



The role of substrata width and millimeter scale surface micro-structure in the attachment of juvenile mussels

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ABSTRACT

The global mussel aquaculture industry is constrained by the unreliable supply of wild seed mussels and high losses of seed mussels shortly after they are seeded onto coastal farms. The success in the collection of the settling larvae of mussel in the wild and the subsequent on-growing of early seed mussels in aquaculture are largely determined by the settlement and attachment behaviour of the larval and juvenile mussels, which is strongly influenced by the physical structure of their attachment substrata, especially filamentous substrata. This study aimed to identify an ideal set of morphological characteristics of filamentous substrata that would promote the improved attachment of early mussels and contribute to reducing the shortage of seed mussels in the New Zealand green-lipped mussel (*Perna canaliculus*) aquaculture industry. Artificial filamentous substrata with varying branch width and textured surfaces were tested for their ability to promote the attachment of wild juvenile mussels. The attachment of early juvenile mussels to 1.6 mm wide filamentous substrate was six times higher than their wider counterparts (i.e., 3.7, 5.6, 7.4, and 9.5 mm). There was also a higher proportion of small-size mussels (<0.99 mm shell length) on the 1.6 mm substrata. Furthermore, the filamentous substrata with regularly ridged surface contours (1 mm spacings) tended to attract more juvenile mussels. However, this preference was not consistent for small-size mussels on filaments of thinner width (i.e., 0.4, 0.8, 1.6, and 3.7 mm). Overall, the dimensions of filamentous substrates identified through this study will be valuable for improving the design of artificial substrata used in mussel aquaculture for improving its effectiveness for the attachment of seed mussels.

1. Introduction

Capturing and retaining sufficient seed mussels for aquaculture production is a major constraint to the efficiency and growth of mussel aquaculture in many parts of the world (Kamermans and Capelle, 2019). The direct harvest of wild seed attached to the natural substrata and the use of suspended collectors in coastal water to gather settling larvae and early juveniles are still the major sources of mussel seed used for aquaculture globally. More reliable hatchery production of seed mussels, has not yet replaced wild seed as the dominant seed supply due to its higher cost (Kamermans et al., 2013; Smaal et al., 2019). In New Zealand, the green-lipped mussel (*Perna canaliculus*) aquaculture industry (i.e., Greenshell™) relies heavily (around 80 % of the needs) on the harvesting of large numbers of wild seed mussels that are attached to

drifting filamentous seaweed and other material that washes up at one beach in the north of the country (Alfaro et al., 2010; Alfaro and Jeffs, 2003; Jeffs et al., 1999). This wild source of mussel seed is unreliable and fluctuates year-to-year with environmental conditions including 1) the location and availability of algal material (Alfaro and Jeffs, 2002); 2) the nearshore wind, currents, and their interaction which determine the subtidal water flow dynamics (Alfaro et al., 2010); 3) the temperatures and nutrients which could affect mussel spawning events (Jeffs et al., 2018), and 4) the occurrences of storm events, which are likely to dislodge seaweed, enabling them tumble near bottom of water column and further increase their probability of being transported to the beach (Alfaro et al., 2010). Also, after beach harvesting, the collected drift seaweed and other debris along with attached wild mussel seed is placed alongside a polypropylene nursery rope which is then enclosed in a

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biodegradable cotton socking to hold it in place and then seeded rope with the cotton socking outer is suspended beneath the backbone lines on a coastal mussel farm (Dawber, 2004; Hickman, 1978; Jeffs et al., 1999). The uncertainty of this wild seed supply is therefore further exacerbated by the high losses (typically exceeding 60 % and on occasions 100 %) of mussel seed after this subsequent seeding practice on mussel farms (Sim-Smith, 2006; Skelton et al., 2022; Skelton and Jeffs, 2021; South et al., 2021). A major cause of this loss of mussel seed appears to relate to the suitability of their attachment environment at seeding leading to their off-migration, although other factors have been identified, including predation, natural mortality, disease, and starvation (Carton et al., 2007; Jeffs et al., 1999; South, 2018). Early juvenile mussels of this species remain highly mobile and will detach, move, and then re-attach numerous times after their primary larval settlement in an effort to locate better attachment sites, a process known as secondary migration/settlement (Bayne, 1964; Buchanan and Babcock, 1997). The juvenile mussels can migrate by crawling, or by drifting in the water column by secreting a buoyant mucous thread, a process known as byssopelagic migration, or mucus drifting (Alfaro, 2006; Alfaro and Jeffs, 2003). This migration behaviour is most pronounced in mussel seed between 0.5 and 5.0 mm in shell length (Supono et al., 2020). This characteristic migration behaviour suggests a likely relationship between the high losses of mussel seed following seeding out and the unsuitability of the attachment substratum (South, 2018). The presence of such a relationship provides the opportunity to increase the efficiency of wild mussel seed collecting and tackle the poor seed retention on farms through experimentally exploring the substrate preferences of juvenile mussels.

