Settlement and metamorphosis in the veliger larvae of the South African abalone *Haliotis midae* exposed to ambient grown biofilms treated with conspecific mucous

A thesis submitted in the fulfilment of the requirement for the degree of

MASTER OF SCIENCE

of

RHODES UNIVERSITY

by

Jefferson Luke van Staden

Supervisor: Prof. Horst Kaiser
Co-supervisor: Dr Clifford Jones

March 2021

ABSTRACT

The South African abalone, *Haliotis midae*, is a commercially important species of mollusc which contributes significantly to the value of the South African mariculture industry. One of the primary challenges experienced by abalone farmers is the consistent production of juvenile abalone (spat) in sufficient volumes to keep stocking farms and facilitate expansion of the industry. One of the key production bottlenecks of *H. midae* is achieving adequate levels of larval attachment and metamorphosis (settlement). The larvae of *H. midae* are settled on polycarbonate plates which have been pre-conditioned with biofilms in seawater which is pumped ashore onto farms. The seasonal variability in settlement success reported by hatchery managers in South Africa is hypothesised to be as a result of different diatom species compositions colonising the settlement plates at different times of the year, with settlement success being lowest during the winter months.

The following study investigated whether the addition of conspecific mucous to biofilms could result in elevated settlement success, and whether there was potential for sterilisation of this mucous. A novel method of mucous application, spraying it onto the plates as opposed to pre-grazing, was tested in settlement assays and the trials revealed the following results:

- The addition of *H. midae* mucous induced significantly more larvae to attach to settlement plates, when mucous was harvested around the spawning season.
- Elevated attachment of larvae on mucous treated plates did not result in more post-larvae occupying the plates at the end of trials, and increased mortality is likely attributed to introduction of pathogens in conjunction with mucous.
- No increase in the final proportion of settled larvae which had metamorphosed or the rate at which they metamorphosed was observed between mucous application treatments and biofilm only treatments.

Subsequent trials assessed whether methods of mucous handling could reduce the biosecurity risk associated with mucous use, and so mucous was either UV irradiated or autoclaved. These trials revealed the following findings:

- No difference in attachment was seen between any treatments, including the untreated mucous. This is contrary to the findings of our initial experiments and illustrates that the attachment-inducing properties within mucous may be seasonally expressed.

- Numbers of observed larvae/post-larvae on plates applied with UV and autoclaved mucous where less stable than biofilms only, especially in the second trial, illustrating that mucous still presents a biosecurity risk even after undergoing these handling methods as it may act as a substrate on which pathogenic bacteria could colonise.

Key words: Haliotis, larvae, settlement, attachment, metamorphosis, mucous, aquaculture, biosecurity

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ACKNOWLEDGEMENTS

This research was supported and funded by HIK Abalone Farm (Pty) Ltd. and the Department of Science and Technology (DST).

I owe the biggest thanks to my supervisors, Prof. Horst Kaiser and Dr Cliff Jones for their patience, sound advice and unwavering guidance in completing this thesis. Each of you brought amazingly positive support in sometimes vastly different ways, but ultimately ensuring we made a success of this thesis. Words truly cannot express my gratitude towards the both of you.

I wish to express my sincerest appreciation to the hatchery staff at HIK Abalone Farm (Pty) Ltd., without your assistance this research would not have been possible. In particular, I would like to thank Matt Naylor, Devin Ayres and Sally Paulet for allowing me to learn some lessons the hard way but always being there when needed and encouraging me to succeed. You were there for the good, the bad and the ugly; your confidence in me and patience with my stress levels throughout this project has not gone unnoticed.

I would also like to thank my family for always believing that I could get it done, even when I myself could not see a light at the end of the tunnel. You can stop asking now, it’s done.

To the “Honde”, your support and encouragement was constant. You are a special group of characters who have left a lifelong impression on me, I will cherish our varsity memories to the end.

Lastly, I would like to dedicate this thesis to the loving memories of Richard Christian Sorensen (Grandpa), Bernice Cooper (Aunty Ber) and Travis Bradley Cooper (Trav). The loss of you all during the years it took to finish this thesis was truly devastating, my only wish is that the times we missed out on weren’t in vain and that the completion of this thesis can provide some comfort to the bitterness of opportunities missed.

Chapter 1 – General Introduction

Abalone are a high value genus of mollusc belonging to the family *Haliotidae*. Their value is attributed to the high demand for them as a seafood delicacy (Zhou, 2004; de la Cruz and Gallardo-Escarate, 2011). The commercial scale production of abalone has been the subject of much research, with specific emphasis on improving production consistency (Moss and Tong, 1992a; Sales and Britz, 2001). One of the critical limiting factors in the commercial production of abalone, and in particular for the South African Abalone *Haliotis midae*, is the consistent survival of abalone in the settlement phase. Settlement can be defined as a two-step process in the life cycle of *Haliotis* species, i.e., attachment and metamorphosis. Attachment is the process whereby free-swimming larvae leave the water column and attach onto a substrate, whereas metamorphosis describes the anatomical transition from attached larvae to a post-larval juvenile abalone. Traditional methods of settling larvae onto acrylic or plastic plates with a benthic diatom biofilm are highly variable and settlement success can be as low as 2% (Moss and Tong, 1992b; Gallardo and Buen, 2003). A better understanding of the drivers and inducers of settlement and metamorphosis is needed to achieve survival rates that are more commercially viable and consistent if abalone culture is to advance (Moss and Tong, 1992b).

The diatom plates used in commercial hatcheries display variability in species composition, density and abundance (Suzuki et al. 1987). This is attributed to seasonal changes in light intensity (Watson et al. 2004) and water temperature. The diatom film on plates is an important factor in determining the success of attachment, as filamentous diatoms may inhibit attachment by becoming attached to the larvae itself while prostate species at higher densities are preferred (Kawamura and Kikuchi 1992). Capinpin (2015) observed that in settling the larvae of the tropical abalone *Haliotis asinina,* initial attachment was high across a wide range of diatom species at six hours after inoculation. However, metamorphosis and early shell growth was significantly different between diatom species, suggesting that some diatom species are more suitable than others in maintaining settled abalone larvae. The age of the diatom biofilm is another factor affecting both attachment and metamorphosis during settlement. Roberts et al. (2007) showed that while older diatom films showed moderate settlement success, younger films that were used to induce settlement where diatom populations where still growing exponentially, consistently resulted in low rates of attachment as well as reduced likelihood of metamorphosis completion. Diatoms are the primary food source for post-larvae during the first three months of life until they can be weaned onto artificial diets. The majority of commercial abalone hatcheries rely solely on the incoming water pumped ashore as a source of diatoms, affording little control over the species composition which colonise and grow on the acrylic or plastic plates provided for settlement. Some hatcheries may culture preferred diatom species under controlled conditions and then inoculate settlement tanks using bulked-up stocks (Kim et al. 2013). The drawback to this is that this process intensifies the complexity of hatchery operation as a whole in South Africa; requiring skilled labour to culture and maintain stocks, importation of pure stock cultures to begin growing, specialised laboratory equipment as well as an increased budget to accommodate the purchase of fertilisers and increased electricity consumption necessary to control the environment of an algal laboratory. Should a farm consider this approach, it should be noted that maintaining the desired species composition could be complicated by: overgrazing by pests like Harpacticoid copepods, which compete for diatoms as a food source (De Troch et al. 2005), sloughing off of biofilms at high temperatures or overgrazing by abalone (pers. obs.). In the event that the initial species which were inoculated are lost, due to one or more of the factors mentioned, regrowth of the biofilm will be determined by the incoming species – essentially negating the efforts made to establish a biofilm of the desired diatom species.

Algae are an important determinant of abalone larval settlement. However, the coralline red algae onto which wild abalone settle (Morse and Morse, 1984) cannot be used in commercial hatcheries due to the impracticality of culturing and using such algae.

Not all coralline algae induce settlement and metamorphosis of Haliotis sp. larvae as effectively as others (Daume et al. 1999). While species belonging to *Sporolithon durum* have been found to induce settlement and metamorphosis rates as high as 85% in *H. laevigata* larvae, other species such as *Mesophyllum engelhartii* and *Hydrolithon rupestre* are less effective; with settlement and metamorphosis rates of 10% and 5% respectively. Monocultured species would be required to determine which species of coralline algae are appropriate for *H. midae* larval settlement and metamorphosis induction. In addition to requiring appropriate species identification, coralline algae are generally slow growing (Goldberg and Heine, 2008) and reliance on its development for settlement induction would extend the settlement plate conditioning period between batches, thereby reducing a hatcheries potential output. The coralline algae which induce settlement and metamorphosis of *Haliotis* larvae are encrusting, forming dense crusts comprised predominately of calcium carbonate (Darrenougue et al. 2013). These dense crusts can hinder light penetration between the polycarbonate plates traditionally used in the settlement departments of Haliotis hatcheries; if growth is allowed or encouraged over significant portions of a settlement plate, the resultant shading may hinder diatom growth on inner plates (pers. obs.). This latter point is of particular importance as the diatom biofilms grown on settlement plates not only act as attachment and metamorphosis cues, but serve as the predominant feed for newly metamorphosed post-larvae – while the coralline algae serve only as an attachment and metamorphic cue initially (Takami et al. 1997). Increased labour costs are also incurred with the use of coralline algae in South African hatcheries; as the plate scrubbing efficiency of labourers when scrubbing plates between batches, is reduced due to the highly adhesive encrusting growths of coralline algae (pers. obs.).

Other algal species that are easier to culture have been assessed for their suitability as a settlement-inducing cue for abalone larvae. Courtois de Vicose et al. (2010) showed that the green alga *Ulvella lens* not only induced settlement in *Haliotis tuberculata coccinea*, but that its suitability as a larval attachment attractant surpassed that of conventional benthic diatom films. In a follow-up study Courtois de Vicose et al. (2012) demonstrated that algal age plays a significant role in the suitability of *Ulvella* species as settlement attractants. Where 4-day old *U. lens* and *Ulva rigida germlings* resulted in 14% and 10% settlement, respectively, 45-day-old *U. rigida* and a 45-day-old combination of *U. lens* and *U. rigida* achieved 46% and 52% settlement. Despite the relatively low nutritional value of *U. lens* for newly metamorphosed post larval abalone under 3mm, where grazing efficiency is limited; this species can be used to settle larvae prior to the addition of conventional diatom films for further grazing (Daume, 2006). The maintenance and use of algae such as *U. lens* in a commercial hatchery environment requires even more specialist skill and equipment then culturing single species diatom cultures. *U. lens* is produced through a process of sporulation as opposed to simply splitting cultures to reduce algal density as one would do with diatoms. The sporulation process involves a 14-day dark phase for conditioning of broodstock plates at 10°C, followed by transferal to tanks for sporulation at 20°C in light (Hannon et al. 2014). The upscaling of *Ulvella sp.* cultures works on a surface area approach, whereby a small isolate is induced to sporulate onto a slightly larger surface area, that stock is then conditioned and sporulated onto multiple plates; the process is repeated until a sufficient number of plates is conditioned for commercial use (Hannon, pers. comm). This process may take several months to more than a year before *U. lens* usage can be implemented on a commercial scale (pers. obs.). Plates with new spores also need to be left for 40-45 days until they can be used for successful settlement (Courtois de Vicose et al. 2012). Depending on the size of the abalone hatchery, there may be an opportunity cost associated with allocating settlement tanks to conditioning *U. lens*, as opposed to spat production using traditional methods. In addition to the upscaling challenges, high infrastructural and equipment costs are brought about as a result of the need to maintain large volumes of water at constant temperatures for successful sporulation.

Abalone settlement in the wild is to a large extent driven by cues which indicate environmental suitability (Williams et al. 2009). Wild abalone larvae show a tendency to settle at higher rates in environments abundant with coralline algae. This observation motivated Morse et al. (1979a) to identify a possible biochemical signal which could be responsible for the higher rate of settlement. The neurotransmitter gamma-aminobutyric acid, or GABA, was identified as being a contributing factor to larval abalone settlement through its function as an attachment attractant. Subsequent studies have shown that its use in abalone larval attachment is significantly more successful than conventional diatom-covered plates (Moss and Tong, 1992b). Precise concentrations of GABA are used due to its toxicity at concentrations above 1 µM (Searcy-Bernal and Anguiano-Beltrán, 1998). Although a strong attractant for attachment, GABA alone is not a source of nutrition and as such when used alone does not result in high rates of metamorphosis. For this reason, it is used in conjunction with diatom films to achieve overall success in settlement (Roberts and Watts, 2010). GABA is produced in large volumes for use as food supplements in humans as GABA supplementation has been touted to have positive effects on the management of neurological disorders such as: depression, sleeplessness, cognitive impairment, and memory loss (Ngo and Vo, 2019). Whilst the supply of GABA may not be a concern the costs associated with using a human supplement as an agricultural product may be prohibitive, with GABA costing up to $US 654/Kg (Stewart et al. 2008). Furthermore, it has been suggested by Kaspar and Mountfort (1995) that GABA's efficacy as a settlement induction cue in *Haliotis* larvae is further compromised by the ability of bacteria to rapidly break it down. In order to circumvent this issue, some authors have used antibiotics such as penicillin or streptomycin during their trials in order to maintain its inductive abilities (Morse et al. 1979b). The prophylactic use of antibiotics is frowned upon in South African *H. midae* production systems, and would disqualify a commercial aquaculture facility from achieving environmental or organic certification.

The use of conspecific mucous to settle abalone larvae has also proved useful. Gallardo and Buen (2003) demonstrated that a combination of diatoms and mucous resulted in significantly higher larval attachment and survival over 10 days in comparison to diatoms alone. While mucous alone attributed to a higher level of attachment than mixed diatom cultures, examination of survival to day 10 showed no statistically significant difference in the number of post larval abalone on the plates between the two substrates. The fact that increased attachment is observed in treatments containing conspecific mucous may be as a result of the chemical composition of the mucous. Kuanpradit et al. (2012) identified three proteins in *H. asinina* trail mucous, which when purified and added to seawater elicited a response in the cephalic tentacle of conspecific abalone. This chemical signalling may relay some sort of information between conspecific abalone regarding environmental suitability. Other studies into abalone mucous and its suitability as an attachment-inducing substance revealed that one of the chemicals found in abalone mucous is GABA. Using mass spectroscopy, Laimek et al. (2008) confirmed the presence of GABA, a known inducer of abalone settlement, within dried abalone trail mucous samples. The technique of pre-grazing settlement plates with juveniles may have a two-fold advantage of not only laying down a conspecific mucous trail (Seki and Kan-no, 1981), but also removing excess and unfavourable diatoms (Moss, 1999). The mucous of gastropods comprises of protein and polysaccharide complexes, also known as mucopolysaccharides or proteoglycans (Davies and Hawkins, 1998). Seki and Kan-no (1981) suggest that the mucous may serve as a food source for settled larvae before their rasping radula is fully developed. For currently operating abalone farms, sourcing mucous in the volumes required for commercial scale application could be achieved relatively easily and at minimal additional cost to the operation. The use of mucous could be seen as organic and natural, and preferable to alternatives such as GABA which necessitate the use of antibiotics.

For abalone farms to be financially viable in their operations, economy of scale has a large impact on ensuring viability. Expansion to take advantage of the economy of scale requires greater investment from shareholders, and an increased effort from management to manage and mitigate risks in an effort to protect their shareholders' interests. Such risks include disease, and over the last few decades a number of significant outbreaks of disease have occurred on abalone farms globally. Australia experienced a severe outbreak of Abalone Viral Ganglioneuritis caused by Abalone Herpes Virus during 2005 (Hooper et al. 2007), and Chinese farms have been affected by a syndrome referred to as "Abalone shrivelling syndrome" which leads to the reduction in meat mass and eventually the mortality of stock (Wang et al. 2004). These outbreaks have brought the importance of biosecurity on abalone farms into focus. In South Africa the appearance of Abalone Tubercle Mycosis (Macey et al. 2011), the spread of shell dwelling polychaete worms such as *Boccardia sp.* and *Terebrasabella heterounicata* (Simon et al. 2004; Simon et al. 2006) and concerns over Vibriosis in hatcheries (Dixon et al. 1991) have highlighted the importance of effective biosecurity strategies which not only prevent the spread of disease between farms, but between departments on a single farm.

In light of the growing importance placed on biosecurity, the use of conspecific mucous in a hatchery may be seen as relatively high risk. Studies pertaining to the use of mucous as a settlement inducer in *Haliotis* larvae largely achieve pedal mucous deposition through the pre-grazing of settlement plates prior to the addition of larvae (Seki and Kan-no, 1981; Slattery, 1987; Gallardo and Buen, 2003). Such a method could facilitate direct transmission of pathogens and sterilisation of live abalone, especially the volumes that would be required to pre-graze settlement plates in the South African hatchery context, would prove an immense challenge, if not impossible. In order to mitigate the risks of disease spread, should mucous be used, it would need to be harvested separately from live abalone and undergo some method of sterilisation whilst still retaining its inductive properties. A financially viable method of sterilisation would need: to be simple enough so as to be scalable taking into account the level of training general farm workers have, be achievable through low-cost technology and make it possible to sterilise large volumes at a time.

Mucous and diatom combination treatments have been found to increase settlement in *H. asinina* (Gallardo and Buen, 2003)*, H. discus hannai* (Seki and Kan-no, 1981) *and H. rufescens* (Slattery, 1987). The effect of pre-grazing settlement plates using *H. midae* was investigated by Matthews and Cook (1995), however, the focus of their research was on the resultant diatom species composition after pre-grazing rather than its settlement-inducing potential. To date, there has not been a documented published study on the use of mucous to induce the larvae of *H. midae* to settle. The South African abalone farming industry is the largest outside of Asia, and many of the existing farms are expanding their grow-out infrastructure. In order for the industry to achieve its expansion goals, a consistent supply of high-quality spat will be required. There is potential for this to be achieved at low cost through the use of conspecific mucous, which may aid in reducing settlement variability. Increased spat production from hatcheries would not only afford South African abalone farmers the ability to increase selection intensity for the fastest growing spat, but also provide spat for South Africa to capitalise on its opportunities for ranching (De Waal et al. 1999) and reseeding its heavily poached wild stocks (Lambrechts and Goga, 2016).

The objectives of this study were to determine whether the addition of mucous to ambiently-grown biofilms would affect the attachment, rate of metamorphosis and final yield of post-larval abalone as a low cost and simple alternative to the complex or expensive methods described for improving spat production. Placing this study in context of the production cycle, experiments were run from the end of the 6-day free swimming larval period and ended after 4-6 days once larvae had been given enough time to settle and metamorphose into post-larval juveniles. Grow-out on settlement plates (usually 3 – 3.5 months) and weaning onto formulated feed and grow-out to transfer sized animals of 10mm (usually 3 – 3.5 months), was outside of the scope of this study. Settlement on mucous treated settlement plates has been investigated for other species, but to date no such investigation has been carried out for the species cultured in South Africa, *H. midae*. In addition to investigating this phenomenon in our local species for the first time, the method of mucous application was novel to our study and two methods of mucous handling were investigated for their potential to retain the reported settlement improvement properties, after attempting to reduce the biosecurity risk of using mucous. This has not been possible for other studies on mucous induction of settlement, where live abalone were used to deposit mucous.

