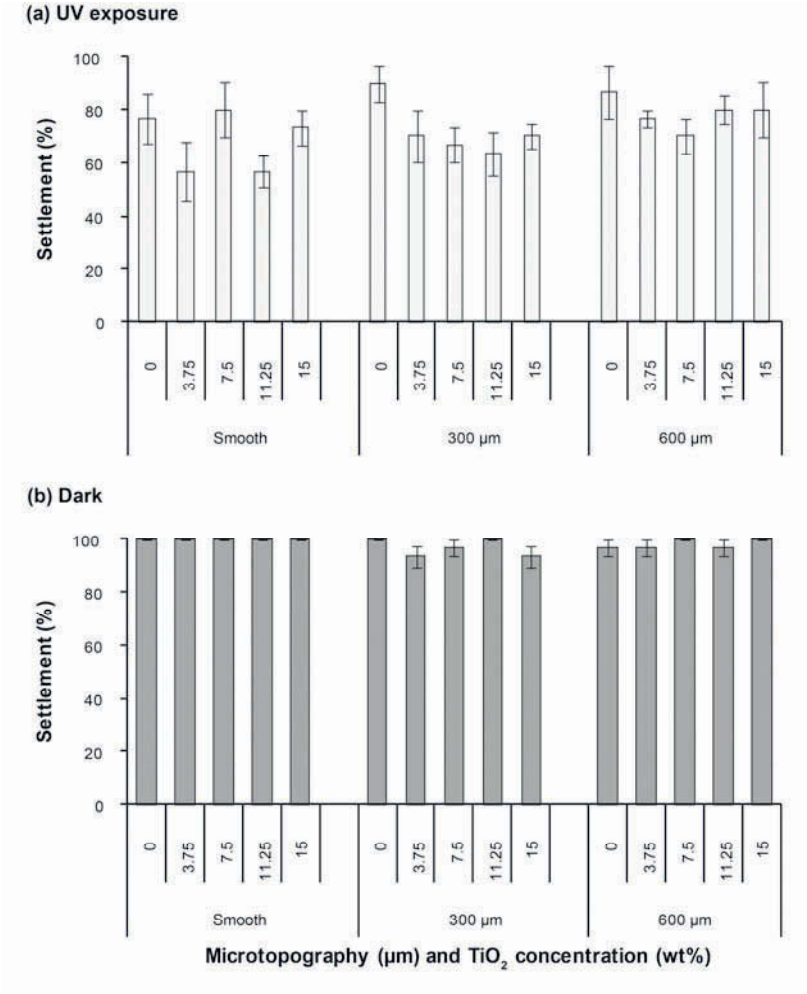

Figure 5.3: Settlement (%) of and plantigrades of *M. galloprovincialis* on textured (300, 600 µm) and smooth PDMS with different TiO_2 concentrations (0, 3.75, 7.5, 11.25, 15 wt%). Assays were conducted (**a**) under UV exposure and (**b**) in the dark. Means ± SE are shown (n = 6).

Table 5.3: PERMANOVA analysis on Bray-Curtis distances for differences in settlement of plantigrades (**a**) under UV exposure and (**b**) in the dark on surfaces with different microtextures (smooth, 300, 600 µm) and TiO_2 concentrations (0, 3.75, 7.5, 11.25, 15 wt%).

Source	*df*	MS	*F*	*p*
(a) UV exposure				
Texture	2	455	2.01	0.136
TiO_2	4	389	1.71	0.147
Texture × TiO_2	8	207	0.91	0.514
Residual	75	228		
(b) Dark				
Texture	2	26.06	2.64	0.083
TiO_2	4	5.49	0.56	0.701
Texture × TiO_2	8	10.63	1.08	0.404
Residual	75	9.88		

5.3.1.3 Adhesion strength

There was no initial settlement of pediveligers on surfaces filled with TiO_2 and photoactivated under UV exposure. Consequently, the differences in adhesion strength among different TiO_2 concentrations could not be determined. Settlement under UV exposure only occurred on control surfaces without TiO_2, and among these pediveligers adhered most strongly to the 600 µm textures with 44.3 ± 13.6% pediveligers remaining after exposure to flow (Figure 5.4a). The number of remaining pediveligers decreased with decreasing feature widths and detachment was highest on smooth PDMS surfaces (5.6 ± 5.6%). However, the effect of microtopography on the adhesion strength of pediveligers settled under UV exposure was not significant (Table 5.4a).

For assays conducted in the dark, TiO_2 had no effect on the adhesion strength of pediveligers, while texture significantly affected adhesion (Table 5.4b). The adhesion strength of pediveligers was significantly weaker on smooth PDMS, with an overall mean of 10.6 ± 2.8% pediveligers remaining on all smooth surfaces. This compared to more than 44% remaining on all microtextured surfaces (Figure 5.4b). Furthermore, adhesion was significantly stronger on microtextures with profiles of 600 µm than on profiles of 300 µm (Table 5.4b).