The settlement and metamorphosis of the pelagic larvae is a critical stage in the lifecycle of most mussel species (Buchanan and Babcock, 1997). Observations of settling larvae consistently confirm that the active exploration and assessment of the surface of substrata prior to settlement is a critically important behaviour for marine benthic invertebrates selecting a settlement site (Eckman, 1990; Keough and Downes, 1982; Lemire and Bourget, 1996). Chemical, biological, and physical cues have been shown to be important factors mediating the settlement behaviour of the larvae of many species of pelagic marine invertebrates (Freckleton et al., 2022; Hu et al., 2024; Shikuma et al., 2014), including mussels (Ganesan et al., 2010; Hu et al., 2024; Liang et al., 2019; Von Der Meden et al., 2010; Yang et al., 2007). In the wild, the settling larvae and early juveniles green-lipped mussels, are commonly associated with natural filamentous substrates, especially fine red seaweeds and erect colonial hydroids, which are thought to provide behavioural cues (a combination of biological, physical and chemical cues) that strongly promote the settlement and attachment of the early stages of this mussel species (Alfaro and Jeffs, 2002). Similar correlations with different macroalgae were also found for larval settlement and metamorphosis in other species of mussels (Yang et al., 2007). Moreover, the smallest juvenile mussels (<0.5 mm in shell length) reportedly prefer to attach to the fine-branching substrata, while coarse-branching substrata had greater numbers of larger juvenile mussels attached, i.e., 1.5–2.0 mm in shell length (Alfaro and Jeffs, 2002). A further study of the structural characteristics of macroalgal substrata with juvenile mussels attached, found that blade width, surface area to volume ratio, and the branching frequency of the materials, which were mostly seaweeds, were the three most important physical cues determining the numbers of attached juvenile mussels (Kelly, 2001). The branch width of substrata had a negative correlation with the number of juvenile mussels attached, while substrata with a greater surface area to volume ratio and higher branching frequency tended to have more seed mussels attached (Kelly, 2001). In addition to the structural characteristics of the substratum, the surface microstructure of substrata has also been found to be another important physical cue in mediating the settlement of many epibenthic invertebrates, including green-lipped mussels (Bourget et al., 1994; Gribben et al., 2011; Pech et al., 2002; Walters and Wethey, 1996). For example, the V-section on

the rough side of plastic cable ties used in an experiment (i.e., the distance between widest portion of V-section is 1 mm) were found to provide a surface microstructure that strongly promoted larval settlement and the subsequent attachment of early juveniles of the green-lipped mussel (Gribben et al., 2011). This finding was consistent with a previous study which elucidated that settling propagules/larvae of marine organisms (e.g., diatom, alga, serpulid tube worm, and bryozoan) tend to prefer surfaces with a microstructure that is slightly larger than their body size, whilst they will avoid those surfaces with a microstructure that is smaller than their body size (Scardino et al., 2008). In addition to these physical cues, several studies have also indicated that natural chemical cues emanating from substrata or biofilms on substrata play a role in the settlement and attachment of larval green-lipped mussels (Alfaro et al., 2006; Ganesan et al., 2010). However, a comparison of the relative importance of chemical cues and surface microstructure of substrate found that the latter was of much greater importance in mediating larval settlement (Gribben et al., 2011).

Based on these previous results, the branch width and the surface microstructure are two important physical cues that appear to play an important role in the selection of substrata for larval settlement and early juvenile attachment in green-lipped mussels (Alfaro and Jeffs, 2002; Gribben et al., 2011; Kelly, 2001). However, these studies are generally based on morphological observations of natural substrates, such as seaweeds and hydroids, which often have many other morphological characteristics (e.g., degree of branching, the presence of nodes, the branching frequency, and the surface area to volume ratio) confounded with these two important physical cues (i.e., branch width and the surface microstructure) (Alfaro and Jeffs, 2002; Gribben et al., 2011; Kelly, 2001). For example, in previous studies, the natural substrata which are associated with a high abundance of attached juvenile mussels, such as colonial hydroids, have a small branch width, but are also consistently associated with other morphological characteristics, such as higher branching frequency, a higher degree of branching, more nodes and larger surface area to volume ratios (Alfaro and Jeffs, 2002; Kelly, 2001). It is therefore unclear how any of these characteristics may individually affect the settlement behaviour and whether the observed attachment behaviour of mussels may be in response to only one, or only a limited combination of some of the morphological characteristics, or the result of synergistic combinations of these characteristics. Such possibilities, as well as the large number of combinations of characteristics, make an experimental assessment of the importance of each morphological characteristic in isolation quite challenging. Consequently, the present study simplified the physical structure model of the attachment substrate to first independently investigate the role of just two physical cues (branch width and surface microstructure) in the attachment of juvenile mussels.

The two selected morphological features are the most fundamental physical cues that are widely recognized, and for which understanding their influence on mussel attachment is a vital precursor to revealing the influence of other complex structures with other physical cues. In so doing, this may help improve the design and performance of spat catching and growing ropes that can promote the attachment behaviour of the mussels, and thus reduce the losses of mussel seed experienced during the early phase of aquaculture production.

2. Materials and methods

2.1. Source of juvenile mussels

Colonial hydroids (*Amphisbetia bispinosa*) and a wide range of species of seaweeds with attached juvenile green-lipped mussels were collected from the rocky intertidal of Maori Bay, northwestern New Zealand (36° 50' 14" S, 174° 25' 35" E) on 7 August 2017 and (36° 50' 13" S, 174° 25' 34" E) on 20 October 2017 respectively. The material was kept moist and at ambient temperature at the time of collection for 2 h while transporting to the Leigh Marine Laboratory facilities. Upon arrival at the

laboratory the juvenile mussels and accompanying substrata were transferred to a 60 l conical plastic tank filled with filtered seawater (5 μm) at 19.5 ± 3.0 °C (S.E.) with vigorous aeration and fed with axenically cultured *Tisochrysis lutea* CS-177 at appropriate concentrations (Sanjayasari and Jeffs, 2019). After 12 h the mussels were detached from their substrata by rinsing the substrata briefly in freshwater over a 38 μm sieve to retain detached juvenile mussels. The mussels were immediately recovered from the handling by returning them to the seawater holding tank with clean seawater.

Three sub-samples of mussels collected in each sampling events were randomly selected, and a series of high-resolution digital photographs were taken for counting mussel number and measuring mussel shell length (maximum distance along the anterior/posterior axis of the shell) using image analysis software (Matlab IMAGEVIEWER version 1.3.0.1, MathWorks, Inc.).