Chapter 2 – General Methods

2.1 Overview of experiments

Two experiments were carried out for the collection of data for this thesis. The first experiment pertains to the aims of Chapter 3, where the effect of adding abalone mucous to ambient-grown biofilms was investigated for its potential to improve settlement induction, metamorphosis rate and proportion of all settled larvae at the end of trials, as well as overall number of post-larvae produced, as compared to the industry standard method of settling larvae onto ambient-grown biofilms without any additives. Four trials of this experiment were carried out, and the results are presented in Chapter 3 (Section 3.3). The second experiment was carried out to investigate the aims of Chapter 4, which were to determine whether the addition of mucous to ambiently grown biofilms would alter its potential for improving settlement success after undergoing novel handling methods in an effort to reduce its biosecurity risk to the hatchery. Two trials of this experiment were carried out, the results of which are presented in Chapter 4 (Section 4.3).

This chapter serves to describe the methods used which were consistent for all trials included in this thesis. Methods, which differed between experiments or trials are described in Chapter 3 (Section 3.2) and Chapter 4 (Section 4.2).

2.2 Experimental site and date

The experiments described in this thesis were carried out on HIK Abalone Farm (Pty) Ltd. This is a commercially operating land-based abalone farm located on the South West coast in the town of Hermanus (34°26’04.35”S; 19°13’12.51”E). The larval settlement induction trials were conducted in the farm’s hatchery; while the diatom biofilm preparation system was located in a well-lit section of the farm’s grow-out platform. System suitability and experimental trials were carried out during the period December 2016 – August 2017.

2.3 Larval source and rearing

Larvae for all trials were provided by the HIK Abalone Farm hatchery. These larvae were excess larvae produced for commercial batches. The wild and F1 broodstock used to produce commercial batches are divided into four groups - A, B, C and D. A different group was used to produce successive batches, until the broodstock in each room had been spawned; the rooms were then cycled again for the upcoming batches. Thus, larvae could not always be acquired from the same room for trials and overall availability was determined by the success of the spawning; where excess larvae were not available a trial had to be postponed to the following spawning. A record of the broodstock rooms that were used to produce larvae for each trial in this thesis was recorded (Table 2.1).

Table 2.1: Broodstock room used to produce the larvae for each experimental trial.

Chapter	Trial	Broodstock room spawned
3	1	C
	2	A
	3	B
	4	A
4	1	B
	2	A

Broodstock were induced to spawn by the addition of dilute hydrogen peroxide which is a successful method for inducing gamete release in ripe *Haliotis rufescens* (Morse et al. 1977). This method is the standard practice for obtaining gametes from ripe *H. midae* in the South African abalone aquaculture industry.

Males were observed to release sperm approximately 4-5 h after the spawning induction protocol was initiated. Soon after the addition of 20-30 ml of sperm to the tanks, the females released their eggs. Once the females stopped releasing eggs, aeration to the tanks was removed, which resulted in the settling of the eggs at the bottom of the tanks so that they could be removed. Eggs were siphoned into 20-l buckets and checked for adequate fertilisation, overall quality and density. Fertilisation of eggs was confirmed by the presence of either polar body formation or the commencement of cell division. Egg quality was determined based on the shape and size of eggs such that eggs presenting with deformed shells or irregular shapes, which could be attributed to polyspermy or insufficient broodstock conditioning time, were excluded from being used. Egg density was calculated by taking 100-µl subsamples from the 20-L buckets in an effort to determine what volume of each bucket would need to be transferred to hatch-out trays to avoid over- or understocking them. Once quality checks were complete, the eggs were loaded into high-density-polyurethane (HDPE) egg trays (840 x 840 x 20 mm) at a density of two million per tray with no inflowing water. The following morning, eggs had hatched into larvae displaying the typical whirlpool spiralling behaviour (Courtois De Vicose et al. 2007). Water was connected to the hatch-out trays and the swimming larvae overflowed into 1000-l conical larval rearing tanks. These conical tanks were supplied with 1.0 µm filtered water at a flow rate of 100 l/h and gentle aeration for the following five days until larvae had developed to the stage where they were deemed competent to settle. Competency to settle was noted by the development of the third tubule on the cephalic tentacle (Hahn 1989) (Figure 2.1). On day four, the day before competency was expected to occur, the water source was switched from the 18 °C heated water to ambient water. This was done to slowly acclimatise the larvae to the temperature at which they will be settled at the following day, without causing a thermal shock.

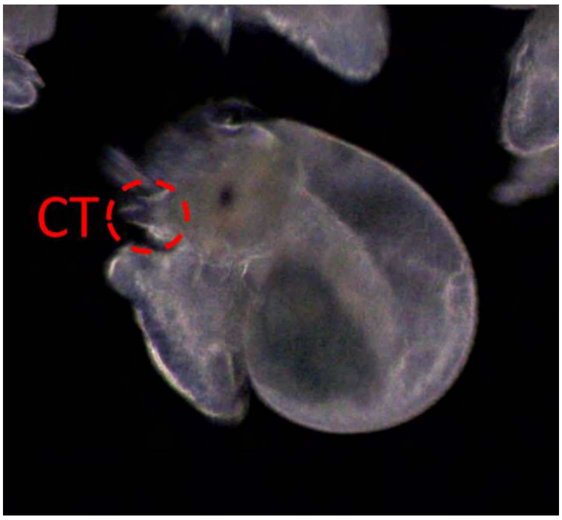


Figure 2.1: Larvae of *Haliotis midae* which have developed to the point of being competent to settle. The branched cephalic tentacle (CT) indicates competency.

2.4 Larval density estimation and preparation for trial

The competency to settle was determined on day five of larval rearing. The larvae were drained out of their rearing cones onto 100-µm sieves and transferred by rinsing the sieve into a 20-l bucket. The larval density within the bucket was then determined.

The bucket of larvae was gently mixed between each density sampling event; this was done as *H. midae* larvae display negative geotaxis (Genade et al. 1988), which results in them distributing unevenly within the bucket over a short time frame.

Mixing of the larvae within the bucket was achieved by holding a 2-l plastic jug horizontally and lowering it to the bottom of the bucket followed by lifting it back through the water column to the top, before finally pouring the contents of the jug out. The jug was then rotated 90° and the process was repeated, until the bucket had been mixed four times between each of the samplings.

Samples (0.5 ml, n=30) were taken using an adjustable pipette (Gilson Pipetman, P1000G - Plastic tip ejector), and transferred into a multiwell plate. These samples were observed using a dissecting microscope (Motic SMZ-171B, Motic Group Co., Ltd, Kowloon, Hong Kong) and the number of larvae in each replicate well was recorded. The mean number of larvae per sample was used to determine the number of inoculations required to achieve the desired stocking density per experimental unit.

2.5 Biofilm conditioning system

To prepare experimental settlement plates for settlement induction trials, a system was designed which would facilitate the growth of an ambient diatom biofilm on the plate surfaces. The system was designed to condition plates oriented vertically, using filtered ambient seawater and natural sunlight, as is standard practice in the industry. Prior to construction the system was modelled in Google SketchUp Pro 2016, to pre-emptively identify any potential design flaws (Figure 2.2).

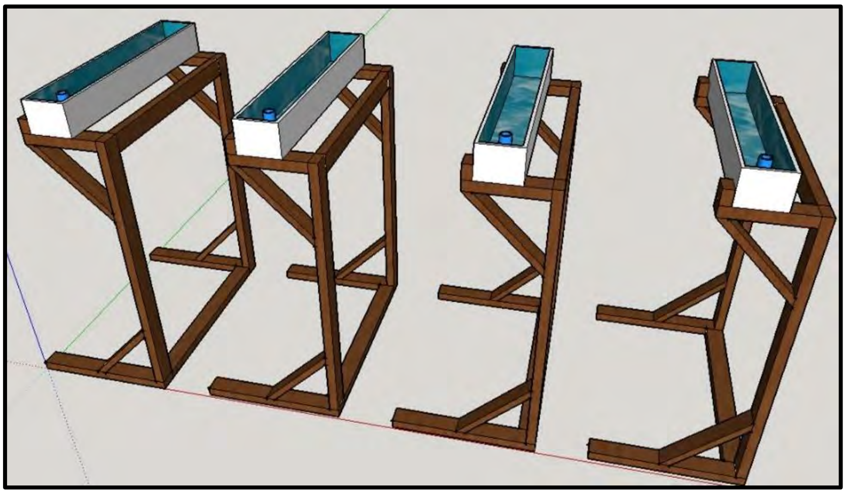


Figure 2.2: Design of the system used to condition experimental settlement plates with ambient diatom biofilms prior to settlement induction.

Square lead-free polyvinyl chloride (PVC) guttering (Marley SGH510) was cut to a length of 120 cm, and sealed at each end using the appropriate stopends (Marley SE501). Marine silicone (Bostik Marine, Permoseal (PTY) Ltd, Cape Town, South Africa) was used to fill the joins between the gutter and stopends to prevent leaking. The gutters were white in colour; as are hatchery settlement tanks, which were intentionally constructed using the reflective properties of white materials to promote even light distribution within the tank for diatom growth. At one end of the gutter a hole was drilled, and a 32-mm stub (32 mm QRD serrated face stub, EFFAST) was attached to the underside of the gutter in alignment with the hole. A 70 mm piece of 32 mm PVC pipe was cut and used as a standpipe for each gutter to maintain the water level in the gutter. The gutter section of the system was attached to a wooden frame using fascia brackets (Marley SK501), and these were attached to each of the frames supporting arms.

A piece of 3-mm thick mild steel was cut to the width of 45 mm and heated using a blow-torch, the heated end was then used to melt 30 pairs of grooves into the strips of plastic which connected the sides of the gutter to the bottom. These grooves were spaced in three-centimetre intervals and served as slots for the base of the settlement plates to slide into, enabling them to stand vertically (Figure 2.3)

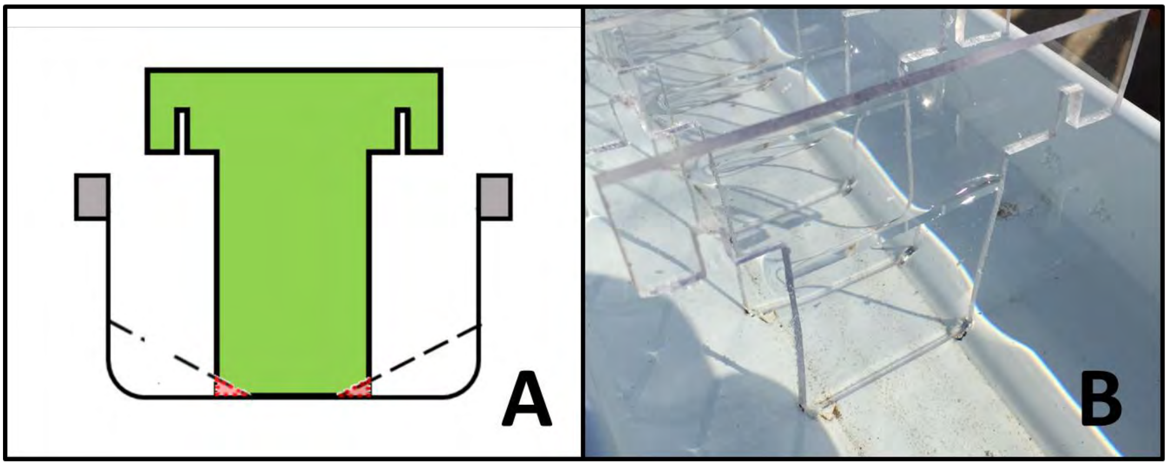


Figure 2.3: (A) Diagrammatic sketch illustrating settlement plate positioning within the gutter portion of the biofilm conditioning systems. Black dashed lines indicate the strips connecting the gutter sidewalls to the bottom, and red markings indicate the slots melted into these strips. (B) A photograph of the transparent experimental settlement plate placed in the white gutter at the start of a biofilm conditioning period.

A piece of 80 % shade cloth was attached to the fascia bracket support blocks. The shade cloth was cut to a size where it could lie over the gutter, blocking out the majority of the incoming sunlight when required. Four plate conditioning trough systems were built and placed in a well-lit area of the farm (Figure 2.4).

Figure 2.4: The ambient biofilm conditioning troughs were placed in a well-lit area of the farm to facilitate diatom growth. The shade cloth covers had not yet been attached to the frames at the time this photograph was taken. (A) indicates the mounted pipe line to supply water for the troughs and rinsing hose.

A standard canvas abalone grow-out tank (3.95 m x 0.875 m x 0.75 m) was used as a sump for the plate conditioning systems. Water was supplied to this tank from the farm's main header tank, where ambient seawater is filtered to 90 µm by two drum filters (Gamco Marine Engineering Services, Gansbaai, South Africa). A 50-µm filter bag (Aquamarine Water Treatment, PP-50-P02E) was hung under the canvas tanks tap to prevent any large particles from fouling the settlement plates. Water was pumped to the conditioning systems using a submersible pump (Sunsun HQB-2500 55W, SunsunGroup Co. Ltd, Zhejiang, China), via 32-mm black irrigation piping. This piping was connected to a wall-mounted 32-mm PVC pipeline (Figure 2.4), which had an outlet for each trough reduced to a 25-mm valve (Figure 2.4). This PVC pipeline terminated in a 32-mm flush valve, which allowed for daily flushing of the supply line and the connection of a hose pipe for *ad-libitum* rinsing (Section 2.6).

2.6 Ambient diatom biofilm conditioning procedure

In preparation for the conditioning of settlement plates the canvas tank that was used as a sump for the plate conditioning system was filled with 2600 l of water. Four litres of sodium hypochlorite (15 % M/V, Protea Chemicals) were added and aeration was supplied to the tank. The following morning the tank was drained, scrubbed, rinsed with sea water and re-filled. This was done to sterilise the tank of any fouling organisms and bacteria which could have grown in the tank between plate conditioning cycles.

While the production tank was filling up after being scrubbed, 30 experimental settlement plates were placed into each conditioning gutter (Figure 2.5). The submersible pump within the production tank was turned on and the valves to each conditioning trough set to a flow rate of 10 l/min. While this flow rate may seem high considering the volume of each gutter, lower flow rates would result in the temperatures within the troughs to rise and negatively affect diatom growth. The settlement plates were then left in these flow-through gutters, allowing for inoculation and growth of ambient benthic diatom films to be used in settlement trials. The conditioning period varied from 10 to 14 days to account for the changes in photoperiod throughout the entire trial period as plates required 14 days of conditioning to develop adequate biofilms in winter when daylength was reduced. The time required to grow a comparative biofilm during summer months was reduced to 10 days.

Figure 2.5: Experimental settlement plates at the start of a conditioning period. Shade cloth covers were removed during winter, as diatom overgrowth was less likely to occur during periods of shorter daylength.

During the conditioning period, rinsing of the settlement plates was required to wash off any particles adhering to the biofilm (Onitsuka et al. 2008) and to rinse off filamentous diatom growths (Seki 1980, Yanagihashi 1986), both of which may negatively affect the settlement of *Haliotis* larvae. Smothering of biofilms by sediment also inhibits light penetration and most likely negatively affects gaseous exchange during photosynthesis through a smothering effect; in the absence of an intervention, like rinsing the biofilms off, this smothering of light and/or restriction of gaseous exchange leads to the sloughing off of the diatom biofilms (pers. obs.)

A 25-mm hosepipe was plugged into the water supply line feeding the production tank and then the standpipe in a conditioning trough was removed. Once the trough had drained, the hosepipe was used to spray both sides of each plate before replacing the standpipe and allowing the trough to refill. Once one trough had been rinsed, the process was repeated for the next trough until all the plates in each of the troughs had been rinsed. This plate rinsing procedure was carried out *ad libitum* throughout the conditioning period and was repeated the day before an experimental trial began, in preparation for settlement.

2.7 Settlement system design

A system was designed to incorporate commercial hatchery settlement induction protocols, thus enabling increased transferability of the information gathered between research and development and the industry. The design had to take the following factors into consideration; space constraints, number of replicates required, similarity to commercial systems, sampling methodology and the provision of environmental conditions suitable for settlement and metamorphosis to occur. In an effort to pre-empt potential system design flaws the system was first modelled in Google SketchUp Pro 2016 before final construction (Figure 2.6).

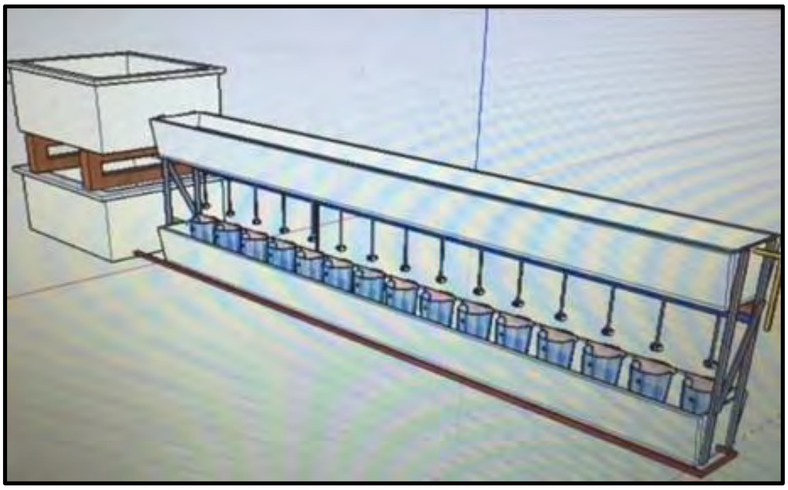


Figure 2.6: Concept design of the systems to be built for the settlement induction trials.

The system consisted of an upper and lower level. The lower level acted as a semi recirculating water bath, which would hold the settlement cups and buffer them from rapid ambient air temperature changes.

A polypropylene meat tray (495 mm x 350 mm x 178 mm) acted as a sump on the lower level, holding a submersible aquarium pump (Sunsun HJ-542 5W, Sunsun Group Co. Ltd, Zhejiang, China) and an aquarium heater (Eheim 3612 Aquarium Heater 50W, Eheim GmbH & Co., Deizisau, Germany). The sump was connected to a rectangular gutter via 25-mm PVC plumbing. The gutter was sealed at each end using the appropriate stopends and marine silicone (Bostik Marine, Permoseal (PTY) Ltd, Cape Town, South Africa).