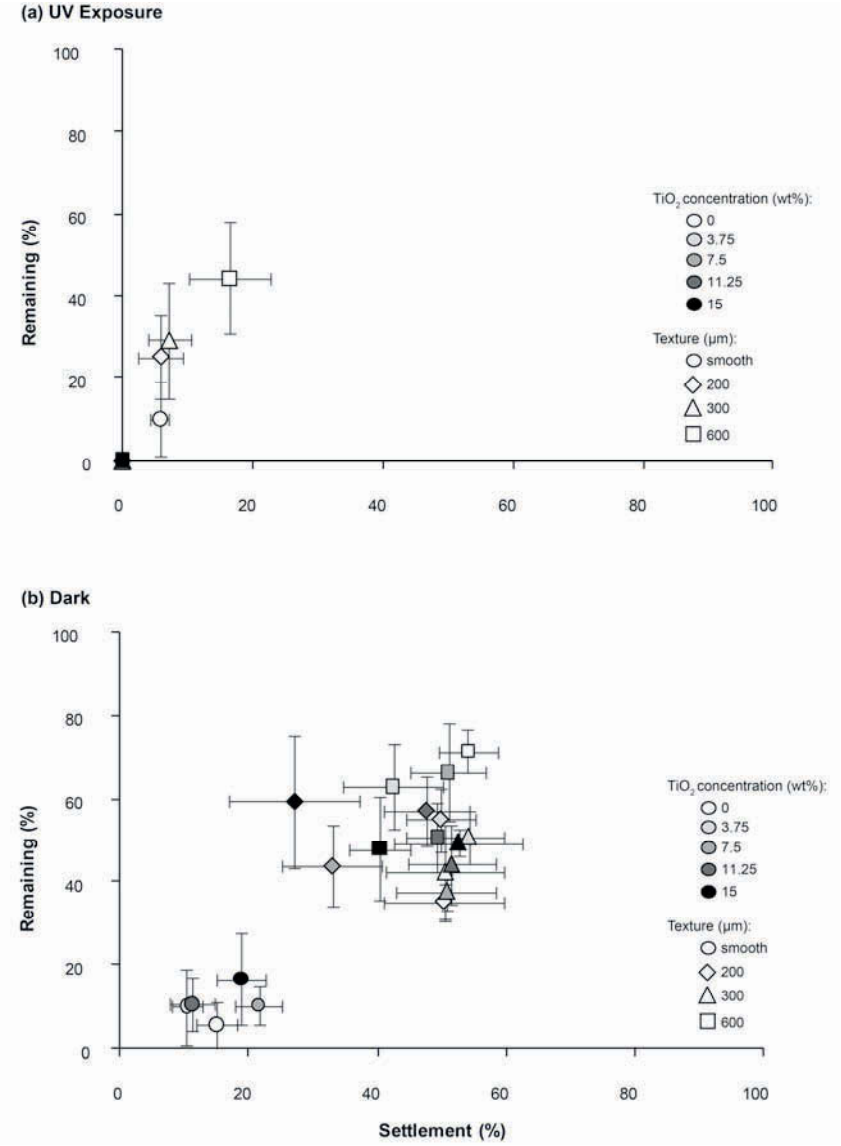

Figure 5.4: Remaining (%) pediveligers on textured (200, 300, 600 µm) and smooth PDMS with different TiO_2 concentrations (0, 3.75, 7.5, 11.25, 15 wt%) after being exposed to a water flow of 4 knots for 2 min. Assays were conducted (**a**) under UV exposure and (**b**) in the dark. The percentage of remaining pediveligers after being exposed to flow is based on the settlement on test surfaces. Means ± 1 SE are shown (n = 6).

Table 5.4: PERMANOVA analysis on Bray-Curtis distances for differences in the number of remaining pediveligers when settled (**a**) under UV exposure and (**b**) in the dark on surfaces with different microtextures (smooth, 200, 300, 600 µm) and TiO_2 concentrations (0, 3.75, 7.5, 11.25, 15 wt%) after the exposure to flow.

Source	*df*	MS	*F*	*p*
(a) UV exposure				
Texture	3	3205	1.14	0.366
TiO_2	0	No test	No test	No test
Texture × TiO_2	0	No test	No test	No test
Residual	13	2808		
(b) Dark				
Texture	3	30053	27.38	< 0.001
TiO_2	4	1213	1.11	0.358
Texture × TiO_2	12	835	0.76	0.741
Residual	98	1098		
Comparison**		*t*		*p*
0 - 200		6.20		< 0.001
0 – 300		6.18		< 0.001
0 – 600		6.38		< 0.001
200 – 300		0.47		0.802
200 – 600		1.33		0.145
300 - 600		1.76		0.026

** Pair-wise *a posterior* tests among microtextures (µm).

For plantigrades settled under UV exposure, adhesion strength on surfaces filled with TiO_2 was significantly weaker than on blank control surfaces (Figure 5.5a; Table 5.5a). On average, more than 82% of plantigrades remained on blank control surfaces exposed to UV, while the mean overall number remaining on TiO_2 filled surfaces ranged between 32.3 ± 4.6% (11.25 wt% TiO_2) and 49.3 ± 7.2% (7.5 wt% TiO_2) (Figure 5.5a).

For assays conducted in the dark, TiO_2 had no effect on the adhesion strength of plantigrades. Significantly fewer individuals remained on microtextures with profiles of 300 µm compared to profiles of 600 µm, with a mean overall remaining of 82.5 ± 4.8% and 94.8 ± 2.7% plantigrades, respectively (Figure 5.5b; Table 5.5b).