In regards to the size of the mussels prior to their placement in the tank, unfortunately, data from the first collection of mussels were lost due to a camera storage failure. However, the overall mean size of the mussels (collected on 7 August 2017) that had settled at the end of the preliminary experiment was 0.60 ± 0.01 mm (S.E.) with 88.2 % mussel spat <0.99 mm, while the mussel spat collected on 20 October 2017 eventually settled on all of the substrata in second experiments had a mean size of 1.03 ± 0.02 mm (S.E.) with 63.0 % of them <0.99 mm. Meanwhile, the mean size of all collected mussels before the follow up experiment was 0.99 ± 0.06 mm (S.E.) (70.5 % of them <0.99 mm), which was not significantly ($t_{1647} = 0.66$, $p = 0.51$) different from the overall mussel size after they eventually settled on all of the substrata (i.e., 1.03 ± 0.02 mm) in the follow up experiment. However, the proportion of mussels smaller than 0.99 mm had significantly decreased after the experiments (possibly due to the daily growth of mussels) by about seven percentage points (from 70.5 % to 63.0 %) ($\chi^2 = 4.09$, $p = 0.04$). Thus, it is reasonable to assume that the mean size of mussels in the preliminary experiment were also around the size of 0.60 mm and about 95 % of them were smaller than 0.99 mm before being placed in the tank.

2.2. Experimental artificial substrata

The preference of juvenile mussels to attach to different branch width of filamentous substrata was tested by using artificial filamentous substrates for which only the branch width was varied. Considering both the shape and the dimension of natural substrata to which seed mussels were attached that were examined in previous studies (Alfaro and Jeffs, 2002; Kelly, 2001), a basic model generated by OpenSCAD software (www.openscad.org) was used as the structural basis for the experimental artificial substrata in this study (Fig. 1.C). It has a 120 mm long rectangular-shaped main stem and six 40 mm rectangular secondary branches which are evenly located on both sides of the main stem with a 20 mm branch distance interval. The main stem and six branches of each model have a unified width (i.e. branch width) which is used as the variable in this study. For the preliminary experiment the importance of branch width was determined by using five variations for this parameter, i.e., branch widths of 1.6, 3.7, 5.6, 7.4, and 9.5 mm (Fig. 1.A). These variations were based on measured widths of the thalli of macroalgae with juvenile mussels attached sampled from Ninety Mile Beach (Kelly, 2001). The variations of branch width chosen in the follow-up experiment were based on the experience and results of the preliminary experiment, in which the optimal width (1.6 mm) for promoting early juvenile mussel attachment that was identified in the preliminary experiment was used again. Additionally, narrower branch widths (i.e., 0.4, 0.8, 1.6, 2.4, 3.2, and 6.4 mm) (Fig. 1.B) were added to determine whether they would further increase the density of attachment of juvenile mussels.

The artificial substrata were cut from an extruded sheet of plastic made from a proprietary blend of polypropylene, amorphous carbon and calcium carbonate which has been developed for the fabrication of high-

performance filamentous green-lipped mussel spat catching ropes (Quality Equipment Ltd., Auckland, New Zealand). The plastic sheet was cut using a Trotec Speedy 300 laser engraving and cutting machine (Trotec Laser GmbH) (Fig. 1.F). Due to the nature of the industrial extrusion process the plastic sheet had a variable thickness (mean thickness of 0.15 ± 0.006 mm (S.E.)) and had a different microstructure on the surface of each side, i.e., one side smooth and the other textured (Fig. 1.D&E). The textured side had evenly distributed ridge lines with a 1 mm interval and less than 0.1 mm height (Fig. 1.E) while the smooth side had no measurable texture (Fig. 1.D). Therefore, the preference on surface microstructure of mussel spat attachment was also experimentally explored accompanying with the branch width. Eight replicates were fabricated for each of the five artificial substrata designs in the preliminary experiment, whilst the twelve replicates of six artificial substrata designs were fabricated for the follow-up experiment. There was a total of 112 substrata tested in two separated laboratory-based experiments (i.e., 40 and 72 respectively) on two different dates (i.e., 9 August 2017 and 21 October 2017).

2.3. The evaluation of artificial substrata in laboratory

At the start of each experiment all the replicates of each design of artificial substrata were attached by the base of the substratum at random positions on a plastic rack (Fig. 2). The rack with the substrata hanging beneath was then placed in a 60 l conical tank (Fig. 2) filled with filtered seawater (5 μm) at 19.5 ± 3.0 °C (S.E.) and aerated vigorously through a concentric series of porous airlines at the base of the tank. An adequate amount of juvenile mussels (around two thousand mussels on 9 August 2017 and ten thousand mussels on 21 October 2017) were then added to the tank that were randomly selected from the mussels that had previously been gathered from Maori Bay and separated from their attachment substrate. The first experiment ran for 5 days from 12:00 h. on 9 August 2017, similarly the second experiment ran for five days commencing at 18:00 h. on 21 October 2017. For each experiment the tank was supplied once a day with axenically cultured *Tisochrysis lutea* CS-177 at appropriate concentrations (Sanjayasari and Jeffs, 2019). After 5 days the artificial substrata were carefully removed from the plastic rack and high-resolution digital photographs taken of both sides of each replicate artificial substratum. Image analysis (Matlab IMAGEVIEWER version 1.3.0.1, MathWorks, Inc.) was used to extract data from the digital images relating to the juvenile mussels attached to each side of the artificial substrata. These data were: 1) the total number of mussels on each side of each replicate substratum; 2) the size of individual mussels as measured by their shell length (maximum distance along the anterior/posterior axis of the shell); 3) the proportion of measured mussels that had a shell length of less than 0.99 mm (i.e., recently settled from larvae) on each side of each replicate experimental substratum; 4) the density of attached juvenile mussels for each side (textured and smooth) of each replicate substratum (i.e., total number of mussels divided by the calculated total surface area of the artificial substratum). To avoid the division error caused by a zero total count of mussels on a small number of replicates, a count of one was added to each replicate experimental substratum after experiment to ensure the computability in the calculation of the percent of mussels.