The strip of plastic connecting the gutters front side-wall to the bottom was removed, thereby allowing sufficient space for the settlement cups to stand levelled when placed inside the gutter. The strip joining the gutter’s rear side wall and bottom was left intact to support the 25-mm PVC spray bar, which fed water into the gutter. The spray bar had two-millimetre holes (n=18) drilled evenly along its length, to provide even distribution of incoming water within the water bath gutter.

The gutter drained via a 32-mm stand pipe positioned at its centre, which redirected water back to the sump. The gutter part of the systems were raised on fibre glass frames to allow for gravity feeding of water back to the sumps.

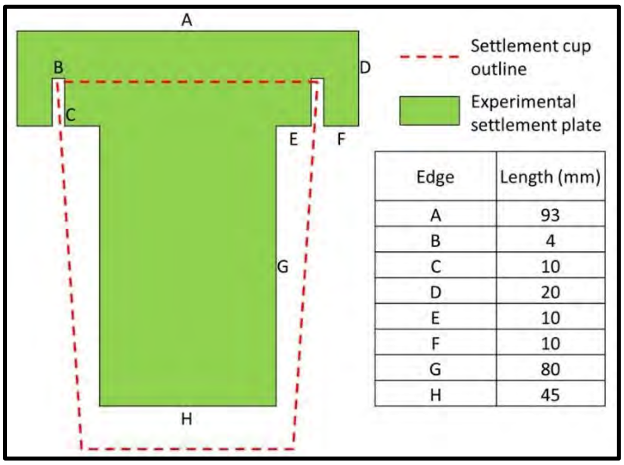
A second meat tray was positioned above the first using wooden supports. The upper meat tray served as a reservoir for the top gutter, supplying water to it via 25-mm PVC piping. Any excess water flowing into the top gutter drained through an overflow pipe attached to the opposite end. The reservoir was filled with 26-µm filtered ambient seawater via a pre-existing supply line at the experimental site. The top gutter was fastened to the frame 18.5 cm above the bottom gutter, and functioned as a water supply source to the settlement cups.

Four-millimetre holes (n=16) were drilled nine centimetres apart in the upper gutter, each hole was threaded and had a threaded barb inserted. A piece of four-millimetre silicone fish tank pipe was attached to the barbs allowing water to travel from the top gutter to each of the 16 cups standing in the bottom gutter. To regulate the flow of water into each cup, an intravenous drip flow regulator (Surgi-Plus I.V Flow Control Regulator 0-250 ml/h, ISO 13485:2003) was attached to the end of each fish tank pipe. Five systems were constructed allowing for 80 settlement cups to act as experimental units (Figure 2.7).

Figure 2.7: Four of the five experimental systems designed and built for the trials carried out for this thesis. This figure depicts the system prior to larval inoculation, when plates were being treated – as indicated by the multicoloured pegs which were used to mark plates and their respective treatments after randomisation had been carried out.

Acrylic cups were used as settlement vessels in which a settlement plate would be placed, similar to what is observed in a commercial settlement tank. The cups were filled with 200 ml of water and a mark was made at the meniscus of this water level. A 6.5-mm hole was drilled at this mark using a wood drill bit to allow for drainage when water was turned on. A 6.5-mm hole was the smallest diameter that allowed water to escape the cup at a steady rate by overcoming surface tension. Smaller diameter holes resulted in irregular drainage as the surface tension could not be overcome by the mass of the water between the rim of the cup and the hole. Any other type of drill bit other than a "wood bit" cracked the acrylic cups.

Experimental settlement plates were designed to be easily removable and hang vertically within the settlement cups. They were designed this way to fulfil the requirement of removal for sampling without dislodging attached larvae through excessive disturbance of the water within the cup. The conditioned section of the plate was supported in the middle of the cup with two notches cut out of the wider area of the plate slid over the rim of the cup, securing the plate in place. The plates were cut out of 3-mm transparent polycarbonate plastic, using a jigsaw, to the specifications seen in Figure 2.8.



Edge	Length (mm)
A	93
B	4
C	10
D	20
E	10
F	10
G	80
H	45

Figure 2.8: Experimental settlement plate design and positioning within an acrylic cup, providing a scaled down vertically oriented settlement substrate once conditioned.

2.8 Settlement procedure

In preparation for settlement the settlement systems were supplied with water. Once the sump was filled, the aquarium heaters and submersible pumps were switched on. Water was then supplied to the upper gutter and the flow regulators were primed, before being shut off. While water was not being fed to the settlement cups, excess water drained via an overflow pipe at the end of the upper gutter. The systems were left running from that point onward.

After larval density within the bucket was determined, the settlement cups were filled with 200 ml of water from the larval rearing system, and placed into their respective positions within the settlement systems. A settlement plate was then placed into each cup and where required it was treated as described in Chapter 3 and Chapter 4.

Once all system and treatment preparations were complete the authors hands were thoroughly disinfected and larvae were added to settlement cups in 0.5-ml inoculations using an adjustable pipette (Gilson Pipetman, P1000G - Plastic tip ejector). Cups were inoculated until a final stocking density of approximately 200 larvae/cup was achieved. This would allow for a settlement density of 2.78 larvae/cm^2 on the settlement plates should 100% attachment be observed. Larvae were added to the cups starting with the cup in position one of system one and finishing with position 16 of system five. After all the settlement cups in each system had been inoculated with larvae, the lights were switched off and the larvae were left to settle overnight. For the remainder of a trial's duration, lights were switched on at 07h30 in the morning and off at 17h00 in the afternoon.

2.9 Settlement plate sampling method

At 08h30 on the morning after larvae had been added to cups, the pre-primed flow regulators were set to 100 ml/h. Sampling began two hours later, after a full exchange of the water in the cups. This flushing period was employed to flush out unsettled larvae, which may stick to settlement plates as a result of surface tension when they are removed for observation (pers. obs.).

When sampled, a plate was removed from its cup, held for five seconds to allow excess water to drip off, and then observed under a dissecting microscope (Motic SMZ-171B, Motic Group Co., Ltd, Kowloon, Hong Kong). The number of successfully settled larvae and the number of metamorphosed larvae were recorded for each plate. Metamorphosis was indicated by the development of peristomal shell (Hahn, 1989) (Figure 2.9).

Figure 2.9: A metamorphosed post-larval *H. midae* observed under dissecting microscope during the system suitability trials in November 2016. Metamorphosis is identified by the growth of peristomal shell (PS), as indicated by the red outline.

Once the total number of larvae and the number of metamorphosed larvae had been recorded for each side of a plate, the plate was then discarded. The resampling of plates was not considered appropriate as a repeated measures experimental design could be confounded by the fact that physical disturbance of a settlement plate, when removing them from a water body after attachment has occurred, can result in the detachment of larvae upon re-submerging it (pers. obs.) Larval survival between sampling events could also have been artificially influenced by potential desiccation of larvae during a prior sampling event if a repeated measures approach to experimental design had been used. Therefore, all experimental units were considered independent of each other.

2.10 Sampling of environmental parameters

Once the plates had been sampled, the following water quality parameters were recorded from the settlement cups: temperature (°C), pH and dissolved oxygen (percentage saturation - %). Temperature was measured using a pocket thermometer (TH310 Pocket Thermometer, Milwaukee Electronics Kft, Szeged, Hungary), while pH and dissolved oxygen were recorded after placing a probe (YSI Inc. Pro Plus Multi-parameter meter, Yellow Springs, Ohio, USA) into the cup and allowing the reading to stabilise.

2.11 Data analysis software

All statistical analyses and tests were performed using Statisca 12 (Statsoft Inc., Oklahoma, USA). The figures and graphs presented in this thesis were generated in Statistica 12, and the tables were edited in Microsoft Excel. Each trial of each experiment was analysed separately to exclude the effects of differences in environmental variables (e.g., temperature), seasonality of algal species composition (Suzuki et al. 1987) and parental source (Deng et al. 2005) on the results obtained in different trials.

Chapter 3 - The addition of conspecific mucous to industry standard settlement substrates, and its effect on the settlement and metamorphosis of *H. midae* larvae

3.1 Introduction

Settlement of Haliotid larvae

Haliotis midae are dioecious broadcast spawners (Wood and Buxton, 1996), which release eggs and sperm to meet, fertilise and develop in the water column. As poikilotherms their metabolic rates are largely governed by temperature (Vosloo et al. 2013), and at a temperature of 18°C the fertilised eggs of *H. midae* hatch into trochophore larvae 12 hours post fertilisation (Visser-Roux, 2011). The larvae of haliotids are lecithotrophic, sustaining themselves using their yolk reserves while passing through a number of developmental stages (Courtois De Viçose et al. 2007). During the larval phase haliotids are planktonic, and develop within the water column until they are ready to settle. After developing from newly hatched trochophores into veliger, which are competent to settle, the larvae begin testing benthic surfaces using their cephalic tentacles in order to find a suitable surface on which to settle, shed their velum, metamorphose and adopt a benthic lifestyle for the remainder of their lives (Morse et al. 1980).

Settlement is a process which can be broken down into two distinct phases, namely attachment and metamorphosis (Roberts and Nicholson, 1997). Newly settled larvae will often attach to a surface in close proximity to crustose coralline algae that produce the neurotransmitter GABA, which has been identified as an inductive cue for the attachment phase of settlement (Morse et al. 1979a). Once attached to a suitable surface, the larvae of haliotids metamorphose into juveniles known as spat, as indicated by the development of peristomal shell and the commencement of feeding, grazing on diatoms in the early stages of their life (Shepherd and Daume, 1996).

Captive propagation of Haliotids

In captivity, *Haliotis* species are induced to spawn by a number of methods such as; exposure to hydrogen peroxide (Morse et al. 1977), thermal shock (Ino, 1952), exposure to UV-irradiated sea water (Kikuchi and Uki 1974, Ebert and Houk 1984) and desiccation followed by re-immersion (Carlisle, 1945). Once spawned, the eggs are fertilised and loaded into hatch-out trays. Depending on the species trochophore larvae will emerge from their eggs after as few as 6 hours in tropical species such as *Haliotis diversicolor supertexta* (Oba, 1964), or after as long as 24 hours in temperate species like *Haliotis rufescens* (Owen et al. 1984). The larvae are then reared until they are competent to settle, as indicated by branching of the cephalic tentacle and commencement of creeping behaviour (Stewart et al. 2008).

Once competency to settle has been determined abalone farmers will transfer larvae from their rearing tanks into settlement tanks. Settlement tanks traditionally contain vertically oriented plastic plates or sheets, which have been pre-conditioned for settlement by allowing them to develop biofilms composed of diatoms. Competent veliger larvae then settle on these diatoms-coated plates, shed their velum, develop peristomal shell and begin feeding (Takami and Kawamura, 2003). Post-larvae graze on diatoms until they reach a size where they are deemed large enough to be anaesthetised and de-plated into weaning tanks to begin transitioning them from a diatom feed source onto artificially formulated feeds and/or macroalgae (Dlaza et al. 2008).

Settlement and metamorphosis challenges

Settlement of larvae in the aquaculture setting is generally variable, with settlement rates as low as 0-10 % being recorded (Li et al. 2006). One of the primary challenges in post-larvae production is achieving attachment of an adequate number of larvae, without which spat production is not financially viable.

Attachment can be negatively influenced by transferring larvae to settlement tanks at an inappropriate stage of development (Moss and Tong, 1992a), larval deformation (pers. obs.), provision of unsuitable water quality (Tahil et al. 2015), and most noticeably through the failure to provide a biofilm which is deemed suitable by the larvae. Ko and Hur (2011) found that certain species of microalgae induced significantly more larvae of *Haliotis discus hannai* to attach than others. After 24 hours *Rhaphoneis sp*. had induced 98.3 % of larvae to attach, whereas *Cylindrotheca closterium* had only induced 46.2 % of larvae to attach. It has also been demonstrated by Baek et al. (2003) that overstory diatoms such as the stalk forming *Licmophora flabellata* are particularly ineffective at inducing attachment as compared to prostate species such as *Cocconeis scutellum* in *H. discus hannai*, with settlement rates of 16.3 and 43.1 %, respectively after three days.

In the event that adequate attachment is initially observed the second stage of settlement, namely metamorphosis, can also be negatively influenced by the species composition of the biofilm (Roberts et al. 2007). Post-larvae feed by rasping their radula over diatoms to break open the frustule and release the cellular contents, while the ability to effectively break open a diatom frustule is dependent on the shape of the diatom species, attachment strength and size (Kawamura et al. 1995). Without an adequate feed source, newly attached larvae may settle but not complete metamorphosis or die after metamorphosis due to energy depletion (Roberts et al. 2001).

The control over which species of diatoms colonise settlement plates is limited in abalone hatcheries, with many farms settling larvae on biofilms composed of whichever species is pumped ashore. Diatom species composition fluctuates throughout the year (Suzuki et al. 1987), and this is considered to be a primary contributor to the inconsistency of spat production in South African hatcheries.

A number of chemicals, microalgal species and the presence of conspecific mucous show potential for improving settlement success (Chapter 1). The following study assesses the potential for improved settlement success in *H. midae* through the use of conspecific mucous for the following reasons: (i) it is freely available on abalone farms at almost no extra cost, unlike chemicals such as GABA; (ii) it is does not require the capital outlay a microalgal production unit would; (iii) it would not require the skilled labour a microalgal production unit would require to be run and maintained and (iv) the potential for mucous to improve settlement success has not yet been evaluated for *H. midae.*

Aims

The aims of this study were to determine whether a) the addition of conspecific mucous to ambiently grown biofilms would yield greater attachment of larvae than ambient grown biofilms alone, b) whether mucous-treated biofilms result in more larvae than untreated biofilms at the end of a trial, c) whether the addition of mucous affects the proportion of settled larvae which metamorphosed by the end of a trial, d) whether the number of larvae observed on plates of mucous-treated biofilms and untreated biofilms can be comparable throughout the course of the trial and e) whether mucous addition affects the rate of metamorphosis. These aims were investigated through testing the following null hypotheses:

- H_{01} – An equal number of larvae will be attached to both ambient grown biofilms and those treated with conspecific mucous.
- H_{02} – An equal number of larvae will be counted on both ambient grown biofilms and those treated with conspecific mucous at the end of the trials.
- H_{03} – An equal proportion of larvae on both ambient grown biofilms and those treated with conspecific mucous will have initiated metamorphosis by the end of the trials.

3.2 Methods

Aspects of the methods applying to experiments in this chapter which were described in Chapter 2 include: experimental site (Section 2.2), larval source and rearing (Section 2.3), larval density estimation and preparation for trial (Section 2.4) biofilm conditioning system (Section 2.5), ambient diatom biofilm preparation procedure (Section 2.6), settlement system (Section 2.7), settling procedure (Section 2.8), settlement plate sampling method (Section 2.9), sampling of environmental parameters (Section 2.10) and the software used to analyse the data (Section 2.11).

3.2.1 Experimental design

Trial 1 and 2

For trials 1 and 2, either of two settlement substrates were provided for larvae to settle on. These treatments were diatom biofilms prepared as per Chapter 2 (Section 2.6) and mucous-treated diatom biofilms, prepared as per Chapter 3 (Section 3.2.5). Twenty-six experimental units of each treatment were prepared, which allowed for 13 replicates of each treatment to be sampled for larval settlement and metamorphosis on each sampling day. Sampling was carried out the morning after larvae were inoculated into cups (day 1) and on the last day of the trial (day 6). These were independent samples as all settling plates were used only once.

Trial 3 and 4

Trials 3 and 4 were carried out in the same way as trials 1 and 2, except for the sampling frequency and number of replicates sampled per day. Sampling intervals and replicates per treatment per day differed from trials 1 and 2 such that n=10 per treatment per day for days one and two and n=5 for days three to six respectively.

Trials 1 and 2 were designed to initially investigate dependent variables at specific times with higher replication per sampling; once this initial investigation was complete, trials 3 and 4 were designed to assess changes in the dependent variables over time with a lower number of replicates per sampling as a result of the experimental systems capacity.

In all trials conducted there was no mucous only treatment, mucous was either added to biofilms or biofilms were used without mucous addition. Conspecific mucous is not a suitable exclusive food source in the absence of diatoms for *Haliotis* post-larvae, as reported by (Takami et al. 1997) where the larvae of *H. discuss hannai* settled on mucous only plates – but experienced 100 % mortality after 3 weeks at a size of 711.9 µm, whereas those settled on a combination of mucous and *Cocconeis scutellum var. parva* survived for the entire 4-week trial, attaining a size of 1.4 mm. The provision of a treatment which would ultimately not be able to yield any post-larvae in a commercial setting was deemed unnecessary.

3.2.2 Randomisation

Block randomisation was used such that an equal number of plates from each diatom trough were allocated to each settlement system, the cup position to which each plate was allocated to within a particular settlement system was also randomised. Each settlement cup position was then randomly assigned a treatment, such that each system had an equal representation of each treatment for each sample day. Sampling day was then randomised in such a way that an equal number of plates were sampled for each treatment on each sampling day.

This degree of randomisation was used to avoid the potential for differences in biofilm characteristics of plates grown in different troughs to affect settlement and metamorphosis within a particular trial. The same method of randomisation was used for all 4 trials, taking into account the difference in sampling regimes between the first two trials (trials 1 and 2) and the next two trials (trials 3 and 4).

3.2.3 Mucous source

Trial 1

At HIK Abalone Farm (Pty) Ltd abalone are transferred from the hatchery for on-growing in plastic baskets, these plastic baskets contain HDPE racks comprised of plates which provide surface area for the animals to occupy. At the end of a 120-day period within a basket, a batch of abalone are anaesthetised and either split or graded to reduce density as the surface area per individual has reduced. Splitting is a quick density reduction process whereby all animals in a basket are anaesthetised by placing the basket into a holding tank saturated with CO_2, placed into tubs and used to stock new baskets at a target stocking density. Grading differs from splitting in that the abalone are placed onto a sorting table and are graded into size and shell quality categories before being stocked into new baskets with comparatively sized animals of the same shell quality.

Due to the red tide event during December 2016 – February 2017, all baskets were split en-mass to quantify stock loss on the farm. As such, there was a period where grading did not occur, as all the baskets had already been split.

The mucous for trial one was collected during the post-red tide splitting sessions. Whilst the abalone were still being split, they were not being graded; thus, the mucous was collected as “drip mucous”. This was defined as mucous which was poured out of the tubs in which the abalone were weighed, as opposed to collecting mucous by scraping off the grading table as was done in pre-study trials.

Trial 2

Mucous collection for trial 2 fell during the period directly after the mass splitting, where animals were neither being graded nor split. In order to maintain consistency with the use of “drip mucous” in trial 1, mucous in trial 2 was collected by suspending abalone in mesh bags over buckets. Abalone were removed from their baskets in the grow-out tanks, gently shaken to remove excess water, and placed into 5 mesh bags. Eight animals, ranging in size from 80-100 g, were placed into each bag before the bag was hung over a bucket for 10 minutes. This period of time allowed for sufficient volumes of mucous to be collected, without causing mortality of the animals. The mucous from all eight buckets was then pooled into a 5-L plastic tub for use in the trial.