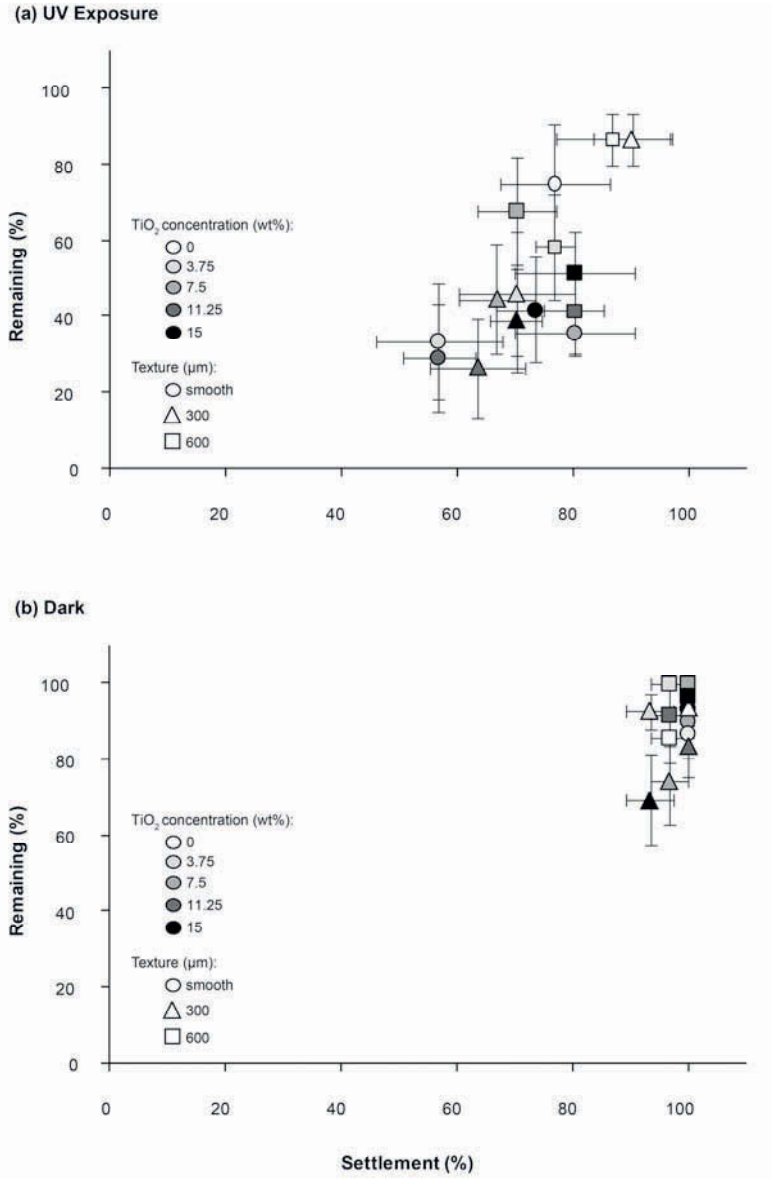

Figure 5.5: Remaining (%) plantigrades on textured (300, 600 µm) and smooth PDMS with different TiO_2 concentrations (0, 3.75, 7.5, 11.25, 15 wt%) after being exposed to a water flow of 4 knots for 2 min. Assays were conducted (**a**) under UV exposure and (**b**) in the dark. The percentage of remaining plantigrades after being exposed to flow is based on the settlement on test surfaces. Means ± 1 SE are shown (n = 6).

Table 5.5: PERMANOVA analysis on Bray-Curtis distances for differences in the number of remaining plantigrades when settled (**a**) under UV exposure and (**b**) in the dark on surfaces with different microtextures (smooth, 200, 300, 600 µm) and TiO_2 concentrations (0, 3.75, 7.5, 11.25, 15 wt%) after the exposure to flow.

Source	*df*	MS	*F*	*p*
(a) UV exposure				
Texture	2	4890	2.67	0.060
TiO_2	4	4610	2.51	0.031
Texture × TiO_2	8	1063	0.58	0.851
Residual	75	1834		
Comparison *		*t*		*p*
0 – 3.75		2.09		0.020
0 – 7.5		2.34		0.001
0 – 11.25		2.87		0.003
0 – 15		2.15		0.044
3.75 – 7.5		1.16		0.267
3.75 – 11.25		0.69		0.559
3.75 – 15		0.43		0.758
7.5 – 11.25		1.78		0.091
7.5 – 15		0.78		0.590
11.25 – 15		1.03		0.351

Source	*df*	MS	*F*	*p*
(b) Dark				
Texture	2	520	3.94	0.013
TiO_2	4	95	0.72	0.622
Texture × TiO_2	8	190	1.44	0.165
Residual	75	132		
Comparison**		*t*		*p*
0 – 300		1.81		0.062
0 – 600		1.11		0.282
300 - 600		2.37		0.012

* Pair-wise *a posterior* tests among TiO_2 concentrations (wt%).

** Pair-wise *a posterior* tests among microtextures (µm).

5.3.1.4 Correlation of settled and remaining pediveligers and plantigrades

In general, higher numbers of pediveligers remained on surfaces with high initial larval settlement (Figure 5.4a,b), with a significant correlation between settled and remaining pediveligers. This association was stronger when the settlement assay was conducted under UV exposure (Pearson's product-moment correlation: $r = 0.797$, $p < 0.001$; Figure 5.4a) compared to the dark ($r = 0.511$, $p < 0.001$; Figure 5.4b). For assays conducted in the dark, the low settlement and weak attachment of pediveligers settled on smooth PDMS is clearly seen with less than 20% of attached individuals remaining after the exposure to flow (Figure 5.4b). In a similar manner to pediveligers, the number of remaining plantigrades was also significantly correlated to settlement (Figure 5.5a,b). However, in contrast to assays conducted with pediveligers, this association was stronger when the assay was conducted in the dark ($r = 0.493$, $p < 0.001$; Figure 5.5b) and weaker for assays conducted under UV exposure ($r = 0.254$, $p = 0.020$; Figure 5.5a).

5.3.2 CNTs in PDMS

5.3.2.1 Surface characterisation.

The incorporation of CNTs resulted in the addition of surface features (topography) to the PDMS base material at the scale of microns (Figure 5.6a,b), with more surface topographic features at higher concentrations. Cross-sections of nanocomposites show large aggregates of CNTs throughout the matrices at all concentrations (Figure 5.6d), indicating an uneven dispersion of the CNTs. Although CNTs formed aggregates, there was dispersion of CNTs throughout the matrices as evidenced by the even black shade across each nanocomposite cross-section. The orientation of CNTs appeared to be random regardless of the aggregates (Figure 5.6e,f). CNTs were only exposed from the base material when cut as micrographs of cross-sections show (Figure 6d-f). Cut surfaces with exposed CNTs were not tested.