2.4. Statistical analyses

A split-plot ANOVA analyses was used to compare the mean density of mussels attached to artificial substrata with different branch widths, with the split plot being used to account for any differences in juvenile mussel attachment between the textured and smooth sides of the artificial substrata. To meet the assumptions of homogeneity of variance and normality of data required for ANOVA, the mussel density data were transformed appropriately and re-checked with a Bartlett's test and a Shapiro-Wilk's test to confirm compliance with the underlying assumptions. Where the ANOVAs identified significant overall differences,

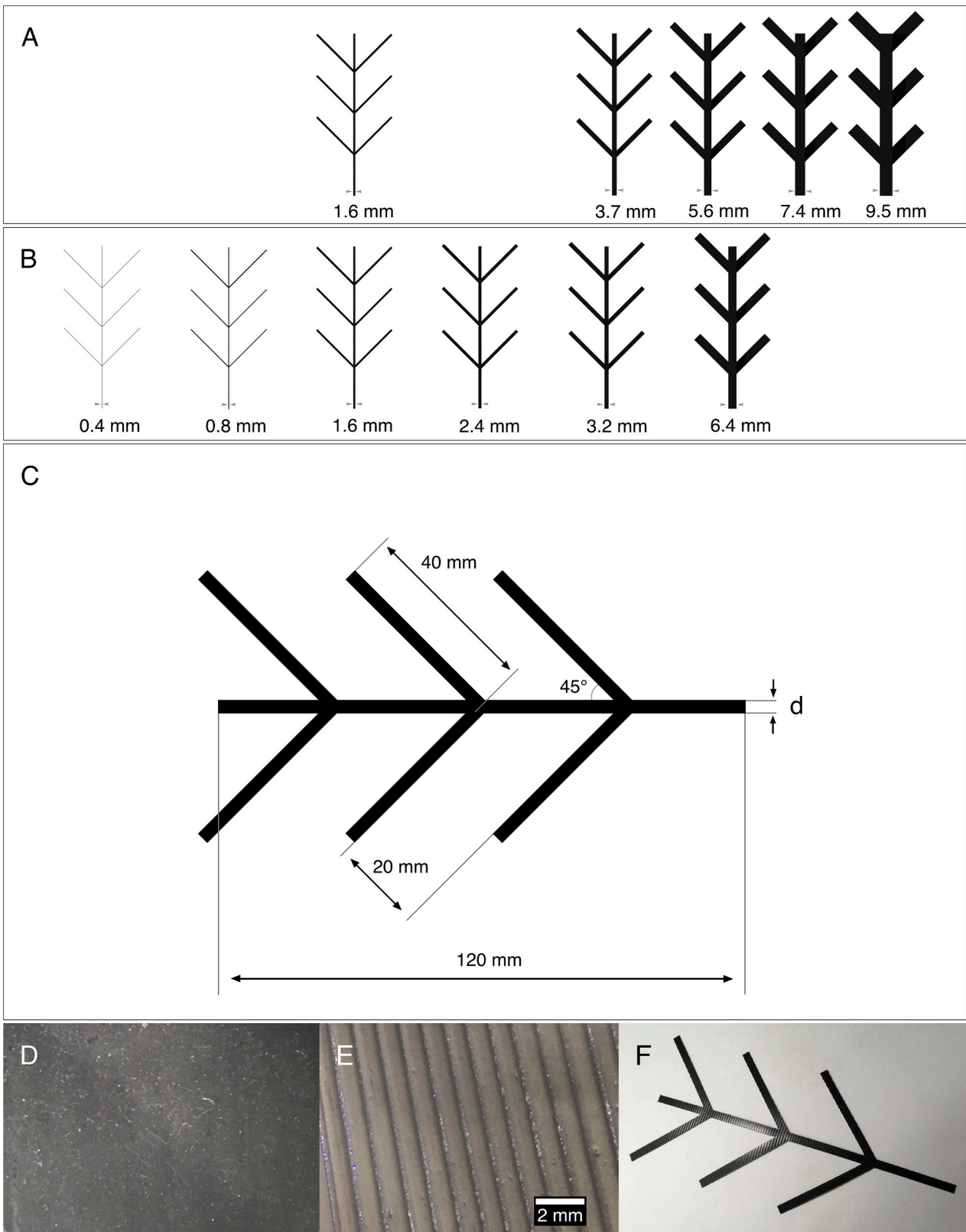


Fig. 1. Diagram of the models of branched artificial substrata generated by computer used for experiments testing the attachment of larval and early juvenile mussels showing the variations in branch width; A) the preliminary experiment, five variations, and B) the follow-up experiment, six variations; Details for the design variables of the branched artificial substratum (Stem length = 120 mm, branch distance = 20 mm, branch angle = 45°, branch length = 40 mm, and branch width – d) (C). Photomicrographs at the same magnification of the surface of the two sides of the extruded plastic sheet used for fabricating the artificial substrata; D) smooth side, and E) textured side; A photograph of an example of the artificial substratum with a branch width of 3.7 mm (textured side is facing upward) that was manufactured from the plastic sheet using laser cutting (F).

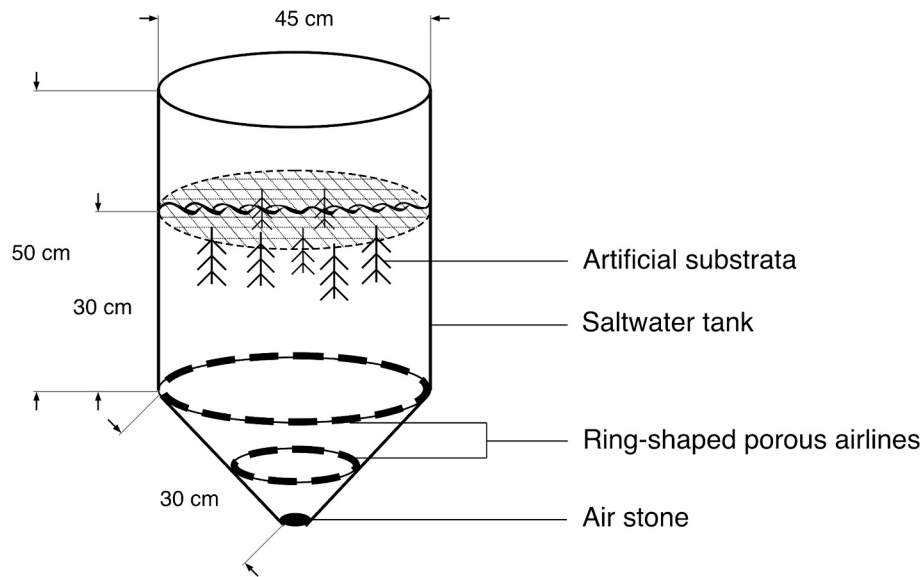


Fig. 2. A diagram of the experimental set up to determine patterns of attachment of juvenile mussels to artificial substrata.