Trials 3 and 4

The day before larvae were to be settled, mucous was collected from abalone in the grow-out section of the farm being “split and graded”, they ranged in size from 80-100 g. In trials 3 and 4 mucous was collected by means of scraping the mucous into a 5-l ice-cream container using a 11 cm x 11 cm piece of 3.0 mm polycarbonate sheet. The mucous was scraped off a canvas covered table located in one of the farms splitting and grading stations, and was defined as “grading mucous”. Each basket on the farm requires splitting at pre-determined intervals to avoid overstocking, the animals are anaesthetised by means of submerging the basket into a fibreglass tank filled with seawater and gassed by means of CO_2 injection. The abalone become weak and are removed from their basket and placed into a plastic dish, in which, they are weighed before being placed onto the grading table. Mucous collection took place between each new basket being graded. Any excess water on the table was removed once the animals from a single basket had been graded, prior to the next basket’s animals being placed on the grading table. This was done as to maintain the consistency of the mucous.

3.2.4 Mucous handling and storage

The mucous was collected between 014h00 and 16h30, the afternoon before settlement experiments were to begin. The container’s lid was put on and the closed container was sealed in a plastic bag. The sealed containers were then stored overnight in a fridge to prevent degradation of the mucous.

At 04h30 on the morning of a settlement trial, the mucous was poured into a 50-ml syringe with a piece of 100-µm filter mesh attached to the tip and 50 ml was filtered at a time until the entire sample collected had been filtered. The mucous was filtered to remove any debris from the sample (e.g., amphipods, shell fragments, etc.) which was collected with the mucous off the grading table. The mucous was filtered into a household spray bottle, the bottle was then sealed in a plastic bag and placed back in a fridge until it was to be applied.

3.2.5 Mucous application

Once settlement plates had been transferred from the conditioning troughs to their respective settlement system positions, the plates that had been randomly selected to receive mucous addition were treated. This was done by setting the household spray bottle to a fine mist and spraying each side of a settlement plate twice from a distance of 10 cm away. The plate was then gently shaken, allowing excess mucous to drip off before being placed back into its respective cup.

Mucous application was carried out only after larval density and the required number of inoculations per cup had been determined, in order to avoid risking the pre-exposure of all larvae to mucous.

3.2.6 Equipment sterilisation

New spray bottles and 5-l plastic tubs were used for each trial to avoid the mixture of mucous between trials and for general biosecurity measures. All other equipment used to handle the mucous, which could be thoroughly disinfected (e.g., syringes, scrapers and 100-µm filter mesh) was disinfected between trials by submerging and rinsing off the equipment with boiling water. This method of boiling and rinsing with boiled water was repeated three times before any equipment was re-used.

3.2.7 Sampling interval

Sampling was first carried out at approximately 10h30 the morning after larvae were added to settlement cups, this was approximately 18.5 hours after the larvae had been added to their respective cups. Sampling intervals for specific trials can be found under the experimental design section of this chapter (Section 3.2.1).

Plates were sampled in the order they were randomly settled, so that the duration between inoculation and sampling was as equal as possible for all replicates sampled at any particular time.

3.2.8 Statistical analyses

All dependent variables were checked for compliance with the assumptions required for parametric statistical testing for each sampling day. Equality of variance was assessed using Levene's test for homogeneity of variance at a significance level of $p < 0.05$ (Levene, 1960). Normality of residual distribution was assessed using the Shapiro-Wilk normality test at a significance level of $p < 0.05$ (Shapiro and Wilk, 1965).

Trials 1 and 2

The dependent variables were then analysed using T-tests for independent samples (“Student” William Sealy Gosset, 1908). Where data failed to meet the assumption of equality of variance, Welch's unequal variances *t*-tests were used at a significance level of $p < 0.05$ (Welch, 1947).

Trials 3 and 4

In trials 3 and 4 additional tests were conducted. Here, a multifactorial analysis of variance with a test for interactions between main effects was used to test for the effect of treatment and day of sampling on a) the number of larvae counted using data from all days, and b) on the percentage of larvae metamorphosised using only data from day 2 to 6, as no metamorphosis occurred in any treatment on day 1.

3.3 Results

Trial 1

The percentage of inoculated larvae which settled by observation day 1 did not significantly differ between treatments (T-test for independent samples; $t = 1.78$; $p = 0.088$). In average 39.58 ± 1.28 % of larvae had settled by observation day one.

There was no statistically different average number of settled larvae observed between the two treatments on observation day 6 (T-test for independent samples; $t = 0.62$; $p = 0.540$). The average number of settled larvae per plate was 79.04 ± 3.72 on day six.

The proportion of larvae which had metamorphosed by observation day six did not differ significantly between treatments (T-test for independent samples; $t = 0.26$; $p = 0.794$). In average 96.12 ± 0.58 % of the settled larvae observed on settlement plates had metamorphosed by day six.

Table 3.1 Mean (± standard error) settlement, number of larvae observed per plate (± standard error), Proportion of settled larvae which had metamorphosed (± standard error) and water quality parameters in tanks where abalone larvae were settled on either a diatom biofilm (D) or a diatom biofilm inoculated with adult abalone mucous (MD) after either one- or six-days post settlement during trial 1 (T-test for independent samples).

	Time (d)	D	MD	$t_{(24)}$	p
Settlement (%)	1	37.39±1.85	41.76±1.62	1.78	0.088
No. Larvae observed (Total)	6	81.38±6.38	76.69±4.02	0.62	0.540
Metamorphosis of settled larvae (%)	6	96.28±0.85	95.97±0.81	0.26	0.794
Oxygen saturation (%)	1	96.69±0.59	95.75±0.41		
	6	96.84±0.45	96.46±0.44		
Temperature (°C)	1	14.15±0.07	14.11±0.06		
	6	14.06±0.04	14.09±0.04		
pH range	1	7.84 – 7.95	7.86 – 7.95		
	6	7.76 – 7.91	7.77 – 7.91		

Trial 2

The percentage of inoculated larvae which settled by observation day one was significantly different between treatments (T-test for independent samples; t = 2.58; p = 0.017). A greater proportion of inoculated larvae settled on mucous-treated biofilms (x̄ ± SE = 37.48 ± 2.36) as opposed to untreated biofilms (x̄ ± SE = 30.01 ± 1.68) (Fig. 3.1; Table 3.2).

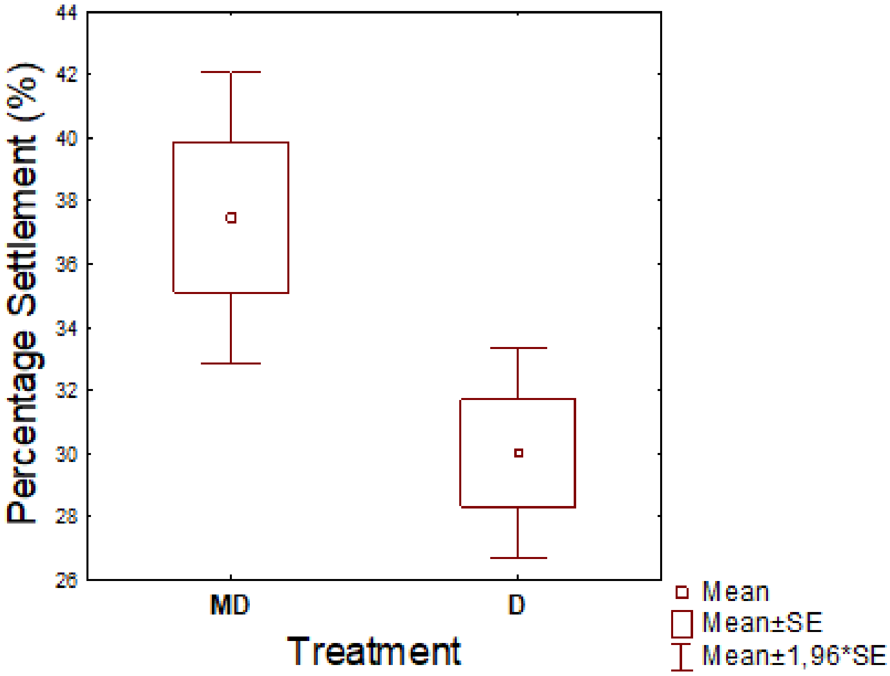


Figure 3.1 The proportion of larvae which had settled on experimental settlement plates by observation day one, in Trial 2. Settlement plate treatments provided for larvae were ambient grown diatom biofilms (D), or ambient grown diatom films treated with abalone mucous (MD). SE= standard error of the mean.

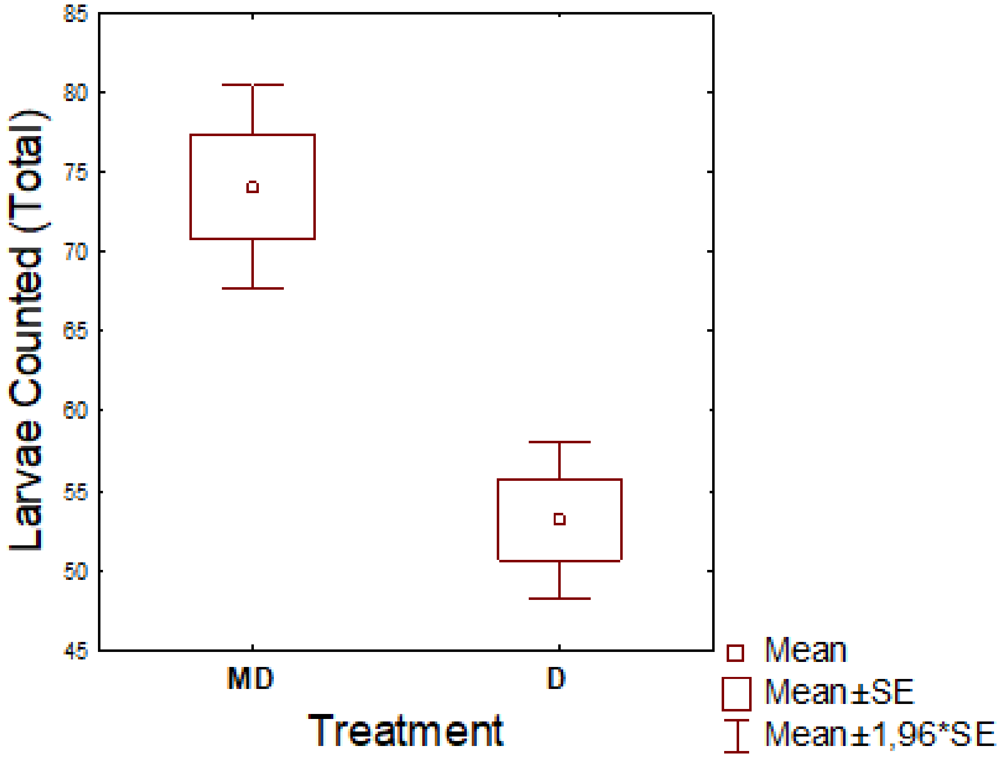


Figure 3.2 The cumulative number of metamorphosed and unmetamorphosed larvae observed on experimental settlement plates, on observation day six, in Trial 2. Settlement plate treatments provided for larvae were ambient grown diatom biofilms (D), or ambient grown diatom films treated with abalone mucous (MD). SE = standard error of the mean

There was a significant difference in the number of settled larvae observed between the two treatments on observation day six (T-test for independent samples; $t = 5.05$; $p<0.001$). The mean number of settled larvae per plate (± SE) was 74.08 ± 3.28 on mucous treated plates, and 53.15 ± 2.53 on untreated biofilms (Fig 3.2; Table 3.2).

The proportion of larvae which had metamorphosed by day six did not differ significantly between treatments (T-test for independent samples; $t = 1.33$; $p = 0.196$) and in average over both treatments 95.86 ± 0.56 % of the settled larvae observed on settlement plates had metamorphosed by day 6 (Table 3.2)

Table 3.2 Mean (± standard error) settlement, number of larvae observed per plate (± standard error), Proportion of settled larvae which had metamorphosed (± standard error) and water quality parameters in tanks where abalone larvae were settled on either a diatom biofilm (D) or a diatom biofilm inoculated with adult abalone mucous (MD) after either one- or six-days post settlement during trial two (T-test for independent samples, p<0.05).

	Time (d)	D	MD	$t_{(24)}$	p
Settlement (%)	1	30.01±1.68	37.48±2.36	2.58	0.017
No. larvae observed per plate (Total)	6	53.15±2.53	74.08±3.28	5.05	p<0.001
Metamorphosis of settled larvae (%)	6	95.13±0.88	96.59±0.67	1.33	0.196
Oxygen saturation (%)	1	94.97±0.58	94.41±0.39		
	6	95.27±0.40	95.84±0.38		
Temperature (°C)	1	16.43±0.06	16.39±0.04		
	6	16.55±0.01	16.54±0.02		
pH range	1	7.77 – 7.91	7.81 – 7.91		
	6	7.80 – 7.88	7.80 – 7.88		

Trial 3

The proportion of larvae which had settled by observation day 1 was significantly greater in the mucous application treatment as compared to the biofilm treatment lacking mucous application (T-test for independent samples; t = 5.47; P<0.001). In this trial, 22.62 ± 1.89 % of the larvae added to cups containing mucous treated plates settled by observation day one, whereas only 9.59 ± 1.45 added to cups with unaltered ambient biofilms settled by day one (Figure 3.3).

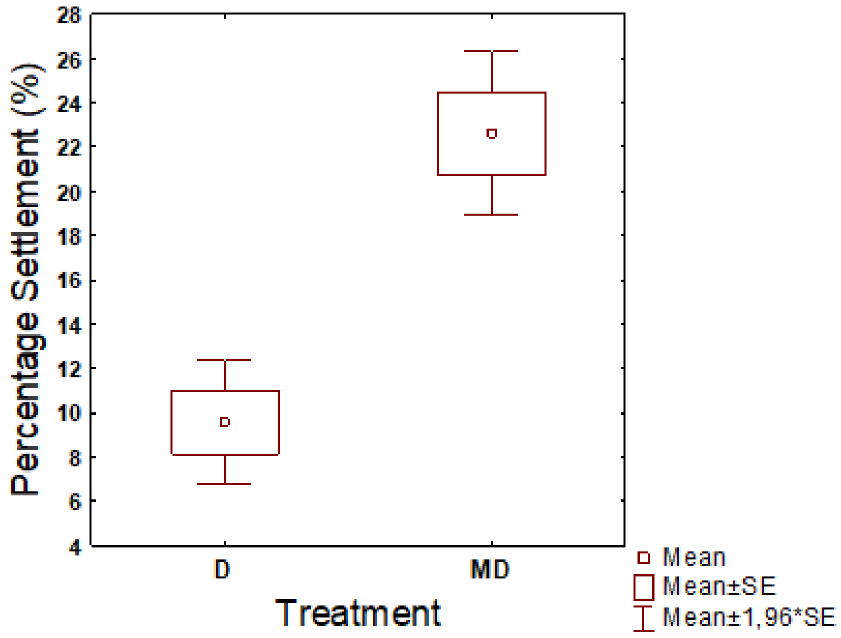


Figure 3.3 The proportion of larvae which had settled on experimental settlement plates by observation day one, in Trial 3. Settlement plate treatments provided for larvae were ambient grown diatom biofilms (D), or ambient grown diatom films treated with abalone mucous (MD). SE = standard error of the mean

The number of larvae observed on settlement plates of the two treatments was significantly higher on the mucous-treated plates on day 1 (T-test for independent samples; t = 5.47; p<0.001), with the mucous-treated plates having 48.1 ± 4.02 larvae per plate whereas the biofilm only plates had 20.4 ± 3.09 larvae per plate. The number of larvae observed on mucous-treated plates reduced over the duration of the trial (factorial ANOVA, F < 0.0001) until the final day of the trial, where the number of larvae observed per plate was not statistically different between treatments (Tukey's post-hoc test, p = 0.99). On the final day of the trial, the number of larvae observed per plate on mucous-treated biofilms and ambiently grown biofilms without mucous addition was 15 ± 6.03 and 8.8 ± 2.25, respectively (Figure 3.4).

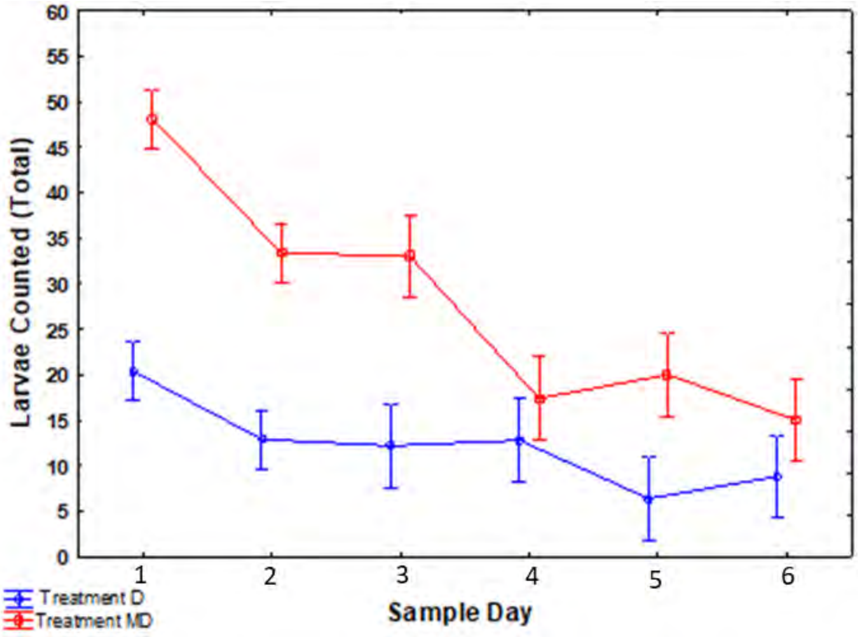


Figure 3.4 Mean number of larvae observed (± standard error) on settlement plates where abalone larvae were settled on either a diatom biofilm (D) or a diatom biofilm treated with adult abalone mucous (MD) one to six days post settlement during trial three.

The proportion of settled larvae which had metamorphosed between the two treatments followed a similar trend over time (Figure 3.5), except for day four, where a significantly greater proportion of settled larvae had metamorphosed on mucous-treated biofilms as compared to untreated biofilms (Factorial ANOVA with a significant interaction between main effects p<0.037, Tukey's post-hoc test for day 4, p < 0.017). The proportion of larvae which had initiated metamorphosis on day four on mucous-treated biofilms and untreated biofilms was 87.38 ± 5.48 and 46.59 ± 9.31 respectively. Despite this, proportional metamorphosis of settled larvae had converged by day six, where the overall proportion of settled larvae which had metamorphosed in both treatments was 73.95 ± 9.90 % (Figure 3.5).