CNTs affected the material properties of the PDMS when embedded, with the mechanical property of hardness increasing with increasing amounts CNTs from 84.8 ± 0.3 Durometer (Scale OO) (blank PDMS) to 89.2 ± 0.5 Durometer (Scale OO) for the highest nanofiller concentration (6 wt% CNTs). In contrast, the change in surface wettability was marginal when CNTs were incorporated as a nanofiller (Table 5.6). All nanocomposites remained hydrophobic with $\theta \geq 110^{o}$.

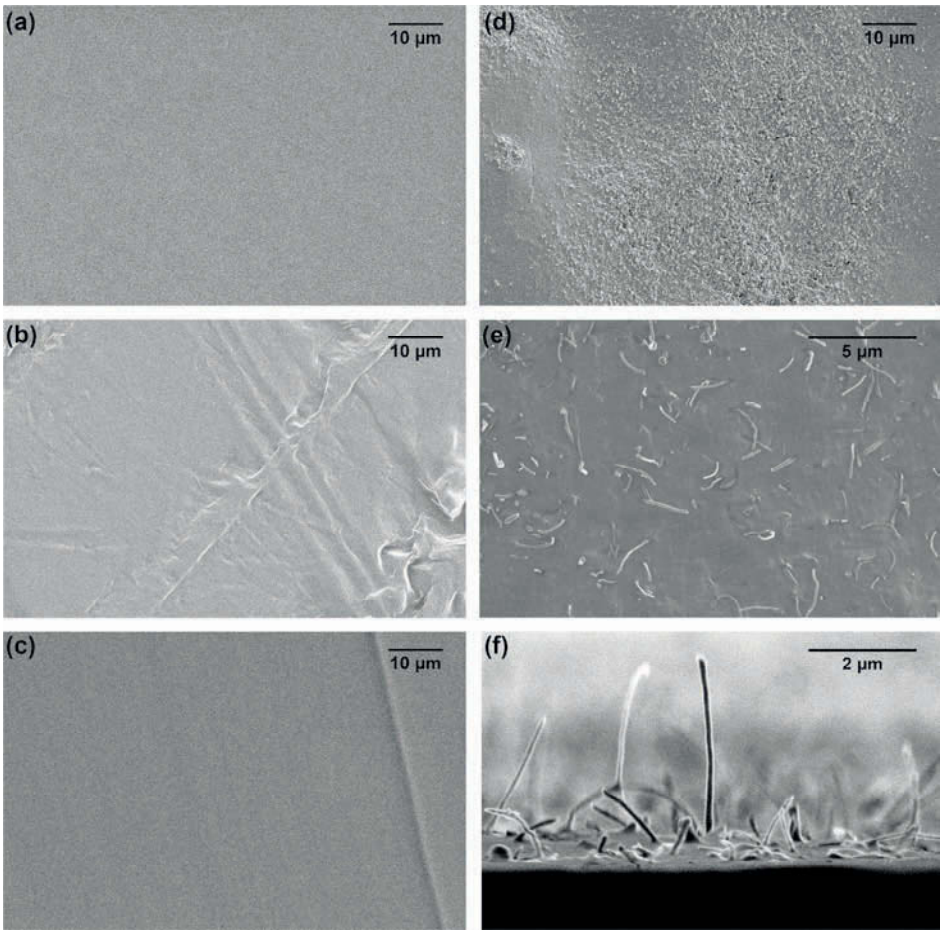

Figure 5.6: SEM images of (**a,b**) the surface and **(c-f)** cross-sections of (**a,c**) blank PDMS and (**b,d-f**) PDMS filled with 6 wt% CNTs. (**d-f**) Cross-sections of filled PDMS show CNTs protruding from the PDMS matrix where cut as illustrated in the (**f**) tilted view of a cross-section.

Table 5.6: Hardness and water contact angles of nanocomposites filled with CNTs.

Nanofiller CNTs (wt%)	Hardness (Durometer Scale OO) ± SE	Water contact angle θ (^{o}) ± SE
0	84.8 ± 0.3	112.4 ± 0.3
0.2	87.8 ± 0.1	110.2 ± 0.5
0.5	87.1 ± 0.4	112.6 ± 0.1
1	87.2 ± 0.3	113.1 ± 0.6
2	87.0 ± 0.0	112.5 ± 0.5
4	88.4 ± 0.2	110.3 ± 0.2
6	89.2 ± 0.5	112.2 ± 0.4

5.3.2.2 Settlement assay

The incorporation of CNTs had no effect on the settlement of either pediveligers (one-factor PERMANOVA: $F_{(6,35)}$ = 1.03, p = 0.404) or plantigrades ($F_{(6,35)}$ = 1.09, p = 0.548). The settlement of pediveligers was below 30% on all test surfaces and there was no consistent trend between settlement and the incorporation of CNTs (Figure 5.7a). The lowest number of pediveligers settled on PDMS surfaces filled with 4 wt% CNTs (17.2 ± 5.9%) and the highest number of pediveligers settled on PDMS filled with 1 wt% CNTs (29.5 ± 4.4%). The overall settlement of plantigrades within 6 h was generally high and ranged from 93.3 ± 4.2% to 100.0 ± 0.0% (Figure 5.7b).