LSD's pairwise comparisons were used to determine significant differences among means. A split-plot ANOVA analysis was also used in the same manner to compare the mean percent of mussels in the smaller size class (<0.99 mm shell length) except and arc-sine transformation was applied to the proportion data prior to testing for conformity to parametric assumptions. All data were analysed using the statistical software R version 3.4.3 through integrated development environment (IDE): RStudio Version 1.0.153.

3. Results

3.1. Mean mussel density

3.1.1. The preliminary experiment with wider branches

The mean density of juvenile mussels was different among the five branch widths of artificial substrata ($F_{(4,28)} = 22.32$, $p = 2.27 \times 10^{-8}$) (Fig. 3.A). The mean density of mussels for the narrowest branch width (1.6 mm) was around six times higher than for all other branch widths tested, i.e., 3.7, 5.6, 7.4, and 9.5 mm ($p < 0.05$). Mean mussel density tended to decrease rapidly with progressive increases in experimental branch width. Overall, there was a higher density of juvenile mussels on the textured versus the smooth side of the experimental substrata regardless of branch width ($F_{(1,35)} = 8.04$; $p = 7.50 \times 10^{-3}$) (Fig. 3.A). Furthermore, there were no interactive effects between the two experimental factors, branch width and sides ($F_{(4,35)} = 0.89$, $p = 0.48$).

3.1.2. The follow up experiment with narrower branches

The mean density of juvenile mussels among six branch widths of artificial substrata was different ($F_{(5,55)} = 10.16$, $p = 6.20 \times 10^{-7}$) (Fig. 3.B). There was no overall significant difference in the density of juvenile mussels on the textured versus the smooth sides of the substrata ($F_{(1,66)} = 0.61$, $p = 0.43$). There was no interactive effect between the microstructure of the sides of the substrata and the six experimental branch widths ($F_{(5,66)} = 0.99$, $p = 0.42$). There was an overall trend for mussel density to increase with decreasing experimental branch width with pairwise comparisons of means confirming that artificial substrata of 2.4, 1.6, 0.8, and 0.4 mm branch width had significantly higher mussel density than the artificial substratum of 6.4 mm branch width (Fig. 3.B). Despite the trend in the data and the highly significant effect of branch width on mussel density, no other significant differences between branch widths could be discerned among the individual means.

3.2. The mussel size-frequency distribution

3.2.1. The preliminary experiment with wider branches

There were differences in the mean proportion of small-size mussels (<0.99 mm shell length) among the five branch widths regardless of the sides of the substrata ($F_{(4,28)} = 5.27$, $p = 0.0027$). A higher proportion of small-size mussels (<0.99 mm shell length) were attached to the artificial substratum treatment with the narrowest branch width (i.e., 1.6 mm) compared with the artificial substrata with broader branch widths (i.e., 5.6, 7.4, and 9.5 mm) ($p < 0.05$) (Fig. 4.A). The different microstructure of the surface of each side of the substrata also had a significant effect on the proportion of small-size mussels attaching to the different sides of each artificial substratum ($F_{(1,35)} = 6.15$, $p = 0.018$), with a higher proportion of the small-size mussels attached on the textured sides of the substrata versus the smooth sides. However, there was also a significant interaction between branch width and the microstructure of the sides of the substrata ($F_{(4,35)} = 3.18$, $p = 0.025$) with the preference of small-size mussels (<0.99 mm shell length) to attach to smooth versus textured sides varying among the five branch width treatments (Fig. 4.A), in which only 5.6 and 7.4 mm width group has significantly higher proportion of small-size mussels on textured sides than the smooth side.

3.2.2. The follow up experiment with narrower branches

There was no significant difference ($F_{(5,55)} = 0.62$, $p = 0.68$) in the mean percent of small-size mussels (<0.99 mm shell length) attached to the artificial substratum treatment among the six branch widths. There was no significant difference in the mean percent of small-size mussels on the textured versus the smooth sides of the experimental substrata ($F_{(1,66)} = 1.67$, $p = 0.20$). Furthermore, there was also no interaction found between branch width and the microstructure of substrata ($F_{(5,66)} = 0.90$, $p = 0.49$). Overall, the proportion of the small-size mussels (<0.99 mm shell length) attached to the artificial substratum in this follow-up experiment was typically less than 50 % (Fig. 4.B).