Table 3.3 Mean (± standard error) Oxygen saturation (%), Temperature (°C) (± standard error) and pH range in tanks where abalone larvae were settled on either a diatom biofilm (D) or a diatom biofilm inoculated with adult abalone mucous (MD) one to six days post settlement during trial 3.

	Time (d)	D	MD
Oxygen saturation (%)	1	88.73±01.03	85.87±02.28
	2	90.03±00.94	90.34±01.24
	3	90.72±02.34	89.78±01.90
	4	92.88±00.96	92.98±01.20
	5	94.80±01.06	95.74±01.15
	6	97.22±00.54	96.74±00.93
Temperature (°C)	1	18.89±00.17	18.92±00.17
	2	16.05±00.07	16.05±00.08
	3	16.24±00.19	16.18±00.20
	4	18.16±00.15	18.16±00.17
	5	18.60±00.34	18.52±00.38
	6	18.72±00.35	18.74±00.34
pH range	1	7.80 – 7.94	7.70 – 7.94
	2	7.98 – 8.07	7.88 – 8.07
	3	7.76 – 8.02	7.86 – 7.98
	4	7.82 – 7.89	7.83 – 7.93
	5	7.80 – 7.88	7.79 – 7.89
	6	7.80 – 7.90	7.84 – 7.90

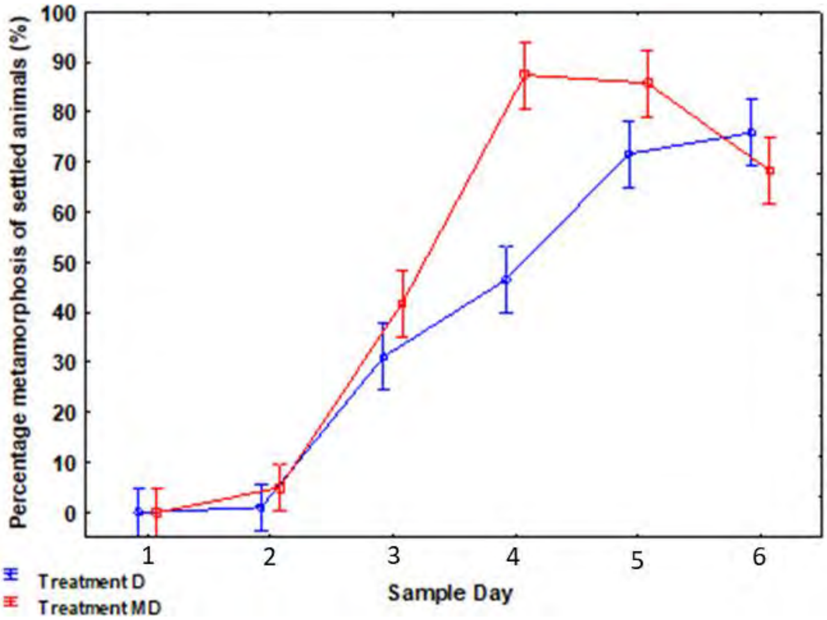


Figure 3.5 Mean percentage of settled larvae which had metamorphosed (± standard error) on settlement plates where abalone larvae were settled on either a diatom biofilm (D) or a diatom biofilm inoculated with adult abalone mucous (MD) one to six days post settlement during trial three.

Trial 4

The proportion of larvae which had settled by observation day one was significantly greater in the mucous application treatment as compared to the biofilm treatment lacking mucous application (T-test for independent samples; t = 4.59; p < 0.001). In this trial 41.39 ± 02.35 % of the larvae added to cups containing mucous-treated plates settled by observation day one, as compared to 27.27 ± 01.98 % (Table 3.4, Fig 3.6.

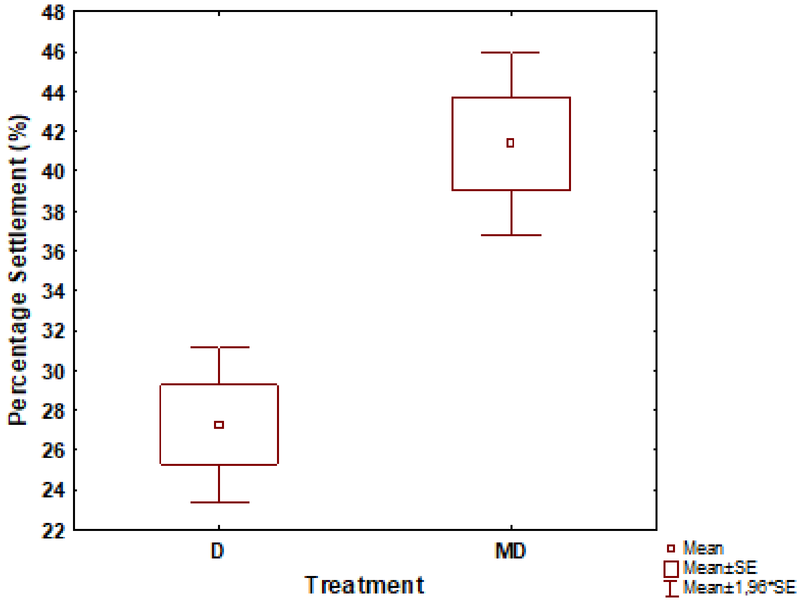


Figure 3.6 The proportion of larvae which had settled on experimental settlement plates by observation day one, in Trial 4. Settlement plate treatments provided for larvae were ambient grown diatom biofilms (D), or ambient grown diatom films treated with abalone mucous (MD). SE = standard error of the mean.

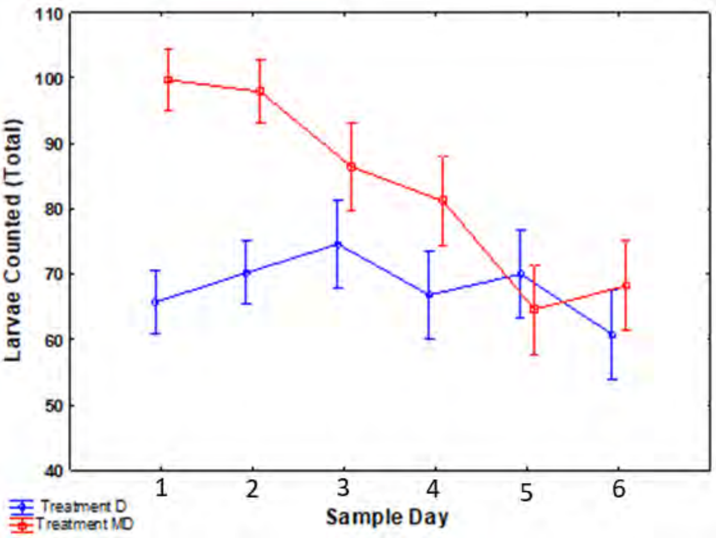


Figure 3.7 Mean number of larvae observed (± standard error) on settlement plates where abalone larvae were settled on either a diatom biofilm (D) or a diatom biofilm inoculated with adult abalone mucous (MD) one to six days post settlement during trial four.

The number of larvae observed on settlement plates of the two treatments was a function of both treatment ($p < 0.001$) and time ($p < 0.001$) with a significant interaction between these two main effects (Factorial ANOVA, interaction term; $p < 0.015$). The values were significantly higher on mucous-treated plates for observation days one and two (Tukey's post-hoc test, $p < 0.05$). Mucous-treated plates yielded 99.70 ± 05.66 and 97.90 ± 03.86 larvae on days one and two respectively; biofilms which were not treated with mucous yielded 65.70 ± 04.77 and 70.20 ± 04.67 larvae on days 1 and 2 respectively (Figure 3.7). The number of larvae observed per plate for mucous treated biofilms decreased throughout the duration of the trial after day two, and no difference between treatments in the number observed per plate was noted by the end of the trial (Tukey's post-hoc test, $p > 0.05$), with an average of 64.5 ± 6.23 larvae being observed on experimental settlement plates on day six irrespective of treatment (Figure 3.7)

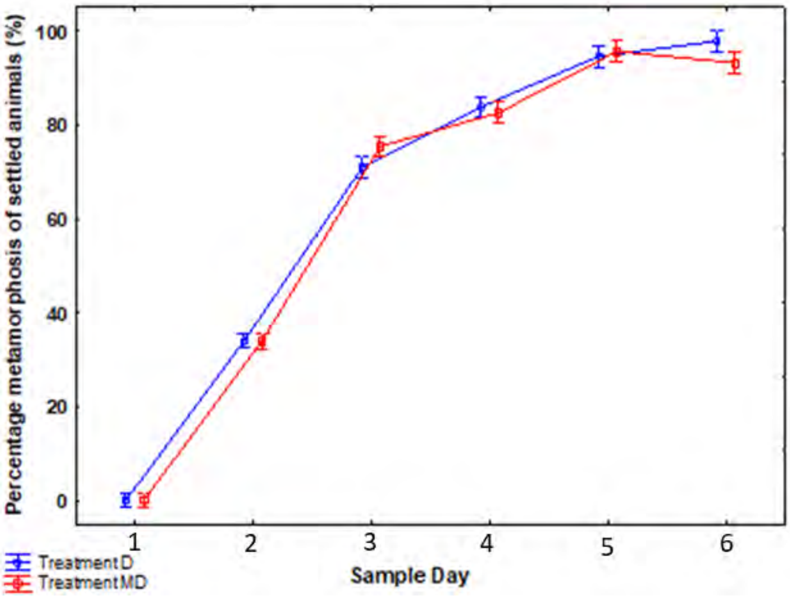


Figure 3.8 Mean percentage of settled larvae which had metamorphosed (± standard error) on settlement plates where abalone larvae were settled on either a diatom biofilm (D) or a diatom biofilm inoculated with adult abalone mucous (MD) one to six days post settlement during trial four.

There was no statistically significant difference between treatments in the proportion of settled larvae which had metamorphosed on any of the observation days (Factorial ANOVA, treatment effect, $p < 0.94$; time effect, $p<0.001$; interaction term, $p = 0.52$). By day six 95.43 ± 01.80% of settled larvae had metamorphosed, irrespective of treatment (Figure 3.8).

Table 3.4 Mean (± standard error) oxygen saturation (%), Temperature (°C) (± standard error) and pH range in tanks where abalone larvae were settled on either a diatom biofilm (D) or a diatom biofilm inoculated with adult abalone mucous (MD) one to six days post settlement during trial four.

	Time (d)	**D**	**MD**
Oxygen saturation (%)	1	88.13±01.52	83.70±01.59
	2	86.19±01.49	83.66±01.53
	3	83.56±01.40	82.00±00.98
	4	84.52±02.21	83.10±03.26
	5	95.98±01.64	90.34±02.57
	6	91.98±01.23	91.52±01.12
Temperature (°C)	1	17.86±00.14	17.86±00.14
	2	18.53±00.16	18.53±00.15
	3	17.90±00.28	17.96±00.29
	4	18.36±00.07	18.40±00.06
	5	18.62±00.19	18.68±00.21
	6	17.30±00.22	17.32±00.22
pH range	1	7.74 – 7.87	7.72 – 7.87
	2	7.70 – 7.87	7.68 – 7.79
	3	7.78 – 7.84	7.74 – 7.86
	4	7.76 – 7.91	7.71 – 7.88
	5	7.80 – 7.99	7.78 – 7.93
	6	7.80 – 7.85	7.81 – 7.83

3.4 Discussion

Settlement

The attraction of competent larvae to settle was influenced by the presence or absence of conspecific mucous on settlement plates in all but one trial. In trial one there was no difference in the proportion of larvae which settled, but significantly more larvae settled on mucous-covered plates in trials 2, 3 and 4.

In trial one larvae were settled at 13.7-14.3°C, which is on the lower end of the spectrum for which *H. midae* larvae have been recorded to settle (Muller, 2003). Trial one also resulted in the highest settlement observed of the trials with 39.58 ± 1.28 % of larvae settling. Cold sea temperatures are usually indicative of upwelling events, which bring nutrients to the surface of water columns (Field et al. 1980). Thomas et al. (1983) demonstrated that diatoms of the same species cultured at differing nutrient levels differed in their proximate composition. Cultures of *Dunaliella primolecta* grown in nitrogen sufficient media produced cells with protein, lipid and carbohydrate proportions of 64.2%, 23.1% and 12.6% respectively, while these ratios were altered when grown in nitrogen deficient media, with protein reducing to 26.9%, lipid reducing to 10.5% and carbohydrates increasing to

50.8%. (Thomas et al. 1983). It is possible that the upwelling prior to the settlement of trial 1 may have resulted in diatom nutrient and species profiles which were favourable to all larvae, thereby masking the effect of mucous-treated biofilms.

Trials 2, 3 and 4 demonstrated that larvae were positively attracted to settle on biofilms treated with conspecific mucous. Increased settlement on plates which have been pre-exposed to conspecific mucous has been described for *H. discus hannai* (Gallardo and Buen, 2003)*, H. rufescens* (Slattery, 1987), *H. diversicolor* (Bryan and Qian, 1998), and while no studies have tested this phenomenon in *H. midae*, the results of these trials suggest that *H. midae* larvae will also settle preferentially on settlement plates which have been pre-exposed to conspecific mucous. Laimek et al. (2008) found that the mucous of *Haliotis rubra* contained the neurotransmitter GABA, and that it was also present in the mucous secretory cells on the foot epithelia of *H. asinina* and *H. diversicolor.* GABA has been determined to be a settlement attractant for a number of species of abalone (Morse et al. 1979a; Gapasin et al. 2004; Roberts and Watts, 2010). Further to this, Kuanpradit et al. (2012) found that the mucous of *H. asinina* contained proteins, which elicited cephalic tentacle responses. Cephalic tentacles are the sensory appendages which Haliotid larvae use during the attachment phase of settlement to determine the suitability of a settlement substrates. It is thus hypothesised that *H. midae* mucous may also contain proteins similar to those found by Kuanpradit et al. (2012), or that it contains GABA, which resulted in the increase in attachment on those plates that were treated with conspecific mucous.

Total number of larvae observed

Gallardo and Buen (2003) found that although mixed diatoms and mucous resulted in significantly more larvae attaching than on mixed diatoms only on day one, by day three the average numbers of surviving post-larvae were not different between these two treatments. A similar trend was found in the trials carried out in this thesis; whilst there was an increase in attachment, lower larval retention after attachment, leading to the similar larval numbers observed between treatments at the end of trials, infers that there was greater mortality on plates treated with conspecific mucous. Conspecific mucous, while potentially acting as a food source for newly settled larvae is not suitable as an exclusive food source (Takami et al. 1997). There are two hypothesised reasons for the observed trends; (i) mucous acted as an initial food source for larvae which were not able to effectively feed on diatoms, and once the mucous had run out survival between the treatments converged as the treatments become more similar (i.e., diatom only food source), or (ii) the addition of conspecific mucous may have introduced pathogens or acted as a substrate for pathogens to proliferate on, which would have negatively affected survival of the increased number of larvae attracted to settle.

The total number of larvae observed at the end of the trials was similar between treatments with the exception of trial two, where more larvae were observed on mucous treated diatom biofilms as opposed to diatom biofilms only. Where it is hypothesized that the introduction of pathogens may have caused the increased mortality on biofilms treated with mucous in the other trials, this may not always be true. Choresca et al. (2010) determined that *Vibrio alingolyticus* comprised 50% of the bacterial community isolated from *H. discus hannai* juvenile and adult mucous, their study was not repeated over time and the results were obtained from a single sampling event where mucous samples were pooled for analysis. It is suggested that either (i) bacterial communities of abalone mucous would change in composition over time in response to either environmental fluctuations or the hosts health, or (ii) that not all abalone have mucous bacterial communities dominated by pathogens. Either of the aforementioned assumptions could lead to the difference in survival observed in trial two but not in the other trials as mucous could have been harvested for trial two at a time when pathogen concentrations were low or from animals which had a lower concentration of pathogens. In future trials microbiome composition of the abalone mucous used should be analysed to assist with interpreting the results of such trials.

Metamorphosis

In trials one and two metamorphosis was quantified only at the end of the trial, and no differences were observed between treatments. Percentage metamorphosis of settled abalone in trials one and two were 96.12 ± 0.58 % and 95.86 ± 0.56 %, respectively. These rates of metamorphosis are to be expected for temperate species of haliotids settling on suitable biofilms as shown by Roberts and Nicholson (1997) who recorded metamorphosis percentages of approximately 75 % after 5 days for *H. iris* larvae settled on an isolated *Nitzschia sp*. film. The various diatom species used by Roberts and Nicholson (1997) revealed that certain species act as stronger metamorphic cues than others, with *Cocconeis scutellum var. parva* also inducing a high percentage of settled larvae to metamorphose, albeit over an extended timeframe compared to *Nitszchia sp. 1*; while *Nitzchia sp. 2* was a weak cue for both attachment and metamorphosis, inducing less than 10 % metamorphosis after ten days. The results of trial one and two thus suggest that the underlying biofilms produced in those trials where favourable for metamorphosis induction in *H. midae*, as determined by the rapid onset of metamorphosis.

If GABA is the chemical within mucous which has resulted in the increased attachment to settlement plates initially, it should be noted that the possible presence of GABA in *H. midae* mucous did not result in an increased metamorphic response by settled *H. midae* larvae. The effect of mucous-associated proteins which elicit cephalic tentacle responses in *H. asinina* has not yet been tested for its efficacy at inducing metamorphosis, but if they are present in the mucous of *H. midae*, it is hypothesised that these proteins provide no added benefit to the induction of metamorphosis.

In trials three and four the proportion of settled larvae which had metamorphosed was tracked for each day of the respective trials. Where trials 1 and 2 looked for absolute differences at the endpoint of the trial, the sampling strategy for trial 3 and 4 was aimed at determining whether the presence of conspecific mucous had any effect on the rate of metamorphosis.

The proportion of settled larvae, which had metamorphosed was similar for any given day in either trial 3 or 4. This is contrary to the findings of Gallardo and Buen (2003), who observed significantly more metamorphosed *H. asinina* post-larvae settled on mixed diatoms which had been pre-grazed by juveniles as compared to mixed diatoms only. The role of diatom species suitability as a feed source was also made evident in the findings of Gallardo and Buen (2003), while significantly more metamorphosed post-larvae were observed on mixed diatoms which had been pre-grazed as opposed to mixed diatoms only, this was not true for treatments which compared mucous presence or absence on a biofilm comprised of *Navicula spp.* only. This suggests than when the settlement biofilm is composed of diatom species which are favourable to post-larval feeding and growth, no increase in metamorphosis may be observed with mucous addition. It is thus hypothesised that the biofilms produced in this trial, which may not have always been suitable for the attachment phase of settlement, were adequate for post-attachment feeding and subsequent metamorphosis.