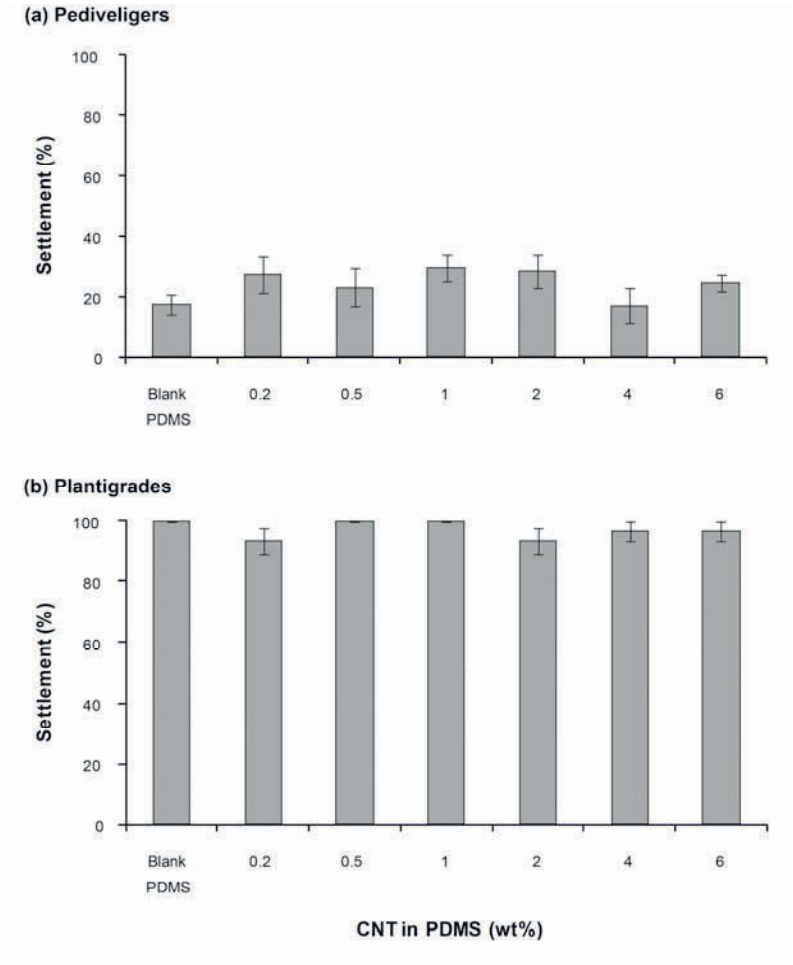

Figure 5.7: Settlement (%) of (**a**) pediveligers of *M. galloprovincialis* after 48 h and (**b**) plantigrades after 6 h on PDMS filled with CNTs (0.2, 0.5, 1, 2, 4, 6 wt%). Means ± 1 SE are shown (n = 6).

5.3.2.3 Adhesion strength

In a similar manner to the results of the settlement assays, there was no effect of the incorporation of CNTs on the adhesion strength of either pediveligers (one-factor PERMANOVA: $F_{(6,33)} = 0.40$, $p = 0.941$) or plantigrades ($F_{(6,35)} = 0.53$, $p = 0.799$). Less than 50% of pediveligers remained on all test surfaces after being exposed to a flow rate of 4 knots, while more than 85% plantigrades remained on all test surfaces (Figure 5.8). Pediveligers adhered most strongly to PDMS surfaces filled with the highest concentration of CNTs (6 wt% CNTs; 49.4 ± 17.1% pediveligers remaining), while the lowest number of pediveligers remained on surfaces filled with 0.5 wt% CNTs (21.3 ± 11.4%). In contrast, the highest number of plantigrades remained on blank PDMS after the exposure to flow (96.7 ± 3.3%) and the lowest number of plantigrades remained on PDMS surfaces filled with the lowest concentration of CNTs (0.2 wt% CNTs; 85.8 ± 4.6%).

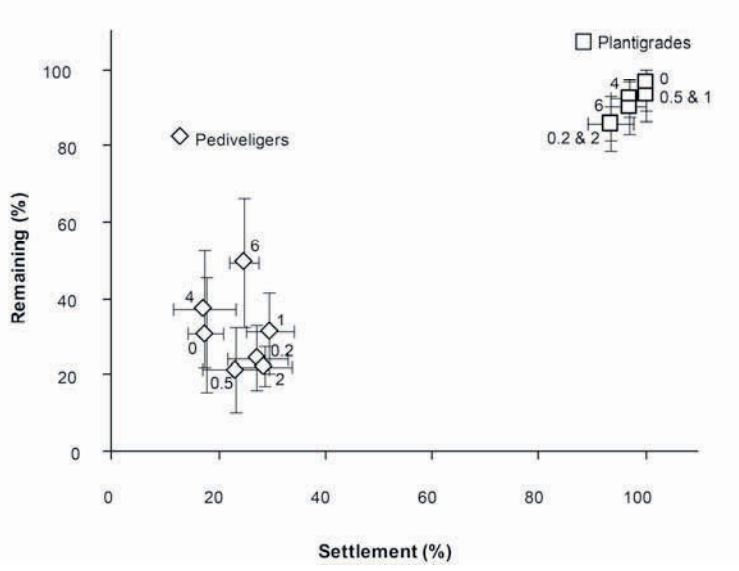

Figure 5.8: Correlation between initial settlement (%) and remaining (%) plantigrades and pediveligers of *M. galloprovincialis* on PDMS filled with CNTs (0.2, 0.5, 1, 2, 4, 6 wt%) after being exposed to a water flow of 4 knots for 2 min. The percentages of remaining pediveligers and plantigrades are based on the settlement on test surfaces. Means ± SE are shown ($n = 6$).

5.3.2.4 Correlation of settled and remaining pediveligers and plantigrades

There was no correlation between the initial settlement and the number of remaining pediveligers (Pearson's product-moment correlation: r = - 0.011, p = 0.947) and plantigrades (r = 0.114, p = 0.474) on surfaces filled with CNTs (Figure 5.8).