4. Discussion

4.1. The effect of branch width

The commercial aquaculture production of green-lipped mussels relies heavily on the collection of wild seed mussels attached to seaweed, hydroids, and other natural filamentous substrata as the predominant seed source for initiating the production cycle for this species in New

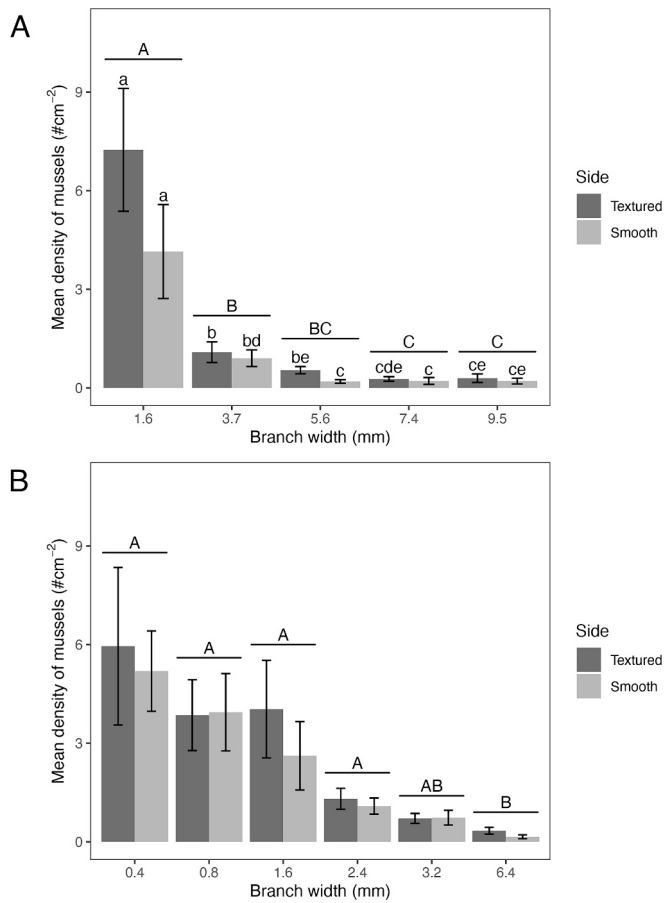


Fig. 3. Mean density (\pm S.E.) of mussels attached to experimental substrata of A) five different branch widths in the preliminary experiment, with each consisting of two sides with different microstructure (i.e., textured and smooth) ($n = 8$ replicates for each experimental substrate), and B) six different branch widths in the follow-up experiment, each also consist of two sides with different microstructure (i.e., textured and smooth) ($n = 12$ replicates for each experimental substratum). Different capital letters indicate differences between branch width treatments. Different lower-case letters indicate pairwise differences of mean mussel density for each side of substrata for every branch width ($p < 0.05$).

Zealand. This natural material, that is covered in mussel seed, is transferred onto mussel farms where it is held alongside plastic filamentous on-growing ropes by a cotton socking and suspended beneath the backbone lines of a mussel farm (Jeffs et al., 1999). Shortly afterwards the natural material and the socking quickly degrades leaving the seed mussels the option of either attaching to the plastic filamentous rope or departing (Skelton and Jeffs, 2021; Skelton and Jeffs, 2020; South et al., 2021). It is during this phase of production that losses of over 60 % of seed mussels typically occur. The retention of the mussel seed during this phase of production has the potential to be greatly improved through optimizing the morphology of the substrate that is offered for attachment by the seed mussels. The branch width is considered to be one of the most important morphological features that affects the attachment of mussel seed on natural substrata, with mussels being found to prefer the highly filamentous and complex settlement substrata. In particular, macroalgae and colonial hydroids, that commonly have narrow branch widths (Harvey et al., 1995; Alfaro and Jeffs, 2002). The overall complex structure of these highly filamentous natural substrates has been suggested to protect spat from predation (Frandsen and Dolmer, 2002) and dislodgement caused by hydrodynamic drag (Brenner and Buck, 2010).

For example, a former field study in juvenile marine benthic

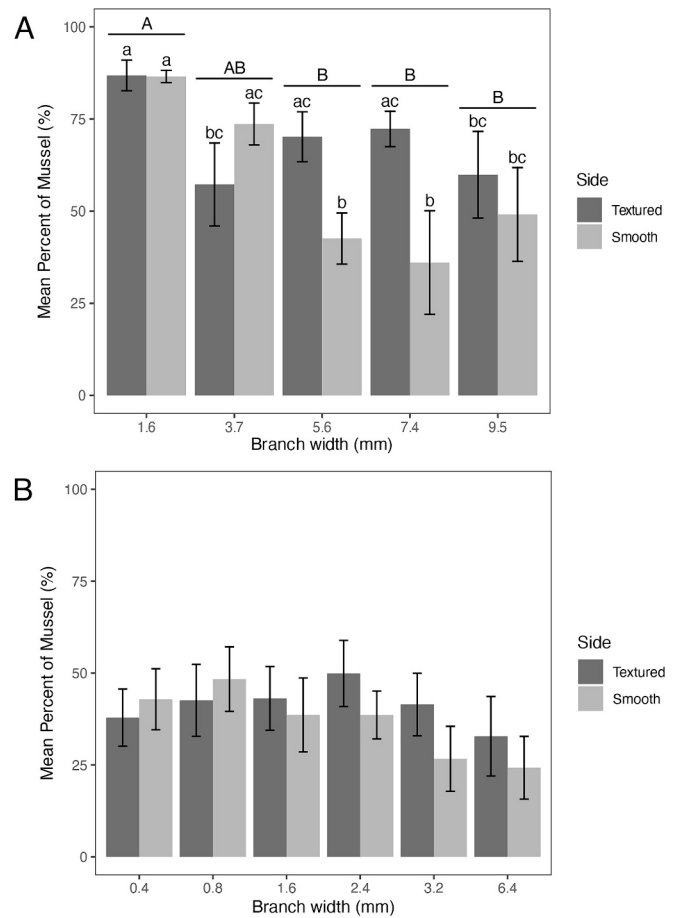


Fig. 4. Mean proportion (\pm S.E.) of total juvenile mussels in the <0.99 mm shell length size class attached to each side (i.e., smooth or textured) of experimental substrata of A) five different branch widths in the preliminary experiment ($n = 8$ replicates for each experimental branch width), and B) six different branch widths in the follow-up experiment ($n = 12$ replicates for each branch width). Different capital letters indicate differences between branch width treatments ($p < 0.05$). Different lower-case letters indicate pairwise differences of mean mussel density for each side of substrata for every branch width ($p < 0.05$).