In both trial three and four, the proportion of settled larvae which had metamorphosed peaked before the end of trial and began to diminish on mucous-treated biofilms but not on untreated biofilms. This suggests that the increased mortality observed on mucous-treated plates was spread across both the attachment and metamorphosis stages of settlement, rather than mortality being isolated to attached larvae or metamorphosed larvae only.

Conclusion

In conclusion the presence of conspecific mucous on biofilms that are used to induce settlement of *H. midae* larvae resulted in increased attachment of competent larvae, however this was only significant under one of the following scenarios: (i) there is something unfavourable with the underlying biofilm’s species or proximate composition, as attachment was only equal between treatments in trial 1 which had the best settlement attachment proportions overall; or (ii) at higher temperatures, as in trial 1, no difference was observed between treatments at the lower end of the spectrum of temperatures at which *H. midae* larvae will settle.

It is further concluded that the presence or absence of conspecific mucous on settlement has no effect on the rate of metamorphosis in *H. midae* larvae, and it is hypothesised that the reduction of larvae to similar numbers observed on biofilms without mucous treatment may be attributed to increased mortality associated with the introduction of pathogens via the conspecific mucous. This should be tested in future experiments.

Chapter 4 - The efficacy of conspecific mucous in inducing *H. midae* larval settlement, after using novel handling methods to reduce potential biosecurity risks

4.1 Introduction

Biosecurity in aquaculture

Biosecurity is an ever-growing concern for aquaculture industries. The Department of Agriculture, Forestry and Fisheries of South Africa (renamed Department of Environment, Forestry and Fisheries as of June 2019) require some form of a biosecurity plan to be in place for the issuance of aquaculture rights permits, without which an aquaculture facility cannot begin operations (DAFF, 2017). Should a farm wish to have their products endorsed by a certification body, biosecurity protocols and disease management are some of the audited aspects for certification of a farm, especially when dealing with larger, well established and recognised certification bodies such as the Aquaculture Stewardship Council (ASC, 2019).

Other than obtaining better prices for their products through certification (Xuan, 2021), biosecurity management is crucial to aquaculture operations who aim to maintain a financially viable business. Aquaculture operations, especially land-based mariculture facilities, often make use of intensive farming practices as the high operating expenses of such facilities necessitate high stocking densities and feeding practices to produce the biomass required to generate a profit.

Intensive farming strategies increase the risk of pathogen transmission (Handlinger et al. 2006; Robertsen 2011), especially during stressful events where animals' immune systems become compromised (Walker, 2004). A disease outbreak could have dire consequences for an aquaculture business, as exemplified by Hardy-Smith (2006b), who reported on the de-stocking of an entire Abalone farm in Port Fairy Australia when a herpes like virus spread through the farm during 2006.

Pathogens and Parasites in Haliotids

In the abalone farming industry biosecurity risks range from pests and parasites to bacterial, viral and fungal pathogens (Hamilton 1984; Simon et al. 2006; Hardy-Smith 2006b; Cai et al. 2007; Macey et al. 2011).

Parasites in abalone include shell-dwelling polychaete worms. *Boccardia* are a genus of spinoid polychaete worm, which produce shell blisters in abalone shells through boring into the shell after (i) larvae swim into a host's mantle cavity, or settle and begin boring in between where the mantle and shell edge meet, or (ii) larvae settle on the outer surface of the shell and begin burrowing directly into the shell (Blake and Evans, 1972). Boring at the leading edge of the shell where the mantle and shell edge meets can result in blistering of the shell, and in severe outbreaks may lead to mass mortalities of abalone (Lleonart et al 2003). Hindrum (1996) described mortality rates of 40% in experimental abalone held at a sea-based farm in Southern Australia, with shells being characterised as having extensive shell blistering where abalone were not able to replace or heal the blisters devoid of nacre. The extent of this shell blistering could cover up to 50% of the inner shell surface (Radashevsky and Olivares, 2005). Examination of the condition of South African abalone infested with *Boccardia sp*. indicated that cultured abalones ratio of meat to shell weight diminishes as the severity of infestation increases (Simon et al. 2006), demonstrating that while severe infestation could lead to mortality events, mild to moderate infestation can reduce a facility's farming efficiency.

Another parasite of particular concern to South African abalone farmers is *Terebrasabella heterouncinata. T. heterounicata* is a shell dwelling polychaete that is endemic to South Africa (Ruck and Cook, 1998) which is incorporated into shells at the growing edge interfering with normal calcification and development of new shell material (Kuris and Culver, 1999). This species of shell-dwelling worm was accidentally introduced to California, initially resulting in brittle shells and deformed respiratory pores in *H. rufescens* (Oakes and Fields, 1996). Severe infestation of abalone with *T. heterounicata* results in severe shell deformations as abalone continuously attempt to repair their shells (Kuris and Culver, 1999). *T. heterounicata* infestations therefore not only have the potential to reduce the marketability of live abalone, but reduce the farming efficiency of infested farms as the abalone allocate more energy into shell repair as opposed to growth.

Vibriosis is a disease of particular significance to abalone farmers. *Vibrio* is a genus of bacteria which have been reported to result in mass mortalities on abalone farms, particularly at the larval to post-larval stage (Cai et al. 2007). Vibriosis presents in the form of abscessing and ulceration of the mantle, the appearance of white discolouration on the foot and a decrease in the meat mass of abalone, followed by mortality (Cai et al. 2007). *Vibrio* is a genus with global distribution and a number of species have been isolated and determined as the causative agents for mortality in cultured abalone. Auguiano-Beltran et al. (1998) described the pathogenicity of *V. alingolyticus*, demonstrating that 4-day old veliger larvae were highly susceptible to a challenge with *V. alingolyticus*, recording survival rates of 3.7 % after 48 hrs post challenge at a concentration of 10^5 cells/ml. The same result was obtained for post-larvae, albeit at a concentration of 10^6 cells/ml. Nicolas et al. (2002) further reported mass mortality in *H. tuberculata* when exposed to *V. harveyi*, noting that mortality was more likely at elevated temperature and concluded that it was most likely the causative agent for the mass mortalities of cultured and wild abalone observed in France between 1997 and 1999. Extreme care should be taken to avoid the occurrence of a vibriosis outbreak, as *Vibrio harveyi* has been demonstrated to rapidly colonise the epithelial and mucosal tissues of *H. tuberculata* with the gill-hypobranchial gland tissues being penetrated within one hour of contact (Cardinaud et al. 2014).

Another disease of concern to South African abalone farmers is Abalone Tubercle Mycosis (ATM). ATM results after infection by the fungus *Halioticida noduliformans*, and was first described in abalone from isolates taken at a holding facility in Japan between 2004 and 2006 (Muraosa et al. 2009). One of the species in which mycosis was observed was *H. midae*, and animals displaying symptoms of ATM were reported from abalone farms in South Africa during 2006. Mortality in juvenile abalone was particularly high, with mortality rates of 90% in juveniles and 30% in larger abalone being reported. ATM presents with both behavioural as well as morphological symptoms, and diseased animals were recorded as having a tendency to aggregate at the top of their holding baskets while animals in the later stages of the disease presented with necrotic tissue and ulcerative lesions in localised areas of epithelial tissue and ultimately mortality (Macey et al. 2011).

Aims

Existing aquaculture biosecurity practices such as batch management, access control, compartmentalisation and sterilisation of farming equipment can help to reduce and prevent the spread of pathogens and parasites. However, when studying the use of conspecific by-products, such as abalone mucous, to improve current productivity batch management, access control, compartmentalisation and sterilisation are negated. Mucous may be sourced from one batch of abalone held in a different section of the farm, transported into a hatchery and used in the creation of a new batch. As such, the conspecific abalone mucous, would need to undergo some form of sterilisation itself to reduce and potentially eliminate the risk of pathogen and parasite transference between not only batches, but departments within a farm as a whole.

UV sterilisation has been used to prevent mucous-associated microbial outgrowths in coral mucous samples used to study the anti-microbial properties of their mucous (Ritchie, 2006).

Autoclaving has long been accepted as the standard for sterilisation of equipment for the production of micro-organisms or micro-algae, such as in diatom culturing labs. Excessive heat and pressure are also known to kill most pathogenic bacteria and viruses which affect aquaculture facilities, and has been used by Bactol et al. (2018) to kill *Aeromonas hydrophilla* in a study assessing methods to produce whole cell vaccines for Nile tilapia, *Oreochromis niloticus*. It is, however, possible that autoclaving is likely to change the composition of mucous (Ritchie, 2006).

The aim of this study was to assess whether mucous which had been UV treated or autoclaved, to reduce biosecurity risk, would still result in the increased larval settlement attraction reported to be associated with the presence of conspecific mucous in *Haliotis* (Seki and Kan-no 1981; Slattery 1987; Gallardo and Buen 2003). This aim was achieved through trials designed to test the following hypotheses:

- H_{01} – An equal number of larvae can attach to both ambient grown biofilms and those treated with conspecific mucous, irrespective of the mucous handling method.
- H_{02} – An equal number of larvae will be counted on both ambient grown biofilms and those treated with conspecific mucous, irrespective of the mucous handling method, at the end of trials.
- H_{03} – An equal proportion of larvae on both ambient grown biofilms and those treated with conspecific mucous will have initiated metamorphosis by the end of the trials, irrespective of mucous handling method.
- H_{04} – There will be no statistically significant interaction between treatment and time if larval numbers are the dependent variable.
- H_{05} – There will be no statistically significant interaction between treatment and time if the proportion of settled larvae which had initiated metamorphosis is the dependent variable.

4.2 Methods

Aspects of the methods applying to trials in this chapter which were previously described in Chapter 2 include: experimental site (2.2), larval source and rearing (Section 2.3), larval density estimation and preparation for trial (Section 2.4) biofilm conditioning system (Section 2.5), ambient diatom biofilm preparation procedure (Section 2.6), settlement system (Section 2.7), settling procedure (Section 2.8), settlement plate sampling method (Section 2.9), sampling of environmental parameters (Section 2.10) and the software used to analyse the data (Section 2.11).

4.2.1 Experimental design

An experiment was designed where larvae were inoculated into cups containing 1 of 4 experimental settlement substrates to test their effect on settlement success, as outlined in the aims section of Chapter 4's introduction (Section 4.1). The four experimental treatments were; ambient diatom biofilms without mucous addition (D), ambient diatom biofilms with unaltered mucous addition (RAW), ambient diatom biofilms with UV treated mucous addition (UV) and ambient diatom biofilms with autoclaved mucous addition (AC). Twenty plates of each treatment were prepared for each trial run, allowing for 5 replicates of each treatment to be sampled on each of the 4 sampling days.

4.2.2 Randomisation

Randomisation for the trials in Chapter 4's experiment was carried out as described for Chapter 3 (Section 3.2.2), taking into account the difference in sample size and trial duration.

4.2.3 Mucous collection

Mucous was collected using the methods described for trials three and four of Chapter 3 (Section 3.2.3). Mucous collection for trials in Chapter 4 was collected and stored over two days, as it was not possible to collect enough mucous for all experimental treatments on the day prior to settlement.

4.2.4 Mucous Handling

UV-Treated Mucous

On the morning a trial was to begin, 1000 ml of the filtered mucous was placed into a UV clarifier (Ultra-Zap UV Clarifier 8W). After the mucous was added, the ends of the UV clarifier were closed off with 50-mm PVC endcaps. The bulb was switched on and the mucous was left to sit in the clarifier for 20 minutes. After 20 minutes the mucous was poured out of the tube into two 900-ml glass beakers, which had been autoclaved at 127°C for a duration of 30 minutes. The mucous was then poured back and forth between these beakers four times to mix the sample. Once thoroughly mixed the mucous was poured back into the UV sterilising tube and left for another 20 minutes. The procedure described was repeated at the end of the second 20-minute sterilising session and the mucous was left to sterilise for a third 20-minute period. This gave a total UV exposure time of 60 minutes. This repeated mixing between UV treatments was done to ensure that the mucous sample was evenly exposed to the UV bulb. At the end of the final sterilising session, the mucous was poured into a new household spray bottle, which was then sealed in a plastic bag and stored in a fridge until further use.

Autoclaved Mucous

Four hundred millilitres of the filtered mucous was poured into a 900-ml glass beaker. The beaker was then covered with a sheet of aluminium foil, placed inside an autoclave (Huxley HL-300 portable Steam Sterilizer, Hung Lin Medical Instruments Co. Ltd, Taipei, Taiwan) and treated for a period of one hour at a temperature of 127°C and pressure of 1.5 kg/cm^2. At the end of the autoclaving cycle, the autoclave was depressurised and the contents were left inside to cool down to room temperature. Once at room temperature, the mucous was poured into a new household spray bottle, sealed in a plastic bag and stored in a fridge until further use.

"Unaltered" Mucous

Four hundred millilitres of mucous were stored and prepared for each trial as per Chapter 3 (Section 3.2.4). This mucous was to be applied to diatom biofilms to serve as an "unaltered" mucous control treatment.

The untreated diatom biofilms acted as a control treatment.

4.2.5 Mucous application

Mucous was applied to settlement plates using the spraying method described in Chapter 3 (Section 3.2.5). The same method of application was used for all mucous treatments, irrespective of how the mucous was handled prior to its application to a settlement plate.

4.2.6 Statistical analyses

The percentage of larvae which settled by observation day one was checked for compliance with the assumptions for parametric testing. Equality of variance was assessed using Levene's test for homogeneity of variance at significance level of $p < 0.05$ (Levene, 1960). Normality of residual distribution was assessed using the Shapiro-Wilk normality test at a significance level of $p < 0.05$ (Shapiro and Wilk, 1965). The data were then analysed using One-Way ANOVA at a significance level of $p < 0.05$ to test for treatment effects. Where treatment effects were observed, Tukey's Honest Significant Difference post-hoc tests were used to investigate which pairs of means differed from each other (Tukey, 1949).

The number of larvae observed on settlement plates were checked for compliance with the assumptions required for parametric statistical testing for each sampling day. Equality of variance was assessed using Levene's test for homogeneity of variance at significance level of $p < 0.05$ (Levene, 1960). Normality of residual distribution was assessed using the Shapiro-Wilk normality test at a significance level of $p < 0.05$ (Shapiro and Wilk, 1965). The data were then analysed using Factorial ANOVA to test for treatment effects, day effects and for interactions between these two main effects, each at a significance level of $p < 0.05$. Where significant effects were observed, Tukey's Honest Significant Difference post-hoc tests were used to investigate which pairs of means differed from each other (Tukey, 1949).

The proportion of settled larvae which had metamorphosed was analysed using the same approach as for the total number of larvae observed, with the exception that only sampling days two, three and four were analysed as no larvae were observed to have metamorphosed on observation day one.

Where significant differences were detected, Tukey's Honest Significant Difference test was used to conduct pair-wise comparisons of means (Tukey, 1949).

4.3 Results

Trial 1

There was no significant difference between treatments in the proportion of inoculated larvae which had settled by observation day one (One-Way ANOVA; F = 0.680; p = 0.577, Table 4.1). In average over all treatment 11.66 ± 0.77 % percent of larvae added to the settlement cups had attached to plates by observation day one (Figure 4.1)

Table 4.1 The effect of treatment on the proportion of larvae which had settled on experimental settlement plates by observation day one, in Trial one (One-Way ANOVA, $p < 0.05$). Larvae were settled on either ambient grown diatom biofilms (D), ambient grown diatom films with untreated abalone mucous applied (RAW), ambient grown diatom films with autoclaved abalone mucous applied (AC) or ambient grown diatom films with ultraviolet-treated abalone mucous applied (UV).

Effect	Degr. of Freedom	SS	MS	F	p
Intercept	1	2720.451	2720.451	216.380	p<0.0001
Treatment	3	25.654	8.551	0.680	0.577
Error	16	201.161	12.573		
Total	19	226.815			

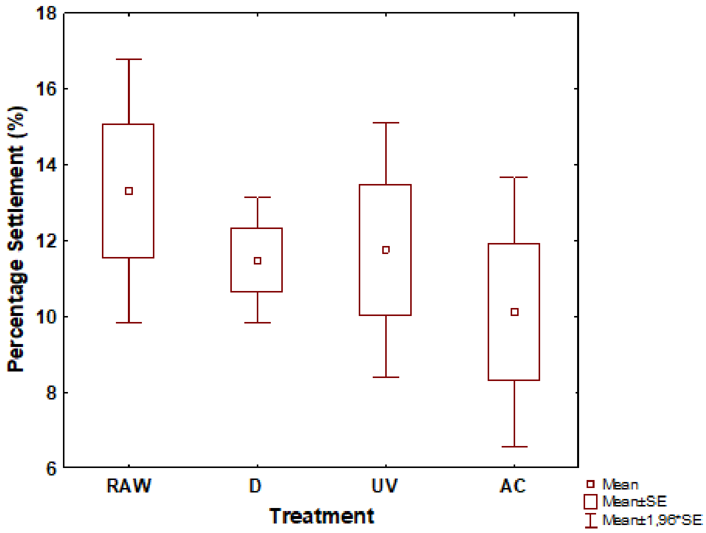


Figure 4.1 The proportion of larvae which had settled on experimental settlement plates by day one, in Trial one. Settlement plate treatments provided for larvae were ambient grown diatom biofilms (D), ambient grown diatom films with untreated abalone mucous applied (RAW), ambient grown diatom films with autoclaved abalone mucous applied (AC) or ambient grown diatom films with ultraviolet-treated abalone mucous applied (UV). SE = standard error of the mean.

The total number of larvae observed on the experimental settlement plates during the trial period was not significantly affected by treatment (Factorial ANOVA; F = 2.51; p = 0.065), Sample day (Factorial ANOVA; F = 0.761; p = 0.520) or the interaction between these two effects (Factorial ANOVA; F = 0.5014; p = 0.868) (Table 4.2, Fig 4.2). The mean number of larvae/post-larvae observed on experimental settlement plates during trial one was 24.19 ± 1.04 larvae/post-larvae per plate.

Table 4.2 Effect of treatment and sampling day on the mean number of larvae observed (± standard error) on settlement plates during trial one (Factorial ANOVA, $p < 0.05$). Larvae were settled on either ambient grown diatom biofilms (D), ambient grown diatom films with untreated abalone mucous applied (RAW), ambient grown diatom films with autoclaved abalone mucous applied (AC) or ambient grown diatom films with ultraviolet treated abalone mucous applied (UV).