5.4 Discussion

This study tested the efficacy of incorporated TiO_2 and CNTs nanofillers in PDMS matrices to improve antifouling and fouling-release properties against the mussel *M. galloprovincialis*. The incorporation of TiO_2 was a highly effective strategy to prevent larval settlement, killing pediveligers and plantigrades and weakening their adhesion strength when photoactivated. Notably, the incorporation of the photocatalyst TiO_2 in a PDMS matrix was highly effective against the settlement of pediveligers regardless of concentration of TiO_2 embedded. No pediveligers settled on any surfaces filled with photoactivated TiO_2 and all pediveligers exposed to photoactivated TiO_2 died after 48 h. Similarly, all plantigrades exposed to photoactivated TiO_2 were dead by the end of the experiment (6 h), despite initial settlement (> 56%) to these surfaces. In addition, the adhesion strength of plantigrades on surfaces with photoactivated TiO_2 was weaker than on blank controls, demonstrating improved fouling-release properties by weakening adhesive bonding to the surfaces. In contrast, the incorporation of CNTs had no effect on either the settlement or adhesion of *M. galloprovincialis*.

In general, fouling-release coatings based on silicone elastomers are inefficient against initial fouling, in particular diatoms (Holland et al. 2004; Molino et al. 2009; Dobretsov and Thomason 2011; Zargiel et al. 2011). The incorporation of TiO_2 presents a promising solution to managing this initial and important stage of fouling. TiO_2 is an ideal photocatalyst due to its wide availability, low production cost, photo-stability, chemical safety, and the high oxidising potential of photogenerated holes (Fujishima and Zhang 2006; Aprile et al. 2008). Silicone elastomers, such as PDMS, are resistant to photodegradation of TiO_2 (Iketani et al. 2003) and transparent to UV light, which in turn allows photoactivation of the incorporated TiO_2 (Gao and Liu 2003). Furthermore, TiO_2 adheres strongly to silicone elastomers ensuring long-term photocatalytic activity (Silva et al. 2009). Overall, the use of a photocatalyst in fouling-release coatings has the potential to minimise the build-up of biofilms, degrade chemical settling cues, prevent larval settlement, compromise adhesion strength, and kill macrofoulers as demonstrated in this study. Moreover, the incorporation of TiO_2 clearly improves the efficacy of fouling-

release coating systems in environments with low shear forces. Importantly, only organisms in direct contact with the activated catalyst surface are exposed to the photocatalytic effects (Gaya and Abdullah 2008) thus reducing risks to non-target organisms. In addition, the risk of suspended photocatalytic nanoparticles causing phototoxicity to microalgae (Aruoja et al. 2009; Gladis et al. 2010; Miller et al. 2012) and adhesion of aggregates to aquatic organisms (Dabrunz et al. 2011) is low because the nanoparticles are incorporated into the PDMS matrix.

However, a limiting factor in the use of TiO_2 as an antifouling technology is the dependence on UV light for photoactivation, which takes place at wavelengths of 300-388 nm when the photocatalytic surface (TiO_2) is illuminated by light with energy above 3.2 eV (Wang et al. 2007; Gaya and Abdullah 2008). UV penetration in water decreases with increasing depth and can be impeded by water clarity (Conde et al. 2000). Therefore, the effectiveness of this antifouling approach is compromised in areas without UV exposure, including the inside of pipes and sea chests.

Non-activated TiO_2 had no impact on the survival, settlement and adhesion strength of pediveligers and plantigrades in this study. For pediveligers, microtopography played a key role in settlement for assays conducted in the dark, while non-activated TiO_2 had no effect. Microtopographic features significantly enhanced the settlement and adhesion strength of pediveligers, which is in accordance with previous studies where there is a positive effect of topographic features on larval settlement and retention (Filgueira et al. 2007; Gribben et al. 2011; see Chapter 3, Section 3.3.2). In contrast, topographies had no effect on the settlement of plantigrades, which agrees with studies where metamorphosed mussels settle on all types of firm substrates within a short period of time (Seed 1976; Gosling 2003; see Chapter 2, Section 2.3.1.2).

Notably, higher numbers of pediveligers and plantigrades settled on blank control surfaces when the settlement assays were conducted in the dark in comparison to assays conducted under UV exposure. While this observation coincides with studies identifying higher settlement rates of adult mussels in the dark (Kobak 2001), it is in contrast to previously reported enhanced settlement of pediveligers under a 12 h light:12 h dark regime compared to complete darkness (see Chapter 2, Section 2.3.2). This is most probably due to the presence of UV light in this study that did not cause mortality but may affect settlement rates.

The increased hydrophobicity of TiO_2 filled surfaces after the exposure to UV contrasts with a photo-induced hydrophilic effect, where hydrophobic surfaces containing TiO_2 become hydrophilic (Fujishima et al. 2008). In this study, the TiO_2 matrices initially generate an

electrostatic charge, which pulls the water droplet to the test surface more strongly, resulting in lower contact angles for statically charged surfaces.

The incorporation of CNTs was ineffective in improving antifouling and fouling-release properties against pediveligers and plantigrades of *M. galloprovincialis*. The embedment of CNTs caused minor topographic effects and resulted in a marginal increase in settlement of pediveligers in comparison to the blank PDMS control. However, CNTs incorporated in the PDMS matrix had no effect on the detachment of neither pediveligers nor plantigrades of *M. galloprovincialis*. Notably, the efficacy of CNTs to enhance fouling-release properties depends on the concentration of CNTs dispersed in the polymeric matrix although effective concentrations differ among species (Beigbeder et al. 2008). This has been demonstrated for the percentage removal of sporelings of *U. linza* by exposure to shear, which was doubled for CNT concentrations in the range of 0.05 to 1 wt% while the incorporation of CNTs at lower or higher concentrations did not result in enhanced fouling-release performance (Beigbeder et al. 2010). CNT concentrations greater than 0.1 wt% are more likely to have bundle-like aggregates resulting in less efficient dispersion and reduced fouling-release performance (Beigbeder et al. 2010). The use of CNT concentrations ≥ 0.2 wt% in the present study resulted in the formation of some aggregates. As a result, the detachment of pediveligers and plantigrades was similar among CNT concentrations tested and the blank control. Nevertheless, the incorporation of CNTs represent a promising approach to enhance fouling-release performance. Cut surfaces in this study (Figure 5.6f) may present a hostile surface for settlement due to the inherent high stiffness of the CNTs (1 TPa; Thorstenson et al. 2001), which may puncture exploring or settling organisms, forming a 'wall of death'. As the properties of CNTs depend on atomic arrangement, morphology, diameter and length of the tubes (Thorstenson et al. 2001), this also provides an opportunity to further manipulate characteristics of CNT spikes.