invertebrate species including bivalve species (e.g., *Mytilus edulis*, *Chlamys islandica*, *Cerastoderma pinnulatum* and *Hiatella arctica*) found the spat density attaching to plastic filamentous material with 0.5 mm branch width was significantly higher than for all other tested branch widths of 0.8, 1.1, 1.4, and 1.7 mm (Harvey and Bourget, 1997). Furthermore, natural substrata on which green-lipped mussels are found to attach in the wild tend to have higher mussel density when they have narrower branch widths (Kelly, 2001). Specifically, those seaweed species present among commercially harvested natural mussel spat material from Ninety Mile Beach with a narrower primary branch width were consistently found to have high densities of seed mussels attached, i.e., *Halopteris novae-zealandiae* (0.48 mm branch width, 42.61 spat cm⁻²), *Ballia callitricha* (0.73 mm, 22.20 spat cm⁻²) and *Scytothamnus australis* (1.40 mm, 12.92 spat cm⁻²). In contrast, those seaweeds with broader primary branch width tended to have lower densities of mussel seed attached, i.e., *Glossophora kunthii* (10.34 mm branch width, 3.59 spat cm⁻²), *Carpophyllum maschalocarpum* (5.80 mm, 2.07 spat cm⁻²) and *Landsburgia quercifolia* (4.74 mm, 0.58 spat cm⁻²) (Kelly, 2001). Furthermore, in laboratory experiments imitation plastic seaweeds with 1 mm branch width were consistently found to attract higher numbers of mussel spat to attach compared to those that were 2, 4, and 8 mm in branch width (Kelly, 2001). This is consistent with the results of our preliminary experiment in which an artificial experimental substratum

with the narrowest branch width of 1.6 mm resulted in a significantly higher density of attached mussel spat compared with equivalent experimental substrata but with wider branch widths (i.e., 3.7, 5.6, 7.4, and 9.5 mm). A branch width of 1.6 mm was considered as optimal based on the results of this preliminary experiment. However, branch widths smaller than 1.6 mm and from 1.6 to 3.7 mm were not tested in this experiment. Moreover, another study using branched plastic structures of varying branch widths (i.e. 0.5, 0.8, 1.1, 1.4, and 1.7 mm) found similar results, with narrower plastic structures having higher total density of attached juvenile bivalves (e.g., *Mytilus edulis*, *Cerastoderma pinnulatum* and *Hiatella arctica*) in a field experiment (Harvey et al., 1995). To further explore the role of branch width of substrata smaller than 1.6 mm in the attachment of juvenile green-lipped mussels, artificial substrata with branch widths of 0.4, 0.8, 1.6, 2.4, 3.2, and 6.4 mm were assessed in the follow-up experiment. The results show that the substrata with branch widths in the range from 0.4 mm to 2.4 mm have significantly higher mussel density than a substratum with a branch width of 6.4 mm. This result is consistent with the results of the preliminary experiment and other previous studies (Harvey et al., 1995; Kelly, 2001). Although the difference of mussel density on artificial substrata with branch width between 0.4 and 3.2 mm in the follow-up experiment is not significant, there was still an apparent trend for artificial substrata with narrower branch width to have higher mean mussel density for both textured and smooth sides. Together these results indicate that juvenile green-lipped mussels prefer to attach to artificial substrata with narrow branch widths, generally narrower than 1.6 mm. These findings can help to guide the design of further experiments examining other structural characteristics of artificial substrata, and provide some useful guidance for improving the design of filamentous spat catching rope and on-growing rope which has the potential to help overcome difficulties with the supply of wild juvenile green-lipped mussels in the New Zealand aquaculture industry. Although the most likely cause for the loss of spat from mussel farms is their migration off the seeded dropper ropes, which is possibly related to the unsuitability of the attachment substratum (South, 2018), the physical characteristics of seeding substrata was also found to have no significant influence on mussel retention in one recent field study (Skelton and Jeffs, 2020). Further research is required to better understand the biology of the early juvenile phase of the lifecycle in the green-lipped mussel and to isolate the possible causes of the extensive loss of mussel seed from mussel farms so that these losses can be mitigated (Hickman, 1978; Zazzaro, 2016).

4.2. The effect of surface microstructure of artificial substrata

Previous studies have suggested a wide range of behavioural cues on the surface of substrata as inducers of settlement of marine invertebrate larvae including surface wettability and microtopography (Carl et al., 2012; Gribben et al., 2011; Yang et al., 2017) as well as many natural chemical cues frequently emanating from sources such as, biofilms, macroalgae, and conspecifics (Li et al., 2014; Liang et al., 2019; Jensen and Morse, 1990; Yang et al., 2017; Yang et al., 2007). However, for green-lipped mussel, surface microstructure of substrate was suggested to be more important than the chemical cues of substrate in mediating larval settlement (Gribben et al., 2011), in which the V-section on the textured side of cable ties (i.e., distance between widest portion of V-section is 1 mm) has been shown to strongly promote the larval settlement of green-lipped mussels (Gribben et al., 2011). The importance of surface microstructure has also been implicated in the settlement and attachment behaviour of many other epibenthic invertebrates (Bourget et al., 1994; Pech et al., 2002; Scardino et al., 2008; Walters and Wethey, 1996). The preliminary experiment in this study also provided evidence that the textured side of the experimental artificial substrata resulted in an overall higher density of attached mussels compared with the smooth side. While the effect of microstructure in this study was not strong, this result suggests some preference by mussel spat for the presence of