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	46802.81	1	46802.81	531.737	p<0.001
Treatment	664.84	3	221.61	2.5178	0.066
Sample Day	200.94	3	66.98	0.761	0.520
Sample Day*Treatment	397.21	9	44.13	0.5014	0.868
Error	5633.20	64	88.02		

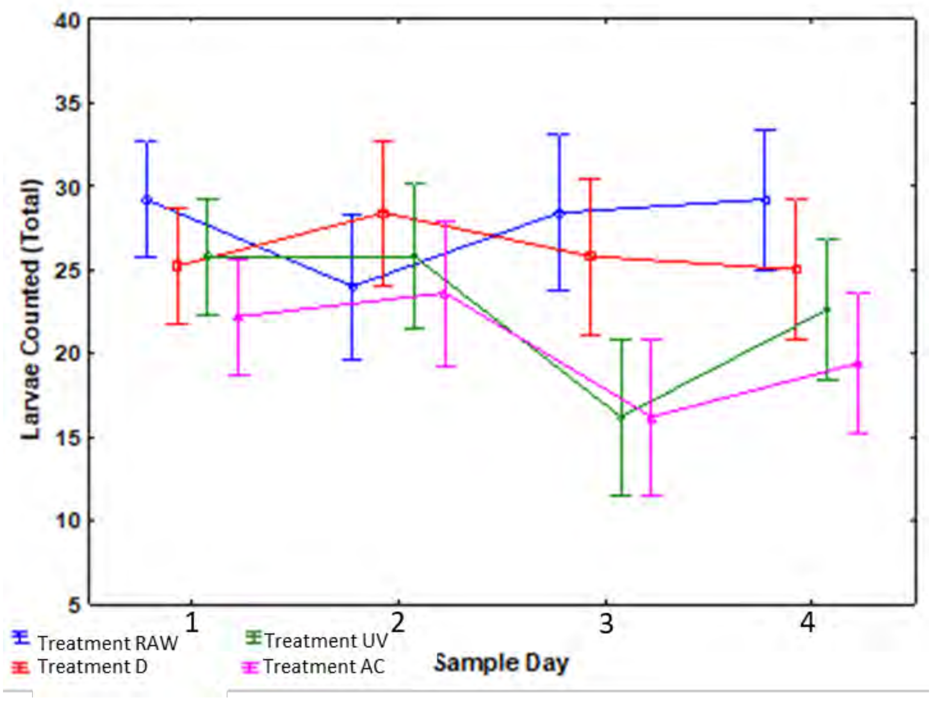


Figure 4.2 Mean number of larvae observed (± standard error) on settlement plates one to four days post settlement during trial one; where abalone larvae were settled on either ambient grown diatom biofilms (D), ambient grown diatom films with untreated abalone mucous applied (RAW), ambient grown diatom films with autoclaved abalone mucous applied (AC) or ambient grown diatom films with ultraviolet-treated abalone mucous applied (UV).

The proportion of settled larvae which had metamorphosed throughout the trial period was not significantly affected by treatment (Factorial ANOVA; F = 1.95; p = 0.129) or the interaction between treatment and sample day (Factorial ANOVA; F = 0.75; p = 0.662) (Table 4.3). The proportion of settled larvae which had metamorphosed was significantly affected by sampling day (Factorial ANOVA; F = 434.06; p<0.001). On observation day two 1.42 ± 0.42 % of settled larvae had metamorphosed, and this increased significantly (Tukey's HSD; p < 0.05) to 10.02 ± 1.67 % on day three. The increase from 10.02 ± 1.67 % on day three to 62.16 ± 2.26 % on day four was statistically significant (Tukey's HSD; p < 0.05).

Table 4.3 The effect of treatment and sampling day on the mean percentage of settled larvae which had metamorphosed on settlement plates during trial one (Factorial ANOVA, p < 0.05). Larvae were settled on either ambient grown diatom biofilms (D), ambient grown diatom films with untreated abalone mucous applied (RAW), ambient grown diatom films with autoclaved abalone mucous applied (AC) or ambient grown diatom films with ultraviolet treated abalone mucous applied (UV).

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	27084.05	1	27084.05	675.07	p<0.001
Treatment	235.69	3	78.56	1.9582	0.1291
Sample Day	52243.01	3	17414.34	434.06	p<0.001
Sample Day*Treatment	270.98	9	30.10	0.7502	0.6618
Error	2567.69	64	40.12		

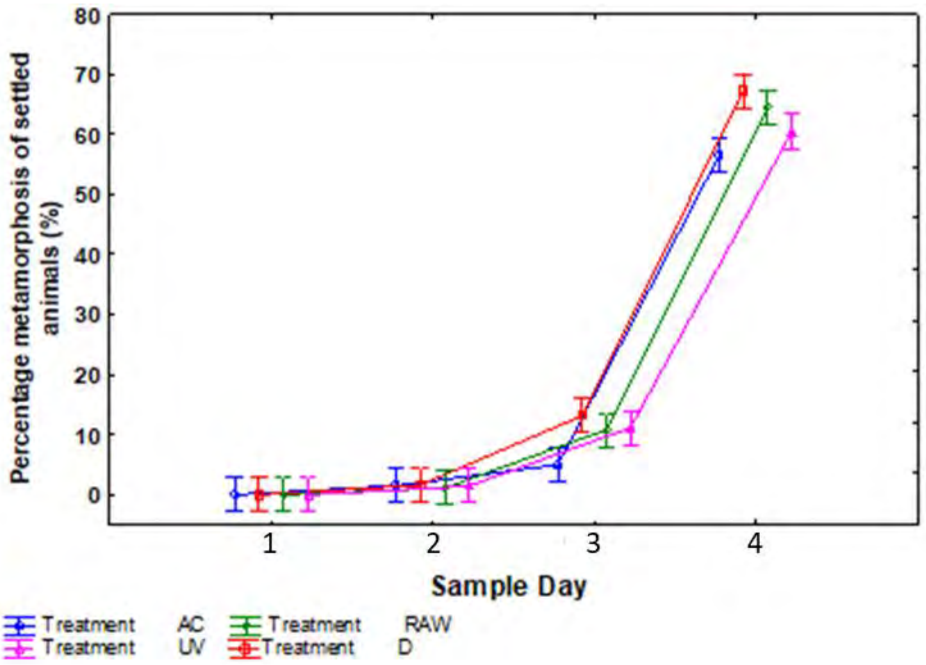


Figure 4.3 Mean percentage of settled larvae which had metamorphosed (± standard error) on settlement plates one to four days post settlement during trial one. Larvae were settled on either ambient grown diatom biofilms (D), ambient grown diatom films with untreated abalone mucous applied (RAW), ambient grown diatom films with autoclaved abalone mucous applied (AC) or ambient grown diatom films with ultraviolet treated abalone mucous applied (UV).

Table 4.4 Mean (± standard error) of settlement, number of larvae observed (± standard error), Proportion of settled larvae which had metamorphosed and water quality parameters in tanks, for one to four days post settlement during trial one. Larvae were settled on either ambient grown diatom biofilms (D), ambient grown diatom films with untreated abalone mucous applied (RAW), ambient grown diatom films with autoclaved abalone mucous applied (AC) or ambient grown diatom films with ultraviolet treated abalone mucous applied (UV).

	Time (d)	**RAW**	**D**	**UV**	**AC**	**All Groups**
Settlement (%)	1	13.30±1.77	11.48±0.84	11.75±1.71	10.11±1.82	11.66±0.77
Number of larvae observed (Total)	1	29.20±3.88	25.20±1.85	25.80±3.75	22.20±3.99	25.60±1.70
	2	24.00±5.83	28.40±4.13	25.80±3.58	23.60±3.36	25.45±2.04
	3	28.40±6.16	25.80±5.89	16.20±2.75	16.20±2.60	21.65±2.49
	4	29.20±4.86	25.00±4.87	22.60±2.18	19.40±4.35	24.05±2.10
Metamorphosis of settled larvae (%)	1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	2	1.12±0.71	1.62±1.10	1.44±0.88	1.49±0.95	1.42±0.42
	3	10.80±3.14	13.15±3.97	11.15±2.97	4.97±3.06	10.02±1.67
	4	64.40±4.51	67.17±3.29	60.49±5.66	56.58±4.24	62.16±2.26
Oxygen saturation (%)	1	94.90±1.48	94.44±1.10	93.96±0.97	94.14±1.49	94.36±0.59
	2	95.12±0.22	95.06±0.54	95.40±0.28	95.22±0.34	95.20±0.17
	3	94.24±1.49	95.48±0.79	95.06±0.75	95.10±0.71	94.97±0.46
	4	92.76±0.74	92.88±0.78	92.38±0.50	92.30±0.53	92.58±0.30
Temperature (°C)	1	15.50±0.03	15.56±0.02	15.56±0.02	15.54±0.02	15.54±0.01
	2	18.08±0.06	18.08±0.07	18.08±0.07	18.12±0.05	18.09±0.03
	3	16.40±0.05	16.34±0.06	16.36±0.07	16.38±0.07	16.37±0.03
	4	16.80±0.00	16.80±0.00	16.80±0.00	16.80±0.00	16.80±0.00
pH range	1	7.76 – 7.88	7.77 – 7.87	7.78 – 7.88	7.79 – 7.87	7.76 – 7.87
	2	7.86 – 7.87	7.86 – 7.87	7.86 – 7.87	7.86 – 7.87	7.86 – 7.87
	3	7.84 – 7.86	7.84 – 7.86	7.84 – 7.86	7.84 – 7.86	7.84 – 7.86
	4	7.86 – 7.87	7.86 – 7.87	7.85 – 7.85	7.85 – 7.86	7.85 – 7.87

Trial 2

There was no significant difference between treatments in the proportion of inoculated larvae which had settled by observation day one (One-Way ANOVA; F = 2.715; p = 0.079, Table 4.5). In average, 12.25 ± 0.84 % of larvae added to settlement cups had attached to plates by observation day 1 (Fig 4.4).

Table 4.5 The effect of treatment and sampling day on the proportion of larvae which had settled on experimental settlement plates by observation day one, in Trial two (One-way ANOVA, $p < 0.05$). Larvae were settled on either ambient grown diatom biofilms (D), ambient grown diatom films with untreated abalone mucous applied (RAW), ambient grown diatom films with autoclaved abalone mucous applied (AC) or ambient grown diatom films with ultraviolet treated abalone mucous applied (UV).

Effect	Degr. of Freedom	SS	MS	F	p
Intercept	1	3000.468	3000.468	271.0953	p<0.001
Treatment	3	90.170	30.057	2.7157	0.079
Error	16	177.087	11.068		
Total	19	267.258			

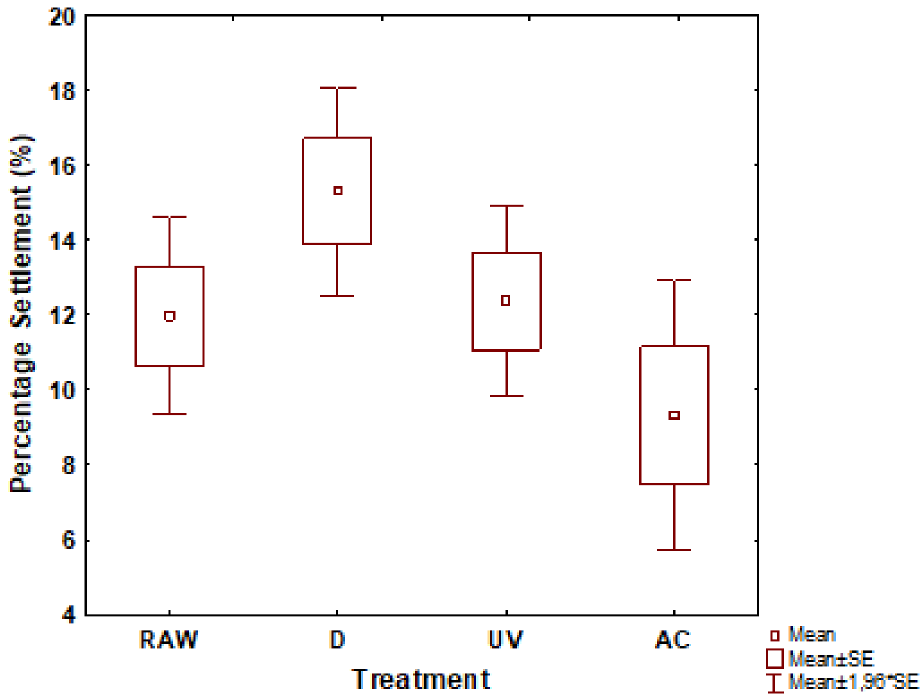


Figure 4.4 The proportion of larvae which had settled on experimental settlement plates by observation day one, in Trial two. Settlement plate treatments provided for larvae were ambient grown diatom biofilms (D), ambient grown diatom films with untreated abalone mucous applied (RAW), ambient grown diatom films with autoclaved abalone mucous applied (AC) or ambient grown diatom films with ultraviolet treated abalone mucous applied (UV). SE = standard error of the mean.

The number of larvae observed on the experimental settlement plates throughout the trial was significantly affected by treatment (Factorial ANOVA; F = 16.340; $p < 0.001$) but not by sampling day (Factorial ANOVA; F = 2.142; p = 0.1036), and there was no interaction between these two variables (Factorial ANOVA; F = 0.7244; p = 0.685) (Table 4.6).

Ambient biofilms without mucous addition yielded 35.20 ± 3.22 larvae per plate on day four, which was significantly greater than the average values for the other three treatments (Tukey's HSD; $p < 0.05$). Biofilms which had untreated mucous applied yielded 15.60 ± 3.93 larvae per plate on day four, while biofilms treated with autoclaved mucous yielded 15.40 ± 1.86. While the number of larvae observed on biofilms treated with autoclaved mucous were not statistically different from biofilm only plates on day four (Tukey's HSD; $p > 0.05$), the numbers observed were relatively low, yielding only 17.40 ± 2.73 larvae per plate.

Table 4.6 The effect of treatment and sampling day on the mean number of larvae observed on settlement plates during trial 2 (Factorial ANOVA, p < 0.05). Larvae were settled on either ambient grown diatom biofilms (D), ambient grown diatom films with untreated abalone mucous applied (RAW), ambient grown diatom films with autoclaved abalone mucous applied (AC) or ambient grown diatom films with ultraviolet treated abalone mucous applied (UV).

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	48363.61	1	48363.61	750.69	p<0.001
Treatment	3158.14	3	1052.71	16.340	p<0.001
Sample Day	414.04	3	138.01	2.1422	0.1036
Sample Day*Treatment	420.01	9	46.67	0.7244	0.6848
Error	2324.70	48	48.43		

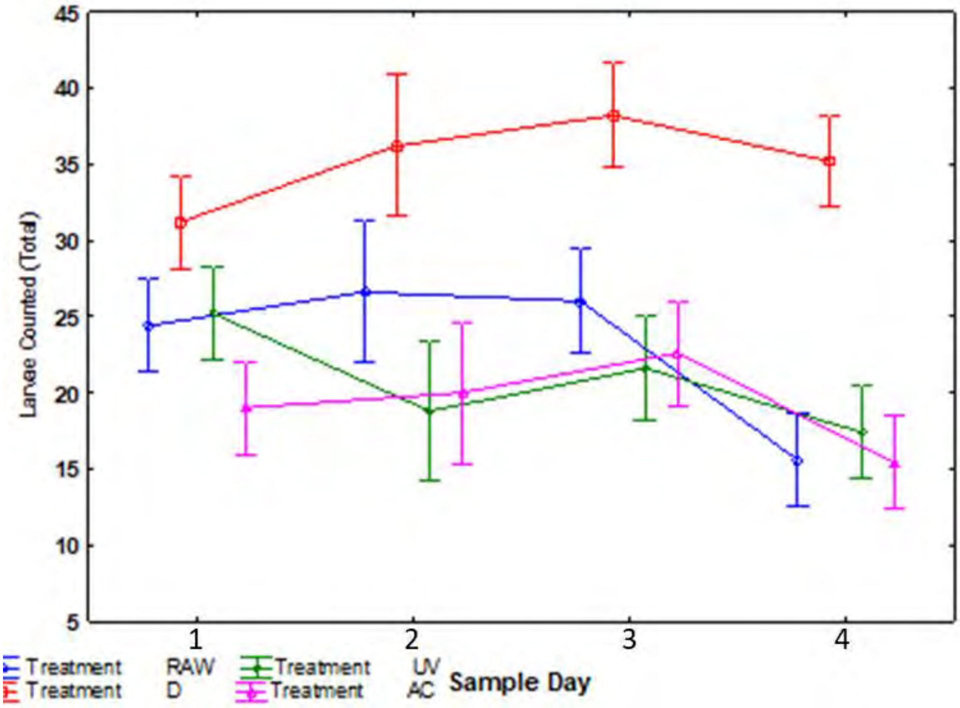


Figure 4.5 Mean number of larvae observed (± standard error) on settlement plates one to four days post settlement during trial two; where abalone larvae were settled on either ambient grown diatom biofilms (D), ambient grown diatom films with untreated abalone mucous applied (RAW), ambient grown diatom films with autoclaved abalone mucous applied (AC) or ambient grown diatom films with ultraviolet treated abalone mucous applied (UV).

The proportion of settled larvae which had metamorphosed throughout the trial period was not significantly affected by treatment (Factorial ANOVA; F = 1.4997; p = 0.2231) or the interaction between treatment and sample day (Factorial ANOVA; F = 1.128; p = 0.3568) (Table 4.7). The proportion of settled larvae which had metamorphosed was significantly affected by sampling day (Factorial ANOVA; F = 360.35; p < 0.001). On observation day two 0.68 ± 0.43 % of settled larvae had metamorphosed, this increased significantly (Tukey's HSD; p<0.001) to 38.42 ± 2.73 % on day three.

The increase from 38.42 ± 2.73 % on day three to 77.38 ± 2.82 % on day four was also statistically significant (Tukey's HSD; p<0.001) (Fig 4.6).

Table 4.7 The effect of treatment and sampling day on the mean percentage of settled larvae which had metamorphosed on settlement plates during trial 2 (Factorial ANOVA, p < 0.05). Larvae were settled on either ambient grown diatom biofilms (D), ambient grown diatom films with untreated abalone mucous applied (RAW), ambient grown diatom films with autoclaved abalone mucous applied (AC) or ambient grown diatom films with ultraviolet treated abalone mucous applied (UV).