In conclusion, the incorporation of CNTs and TiO_2 as nanofillers improved the hardness of a 'weak' silicone elastomer. The use of the photocatalyst TiO_2 in antifouling and fouling-release coatings is promising and effective. The advantages of using TiO_2 are improved mechanical properties of weak coatings, enhanced fouling–release properties, and photocatalytic destruction of a broad range of fouling organisms. The limitations and longevity of the photocatalytic activity, and the retention of TiO_2 within the coating, require delineation and quantification. However TiO_2 has been used successfully in self-cleaning tiles which are effective over a span of 20 years (Fujishima et al. 2008). Overall, the incorporation of TiO_2 into coatings is a stepping stone aimed at improving antifouling technologies.

Chapter 6

Synthesis and discussion

The settlement ecology and preferences of the mussel *Mytilus galloprovincialis* are highly complex and understanding these processes are the first step to facilitate the development of surface-based technologies to either promote or deter the settlement of mussels. Previous studies have demonstrated that the settlement of mussels is associated with biofilms (Satuito et al. 1995; Bao et al. 2007b; Ank et al. 2009; Yang et al. 2011), chemical cues (Eyster and Pechenik 1987; Dobretsov and Qian 2003; Alfaro et al. 2006; Gribben et al. 2011), hydrodynamics (Eyster and Pechenik 1987; Pernet et al. 2003; Alfaro 2005, 2006; Hayden and Woods 2011), noise (Wilkens et al. 2012), and the settlement surface and its chemical and physical properties (Crisp et al. 1985; Eyster and Pechenik 1987; Cáceres-Martínez 1994; Harvey et al. 1995; Walter and Liebezeit 2003; Aldred et al. 2006; Filgueira et al. 2007; Brenner and Buck 2010). However, sufficient information to deliver new surface-based technologies with applications for aquaculture or biofouling are still lacking. The development of these technologies needs to be based on a fundamental understanding of key drivers for settlement and attachment, but the degree and comparative contribution thereof remain undefined. Therefore, the aims of this thesis were to define and understand key surface parameters affecting settlement and adhesion of this economically important species.

The major outcomes of this study are:

1. Reliable and reproducible laboratory settlement assays for *M. galloprovincialis* (Chapter 2). Conspecific density had no effect on the settlement of plantigrades, whereas the number of settled pediveligers decreased with increasing density. Settlement of pediveligers increased drop assays in a 12 h light:12 h dark cycle, while bottom shade had no effect of any magnitude. In addition, settlement was significantly enhanced by storing pediveligers for between 4 and 24 days at 4ºC.

2. The development of surface technologies to enhance settlement and adhesion strength of pediveligers of *M. galloprovincialis* for the aquaculture industry (Chapter 3). Texture was a key factor in the active selection of settlement sites of pediveligers, with a far

greater effect than wettability. Textured surfaces with 400 µm grooves had the highest settlement and attachment strength of pediveligers. Furthermore, settlement to specific topography dimensions was positively correlated to the attachment strength of pediveligers.

3. Establishing that complexity was a key factor in the active selection of settlement sites (Chapter 4). Mussels preferred to settle on increasingly complex surfaces, regardless of ontogenetic stage. The standard commercial methods to settle mussels on ropes showed that pediveligers initially settled within the sub-surface portion of ropes, moving towards the surface as they increased in size. Notably, smaller size classes preferred central or sub-surface sections of rope.

4. Demonstrating the importance of novel antifouling technologies (Chapter 5). The incorporation of the photocatalyst TiO_2 into fouling-release coatings was a highly effective mechanism to prevent initial larval settlement, causing high rates of mortality and weakening the adhesion strength of any settled pediveliger or plantigrade. In contrast, the incorporation of CNTs had no effect on the settlement and bio-adhesion of *M. galloprovincialis*.

Previous laboratory studies have been hindered by low settlement rates of pediveligers (Dobretsov and Qian 2003; Bao et al. 2007b; Yang et al. 2008) and this study provides an optimised and standardised static laboratory settlement assay for mussels. Optimised settlement of pediveligers was achieved with low numbers of individuals in drop assays with a photoperiod of 12 h light:12 h dark. Furthermore, the storage of competent pediveligers at 4ºC between 4 and 12 days enhanced the settlement from 7.1 ± 1.7% to > 70%. The high number of settled pediveligers after refrigeration provides an alternative to chemical settlement inducers in static laboratory assays (Dobretsov and Qian 2003; Yang et al. 2008). Although the effect of cold storage on settlement behaviour such as time of exploration prior to settlement remains unclear, the use of cold stored larvae with high settlement is a useful tool in assessing extremes of deterrence for antifouling purposes.

Furthermore, the cold storage of competent pediveligers is a promising approach to increase the efficacy of the mussel aquaculture industry. Mussel seed is collected from the wild and also produced in closed life-cycle hatchery culture. While the settlement of *M. galloprovincialis* is generally high in hatcheries, the successful hatchery production of the mussels *Perna viridis* (Laxmilatha et al. 2011) and *P. canaliculus* (Ganesan et al. 2010) are hindered by low settlement rates. Therefore, the cold storage of competent pediveligers provides a promising

approach to increase the settlement of cultured species with low settlement rates under hatchery conditions. Further investigations of the effects on the settlement and retention of cold stored pediveligers on ropes is also of interest given the importance of translating laboratory studies into a commercial context.