surface microstructure. However, the follow-up experiments failed to provide any difference in the density of attached juvenile mussels between the two sides. In the follow-up experiment, there was a general trend for a higher density of attached mussels on the textured sides of experimental substrata, however, there was a high degree of variability in mussel density within sides making it difficult to statistically discriminate any differences. The effect of surface microstructure on marine sessile organisms is not unidirectional, it can either enhance (Gribben et al., 2011) or reduce larval settlement and/or juvenile attachment (Berntsson et al., 2000). The influence of surface microstructure varies depending on its scale, contours, and other topographic features (Carl et al., 2012; Pech et al., 2002; Scardino et al., 2008). All these variations in surface microstructure, such as the topographic heterogeneity, can greatly influence the total numbers of marine epibenthic organisms attaching to the surface (Pech et al., 2002). Many settling larvae of marine organisms tend to settle on surfaces with microstructure that offers undulations that are slightly larger than their body size which can serve to protect the settling stage from hydrodynamic forces, unlike surfaces with smaller undulations which are often avoided by the same settling organisms (Scardino et al., 2008). Hence, preferences for attaching to surface microstructure can have distinct specificity that varies by scale, shape, and other topographic features (Myan et al., 2013). In addition, about 95 % of the mussels in the preliminary experiment were smaller than 0.99 mm at the beginning while only about 70.5 % were smaller than 0.99 mm before the follow-up experiment. According to the theory of these previous study (Scardino et al., 2008), the 1 mm ridged surface structure in our experiment may only have a protective/attractive effect on mussels smaller than 0.99 mm. It would probably be the main reason why the results of the second experiment were not significant. The artificial substrata used in this study were cut from extruded sheets of plastic which may have had slight differences in the surface microstructure caused by the unevenness of the sheet of plastic. This unevenness might bring some changes in the scale of surface microstructure and might also result in differences in mean density of attached juvenile mussels between the different experiments in this study, i.e., preliminary experiment versus follow-up experiment. Although no significant effect of surface microstructure on juvenile mussel attachment was found in the follow-up experiment, the role of surface microstructure in mediating attachment behaviour in juvenile green-lipped mussels cannot be ruled out until a wider range of variations in surface microstructure is tested.

4.3. The mussel size-frequency distribution

Previous analyses of natural filamentous substrata (i.e., various seaweed species) from Ninety Mile Beach to which green-lipped mussels were attached found higher numbers of mussels in the 1.5–1.99 mm shell length size classes were attached to coarse-branching substrata (i.e., broader branch width), compared to fine-branching substrata (i.e., narrower branch width) (Alfaro and Jeffs, 2002). In contrast, fine-branching substrata had a higher density of attached mussels within the <0.49 mm size class. These results indicated a distinct preference among juvenile green-lipped mussels of different sizes to attach to substrata of different branch width. Similar results were found in the current experimental study with a significantly higher proportion of small-size mussels (<0.99 mm shell length) attaching to the substratum with the narrowest branch width (i.e., 1.6 mm) compared to substrata with a broader branch width (i.e., 5.6, 7.4, and 9.5 mm).

In addition to the differential effect of branch width on the settlement of the mussels from different size classes, the effect of surface microstructure on the attachment of different marine invertebrates varies based on their sizes, and is highly influenced by relative match of the surface contours with the size of the settlers. The general principle is that increasing the number of attachment points and providing a surface microstructure that is slightly larger than the size of the settling organism will facilitate settler attachment (Callow et al., 2002; Scardino

et al., 2008). Consequently, mussels in the small-size class that are less than 0.99 mm in shell length, which is slightly smaller than the 1 mm interval of ridge lines on the textured surface, may be attracted to attach to more extensive areas of textured surface offered by the experimental substrata with broader branch widths. Therefore, it can be collectively deduced that in the present study, the fine branches (e.g., 1.6 mm), and the 1 mm textured surface are both attractive to small-sized mussels.

The conclusion of the preliminary experiment is in line with this hypothesis, in which the small-size mussels (<0.99 mm shell length) showed a varying behavioural response for attaching to the textured surface of the experimental artificial substrata versus the smooth surface depending on the branch width of the substratum. The proportion of small-size mussels on the textured surfaces of the experimental substrata with the narrowest branch widths (i.e., 1.6 and 3.7 mm) had no difference from the smooth surfaces, whereas for the experimental substrata with wider branch widths (i.e., 5.6, 7.4) there were significantly more small-size mussels on the textured surface (Fig. 4.A).

In the follow-up experiment, although there was no significant difference in the mean percent of small-size mussels among different branch widths and two sides of the experimental substrata, there was a similar trend that the proportion of the small-size mussels attached to the artificial substratum with the narrowest branch widths (i.e., 0.4 and 0.8 mm) were relatively lower than for the smooth surfaces, whereas for the experimental substrata with wider branch widths (i.e., 1.6, 2.4, 3.2, and 6.4 mm) there were significantly more small-size mussels on the textured surface. Moreover, in this follow-up experiment the percent of small-size mussels was typically less than 50 %, while the percent of small-size mussel was mostly more than 50 % especially on the experimental substrata with the narrowest branch widths (i.e., 1.6 and 3.7 mm) in the preliminary experiment. This different trend might be caused by the different size ranges of wild mussels used between these two experiments.

5. Conclusion

This study validates and emphasizes the vital influence of branch width and 1 mm ridged surface microstructure of substrate on the juvenile attachment of an ecologically and economically important New Zealand mussel species. After a step-by-step exploration, their preferred branching widths were identified (i.e., 0.4–2.4 mm), and it was determined that different mussel sizes are attracted to different surface structures and branching, which provides a very basic and strong knowledge background for future research involving the settlement behaviour of this species. Considering the possible different settlement preferences of marine organisms for substrata when comparing results from laboratory assays and field studies (Vucko et al., 2014), additional field studies still need to be undertaken to better understand the influence of these key morphological characteristics identified through the current study on juvenile mussel attachment. Collectively, the study has improved our understanding of the attachment preference of juvenile green-lipped mussels for varying morphological characteristics of substrata. The results also offer a possibility for greatly improving the harvesting of settling larvae and the subsequent retention of juvenile green-lipped mussels for aquaculture.

CRedit authorship contribution statement

Wenjie Wu: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Iain Anderson:** Resources, Methodology, Conceptualization. **Andrew G. Jeffs:** Writing – review & editing, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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