Previous studies identified surface wettability (Gerhart et al. 1992; O'Connor and Richardson 1994; Yamamoto et al. 1997; Dahlström et al. 2004; Aldred et al. 2006) and topography (Bers and Wahl 2004; Scardino et al. 2006; Scardino and de Nys 2011) as key surface parameters affecting the settlement and bio-adhesion of marine invertebrates. This study demonstrated the importance of wettability and microtexture to the settlement of pediveligers of *M. galloprovincialis* and is the first study to demonstrate the positive correlation of settlement preferences and the highest attachment strength of pediveligers on microtextured surfaces. Microtopography has a far greater effect on the active selection of settlement sites of pediveligers than wettability. Textured surfaces with 400 µm grooves have the highest settlement (> 90%) and attachment strength. This is an obvious avenue to develop improved materials for the enhanced settlement and attachment of mussel larvae and provide an opportunity to regulate density and retention of mussels in the aquaculture industry. The preferred feature-grooves can be incorporated in ropes to optimise settlement and retention.

Subsequently, the settlement preferences for increasingly complex surfaces and site-specific settlement sites on and within nursery ropes were determined at an industrial scale. Pediveligers are highly selective and show a clear settlement preference for increasingly complex surfaces. Textured surfaces with 400 µm grooves enhanced settlement but not to the same extent as in laboratory drop assays (Chapter 3). This poorer performance in larger-scale experiments suggests difficulties in translating laboratory results directly to commercial conditions and highlights the selective preference of pediveligers for settlement sites. In addition, differences in the performance of the textured surface between studies (Chapter 3 and 4) may be based on differences in the applied assay types. In static drop assays (Chapter 3), individuals have a limited choice for settlement and exploration, and gravitational effects may further play an important role (Aldred et al. 2010a). In contrast, individuals in volume assays (Chapter 4) are able to explore a larger area and move within the water column. However, static drop assays represent a powerful screening methodology for antifouling technologies due to high settlement pressure, while being less effective in screening settlement enhancing technologies for aquaculture purposes.

In general, complex materials such as ropes used in the mussel aquaculture industry are highly effective settlement surfaces for *M. galloprovincialis* (Chapter 4). Although numerous studies

have tested the performance of a variety of collector and nursery ropes in the commercial context of the mussel aquaculture industry (Alfaro and Jeffs 2003; Walter and Liebezeit 2003; Filgueira et al. 2007; Peteiro et al. 2007; Brenner and Buck 2010), this is the first study to identify settlement distribution of mussels on and within nursery ropes. It is also the first study to track the movement of individuals as they grow. Pediveligers of *M. galloprovincialis* initially settle deep within nursery ropes and move outwards as they increase in size. In contrast, mussels remaining within their initial size class move deeper within the ropes over time, indicating a preference for protected settlement sites. To optimise aquaculture practices, the effect of novel ropes with specifically manufactured texture to optimise the settlement and retention of a range of mussel species will contribute to enhanced supply of wild and hatchery stock for culture. Further investigations of the effect of complexity on other commercial sessile species are of interest and are a promising direction for future research.

Micro-CT was used for the first time as a tool to locate and track individual mussels. It proved to be an excellent non-destructive technique for mapping attachment sites of individuals as early as one day post settlement and represents a powerful tool to understand the settlement ecology and preferences of mussels and other bivalves within aquaculture. Previous methods to determine the number of individuals on collector ropes ranged from simply scraping mussels off the ropes (Filgueira et al. 2007) to immersing the ropes in a 10% sodium hypochlorite solution to facilitate the detachment of individuals, followed by rinsing the ropes under running water and retaining mussels with a sieve (Cáceres-Martínez et al. 1993). These methods can underestimate the number of settled mussels, particularly within ropes, and provide inaccurate results in the performance of ropes for settlement. Although µCT gives an overview of the three-dimensional attachment locations of all settled mussels on and within ropes, only small sections of the rope can be scanned. However, µCT will be most useful in the development of novel materials providing optimal settlement and retention for the mussel aquaculture industry, with application more broadly to bivalve settlement and retention.

Finally, surface technologies to deter the settlement of pediveligers and plantigrades of *M. galloprovincialis* as fouling organism were investigated. The incorporation of TiO_2 in fouling-release coatings was highly successful in controlling the settlement of *M. galloprovincialis* and also enhanced the efficacy of these coatings by minimising bio-adhesion. In general, the use of a photocatalyst is a promising alternative to metal-based antifouling coatings, which have an impact not only on the targeted organisms, but on the broader marine environment (de Nys and Steinberg 2002). When incorporating TiO_2 in coatings, the risks to non-target organisms are reduced as only organisms in direct contact with the activated catalyst surface undergo photocatalytic degradation (Gaya and Abdullah 2008).

However, the use of TiO_2 is limited to the photic zone where enough light is present for activation of the photocatalyst. The outcomes of this thesis will contribute towards the development of innovative and environmentally sustainable antifouling technologies. The next step is to test the antifouling properties of TiO_2 filled coatings against a broad range of fouling organisms in field experiments. Furthermore, the limitations and longevity of photocatalytic activity, and the retention of TiO_2 within the coating, require delineation and quantification.

In contrast, the incorporation of CNTs was not effective in improving antifouling and fouling-release properties against *M. galloprovincialis*. CNTs were only exposed from the base material when cut and this potential 'wall of death' may represent a hostile surface for settling organisms. Cut surfaces with exposed CNTs are obvious non-biocidal solutions against biofouling and it is important to identify the performance of this novel antifouling technology against a broad range of fouling organisms and the longevity of this approach.

Overall, this thesis contributes to an improved understanding of key surface parameters affecting settlement and adhesion and supports the development of innovative surface-based technologies to either enhance or prevent settlement and adhesion of *M. galloprovincialis* for the aquaculture industry, or as environmentally sustainable antifouling technologies.

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