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	67840.57	1	67840.6	900.5	p<0.001
Treatment	338.92	3	112.97	1.4997	0.2231
Sample Day	81439.75	3	27614.58	360.35	p<0.001
Sample Day*Treatment	764.54	9	84.95	1.1276	0.3568
Error	4821.31	64	75.33		

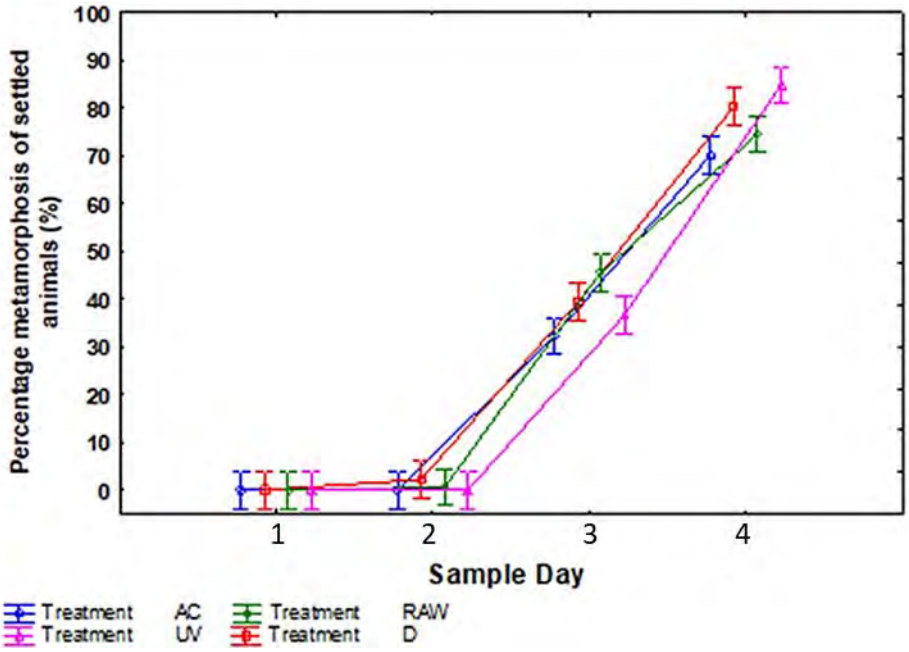


Figure 4.6 Mean percentage of settled larvae which had metamorphosed (± standard error) on settlement plates one to four days post settlement during trial two. Larvae were settled on either ambient grown diatom biofilms (D), ambient grown diatom films with untreated abalone mucous applied (RAW), ambient grown diatom films with autoclaved abalone mucous applied (AC) or ambient grown diatom films with ultraviolet treated abalone mucous applied (UV).

Table 4.8 Mean (± standard error) settlement, number of larvae observed (± standard error), Proportion of settled larvae which had metamorphosed (± standard error) and water quality parameters in tanks, for one to four days post settlement during trial two. Larvae were settled on either ambient grown diatom biofilms (D), ambient grown diatom films with untreated abalone mucous applied (RAW), ambient grown diatom films with autoclaved abalone mucous applied (AC) or ambient grown diatom films with ultraviolet treated abalone mucous applied (UV).

	Time (d)	RAW	D	UV	AC	All Groups
Settlement (%)	1	11.98±1.34	15.32±1.42	12.37±1.29	9.33±1.84	12.25±0.84
Number of Larvae observed (Total)	1	24.40±2.73	31.20±2.89	25.20±2.63	19.00±3.74	24.95±1.71
	2	26.60±4.13	36.20±7.68	18.80±0.49	20.00±3.11	25.40±2.65
	3	26.00±1.87	38.20±4.79	21.60±3.70	22.60±2.58	27.10±2.18
	4	15.60±3.93	35.20±3.22	17.40±2.73	15.40±1.86	20.90±2.36
Metamorphosis of settled larvae (%)	1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	2	0.56±0.56	2.18±1.55	0.00±0.00	0.00±0.00	0.68±0.43
	3	45.56±3.57	39.33±4.75	36.63±6.89	32.16±5.91	38.42±2.73
	4	74.51±8.97	80.22±2.32	84.70±2.53	70.08±5.33	77.38±2.82
Oxygen saturation (%)	1	95.26±0.80	95.64±0.84	94.84±0.77	95.38±0.82	95.28±0.38
	2	95.94±1.25	95.68±1.21	95.46±1.27	95.22±1.28	95.58±0.58
	3	95.84±1.08	95.54±0.90	95.92±0.65	96.30±0.55	95.90±0.38
	4	96.48±1.18	97.10±0.89	97.22±1.04	96.16±1.22	96.74±0.51
Temperature (°C)	1	15.68±0.02	15.72±0.02	15.70±0.03	15.72±0.02	15.71±0.01
	2	15.92±0.04	15.92±0.02	15.92±0.02	15.92±0.04	15.92±0.01
	3	16.40±0.03	16.42±0.02	16.44±0.02	16.42±0.02	16.42±0.01
	4	16.64±0.04	16.68±0.02	16.66±0.02	16.66±0.04	16.66±0.02
pH range	1	7.80 – 7.84	7.81 – 7.82	7.79 – 7.83	7.79 – 7.84	7.80 – 7.84
	2	7.88 – 7.91	7.87 – 7.91	7.88 – 7.91	7.88 – 7.92	7.87 – 7.92
	3	7.85 – 7.86	7.84 – 7.85	7.84 – 7.86	7.84 – 7.86	7.84 – 7.86
	4	7.81 – 7.83	7.80 – 7.83	7.80 – 7.82	7.81 – 7.82	7.80 – 7.83

4.4 Discussion

Settlement

There was no statistically significant difference in the number of larvae which attached to plates of the various treatments by observation day one in either trial one or two. The proportion of larvae added which settled in trials one and two was 11.66 ± 0.77 and 12.25 ± 0.84, respectively. This is considered to be comparable initial attachment success in temperate species of abalone, as Moss (1999) reported attachment rates of 3.2% for *H. australis* on biofilms composed of *Navicula sp.* after 24 hrs. Low initial attachment is likely be attributed to a biofilm composed of unfavourable diatoms, as demonstrated by Roberts and Nicholson (1997) who reported attachment in *H. virginea* to be approximately 90% on *Nitzschia sp. 1* but only approximately 30% on *Nitzschia sp. 2*. It therefore hypothesised that the biofilms produced for these trials where unfavourable for attachment, as reported by commercial hatcheries in South Africa during winter months (Sally Paulet pers. comm.; Devin Ayres pers. comm.).

Untreated mucous applied to ambient grown biofilms did not cause an elevated attachment response in larvae. Conspecific mucous has been found to increase settlement in *H. discus hannai* (Seki and Kan-no, 1981), *H. rufescens* (Slattery, 1987) and *H. asinina* (Gallardo and Buen, 2003), but this was not observed in the trials carried out for *H. midae*. The underlying reasons for elevated attachment are linked to the presence of mucous-associated proteins (Kuanpradit et al. 2012) or GABA (Laimek et al. 2008) in the pedal mucous of Haliotids, which act as positive stimuli for the attachment of larvae during settlement. While the seasonal expression of GABA in haliotid mucous has not been tested, the production of at least one 1 of the 3 Has-MAPS in *Haliotis asinina* mucous has been found to be seasonal (Kuanpradit et al. 2012), and may have been lacking in the mucous at the time of collection for these trials.

Roberts et al. (2007) reported that that highly mobile species of diatoms such as *Nitzschia longissima* inhibit the settlement of *H. iris* larvae as they smother the larvae and that diatoms with long polysaccharide stalks could trap larvae, preventing them from attaching to settlement surfaces with their foot. The results obtained in these trials may thus also be ascribed to larvae initially testing the plates for chemical attachment cues using their cephalic tentacles, and then subsequently swimming away in search of a more suitable surface after encountering a biofilm which may have had unfavourable physical characteristics (filamentous/highly mobile diatoms). As the ambiently grown biofilms were grown as a single batch for each trial, and then randomly distributed amongst treatments, unfavourable underlying biofilms would have been present in all treatments, hence the lack of a difference in attachment between treatments for a trial.

Certain bacteria can also act as attachment cues during settlement as found by Li et al. (2006), where plastics sheets with a bacterial film induced significantly more larvae of *H. diversicolor supertexta* to attach than blank control sheets. It is hypothesised that any beneficial bacteria would have been eliminated or that their numbers were significantly reduced in the UV and autoclaved mucous treatments used in this study to investigate potential reduction of the biosecurity risks associated with using untreated mucous.

Total number of larvae observed

The total number of larvae observed on settlement plates did not differ between treatments or day during trial 1. There was a dip in the number of larvae observed on day 3 from plates applied with UV treated or autoclaved mucous. This dip coincided with the reduction in temperatures from 18.09 ± 0.03 °C on day 2 to 16.37 ± 0.03 °C on day three, which may have caused thermal shock stress. The same trend would have been expected for ambient biofilms and those applied with untreated mucous, but this was not observed. This could be a result of the muco-polysaccharides and proteins contained within gastropod mucous (Davies and Hawkins, 1998) being unstable to UV radiation and high temperatures or pressures. Takami et al. (1997) proposed that the pedal mucous of conspecific abalone acts as a food source to post-larvae until they are of a size where they can effectively graze on diatoms. While the nutrients in the UV and autoclaved mucous may have been altered, this would not be the case for the unaltered mucous. The unaltered mucous is thus hypothesised to have acted as an alternative feed source which may have better equipped post-larvae to survive the environmental fluctuations observed in trial 1, resulting in a difference in more stable numbers of larvae being maintained on the untreated mucous plates compared to the UV and autoclaved mucous-treated plates. The biofilm only treatments may have also retained a more stable number of larvae throughout the duration of the trial for one of the following two reasons: (i) the biofilm only treatments numbers were stable as there was no mucous barrier between the larvae and the underlying diatoms which may have increased ease of access to feed initially, thereby allowing larvae to feed effectively at an earlier time and accumulate enough energy resources to withstand environmental fluctuations; or (ii) reduced potential for opportunistic pathogens to colonise potentially sterile organic matter, as may have occurred in the UV and autoclaved mucous treatments.

In trial two the numbers of larvae observed on settlement plates did not differ between treatments on days one, two and three. However, on the final day of the trial biofilm only treatments yielded significantly more larvae than biofilms with autoclaved or untreated mucous applied. The difference in number of larvae observed on day four between biofilm only treatments and UV treatments were not statistically significant. It is proposed that the mucous treatments either added pathogens (in the untreated mucous treatment), facilitated the proliferation of pathogens through addition of sterile organic matter (autoclaved mucous and UV treated mucous treatments) or acted as a physical barrier between post-larvae and biofilms, thereby resulting in the lower numbers of larvae observed in all mucous treatments on day four.

Metamorphosis

There was no difference in the proportion of settled larvae which had metamorphosed between treatments on a given day for either trial. This suggests that the presence of mucous, whether untreated or potentially sterilised had no effect on metamorphosis induction.

There was, however, a difference in the rate of metamorphosis between trials. Metamorphosis in trial one appeared to be low until day three, where it rapidly climbed from 10.02 ± 1.67 on day three to 62.16 ± 2.26 on day four. This was not observed in trial two, where the proportion of settled larvae which had metamorphosed showed a steady increase between days two, three and four.

Possible explanations for these differences between trials could be the lack of stability in temperatures during trial one, or unfavourable species of diatoms that were conditioned on trial 1's biofilms.

Biofilms act as the primary food source for post-larvae, and based on their growth form may or may not be suitable as feed. Prostate and firmly attaching diatoms provide better nutrition than filamentous or loosely attached diatoms as the post-larvae radula ruptures the cell wall making the cellular contents available, whereas loosely attaching species may be ingested whole and pass through the gastrointestinal (GI) tract intact if grazed at all (Kawamura et al. 1995), providing little to no nutrition. Ding et al. (2017) reported that larvae of *H. asinina* settled on biofilms of *Nitzschia alexandria* induced 15.71 % of settled larvae to metamorphose after 72 hours, whereas biofilms comprised of *Amphora coffeaeformis* only resulted in 10.54 % of settled larvae metamorphosing after 72 hours. Survival rates were similar between the diatom strains after 4 days, which suggests that while *A. coffeaeformis* may have been edible to post-larvae its grazing efficiency or nutritional composition may have been less suitable than that of *N. alexandria* in supporting metamorphosis. The possible presence of high numbers of loosely attached, or extremely firmly attaching species could have resulted in trial 1 in a delayed onset of metamorphosis due to inefficient grazing.

The larvae of *Haliotis* are able to delay the onset of metamorphosis for up to 21 days with no negative effects on subsequent survival (Roberts and Lapworth, 2001). This is suggested to be a mechanism which allows the larvae time to find an appropriate environment in which to settle and metamorphose. It is hypothesised that the unstable temperatures between days two and three may have resulted in the larvae of *H. midae* delaying the onset of metamorphosis in trial one, affording them more time to asses environmental suitability before going through the irreversible process of metamorphosis.

Conclusion

In conclusion it cannot be determined whether the novel handling methods used in an attempt to reduce biosecurity risk effectively retained the properties within mucous which stimulate attachment, as the untreated mucous failed to elicit an increase in settlement over biofilms which never had mucous of any type applied. Despite this, in trial two the number of larvae retained on plates at the end of the trial was either significantly or noticeably lower in all mucous treatments than in biofilm only treatments, suggesting that mucous may continue to pose a biosecurity risk even if it was sterilised.

Mucous presence did not elicit a metamorphic response in the attached larvae of *H. midae*, suggesting that the cues for the attachment stage and metamorphic stage of settlement are likely different. Improving one stage of settlement does not yield an overall improvement in post-larval production if the next stages are not addressed.

Chapter 5 - General Discussion and Conclusion

The trials performed in this study have revealed that the addition of *H. midae* conspecific mucous to biofilms can increase the proportion of larvae which attach to settlement plates. These findings are supported by the research on exotic species of abalone such as *H. discus hannai, H. rufescens* and *H. asinina* which have been found to settle in elevated numbers on biofilms with conspecific pedal mucous deposition (Seki and Kan-no, 1981; Slattery, 1987; Gallardo and Buen, 2003). However, this was not true for all trials, and some degree of seasonality may be involved. Kuanpradit et al. (2012) observed that some of the mucous-associated proteins which stimulate cephalic tentacle responses in *H. asinina* varied in concentration or presence/absence altogether with season; with the highest concentrations and presence of one mucous associated protein being closely linked with the spawning season of *H. asinina*. The abalone on HIK abalone farm display spawning peaks in September to October and January to February (Matthew Naylor, pers. comm.) and the mucous for the trials of Chapter 4 was collected during June to August. The fact that this mucous was collected outside of the spawning season for *H. midae* may explain the differences observed in settlement induction results between the trials of Chapters 3 and 4.

While mucous addition was observed to increase the number of larvae which attach to biofilms, this generally did not result in any increase in the number of viable post-larvae by the end of a given trial. *Vibrio sp.* have been found in the mucous of *H. discus hannai* (Choresca et al. 2010), and this pathogen is associated with the mass mortality of larvae and post-larvae in settlement systems (Cai et al. 2007). It is hypothesised that untreated abalone mucous acted as a vector for the transfer of pathogenic bacteria, which proliferated on the biofilm once added, leading to the observed decline in larval numbers over time. This was not observed for biofilms which were devoid of mucous. Ambient biofilms yielded more larvae per plate at the end of a trial than mucous treated plates where mucous had either been UV-treated or autoclaved. These observations are hypothesised to be attributed to the fact that while the mucous itself may not have introduced excessive pathogen loads, it may have acted as a substrate for these bacteria to proliferate on.

No increase in metamorphosis induction, or the rate at which metamorphosis progressed was observed between treatments. While GABA is a rapid inducer of metamorphosis in Haliotis larvae, and it is present in the mucous of at least three species of abalone (Laimek et al. 2008), it is rapidly degraded by the bacteria found on biofilms (Kaspar and Mountfort, 1995). The trials carried out in this thesis were performed in the absence of any antibiotics, and it is suggested that GABA within the mucous used may have been degraded soon after larval attachment by bacteria on the biofilm leaving the concentrations of GABA too low to induce metamorphosis.

These observations of increased attachment with no metamorphic response to the presence of mucous support the general consensus that the cues for attachment and metamorphosis are separate from one another, and that while a cue may affect the attachment stage of settlement, the same cue may have little to no metamorphosis-inducing properties (Roberts and Nicholson 1997, Roberts 2001).

It should be noted that while many studies on the settlement of *Haliotis* larvae have been carried out in petri dishes (Gallardo and Buen, 2003), acrylic disks placed in beakers (Moss and Tong, 1992a) or on glass slides placed horizontally in beakers (Takami et al. 1997), horizontally orientated settlement surfaces have been found to result in significantly higher settlement rates (41 %) than vertical surfaces (10 %) of the same treatments in *H. australis* larvae (Roberts and Watts, 2010). While studies making use of horizontal surface would have used horizontal surfaces for all treatments in their studies, this approach has the potential to limit the transferability of research between research and development and industry. In order to make a greater impact on industry, research should be conducted in systems which make use of industry methods and practices, but the experimental units must be scaled down sufficiently to allow for replication, as was done for this study.

The differences between the trials performed in this study and other studies pertaining to mucous induction of settlement in Haliotis larvae are not only limited to systems design, but differ in approach to mucous application. The majority of the studies investigating the ability of mucous to induce settlement of Haliotis larvae used live abalone to pre-graze plates and deposit pedal mucous prior to settlement assays (Seki and Kan-no, 1981; Slattery, 1987; Gallardo and Buen, 2003). While this is an effective method for the deposition of mucous, Matthews and Cook (1995) demonstrated that pre-grazing of biofilms removes overstory diatoms which are unfavourable to settlement success and would also reduce the diatom densities on settlement plates. The resultant effect of such a method results in a trial where mucous is not the only variable being assessed, as the underlying biofilms are also different between treatments. This makes it difficult to establish whether increased settlement on pre-grazed plates is due to the presence or absence of mucous, or whether it is simply a factor of a more favourable biofilm being left behind in pre-grazed treatments. Where researchers are not equipped to evaluate the biofilm composition in a study, an approach like spraying the mucous onto plates may be more effective at inferring the effect of mucous presence or absence only as no alteration of the biofilm community composition on a particular treatment occurs. Further to this, pre-grazing leaves no potential for sterilisation and carries with it the risks of pathogen transfer between batches and sites on a farm.

Suggestion for future studies on the settlement induction of *H. midae* larvae should consider the following research objectives:

I. Test the possible presence of mucous-associated proteins within *H. midae* mucous which elicit a cephalic tentacle response and the seasonal expression of these proteins, as has been found for *H. asinina* (Kuanpradit et al. 2012)
II. Test the possible presence of GABA in *H. midae* mucous, and whether its concentration varies seasonally. GABA has been found in the mucous of exotic haliotids (Laimek et al. 2008), but its presence in the mucous of *H. midae* has not been investigated.
III. The contribution of the proteoglycans and mucopolysaccharides, found within mucous, as a dietary food source for newly settled post-larvae should be investigated. Stable isotope marking of mucous and subsequent testing of newly settled larvae may help to determine whether the conspecific mucous is infect incorporated into the diet of newly settled post-larvae, or acts only as a chemosensory cue.
IV. Determine which diatom species act as strong attachment and metamorphic cues in *H. midae*, as the underlying biofilm appears to have a strong effect on overall settlement success whether or not inducers are used. The identification of species and quantification of the biofilm community composition is something which should be factored into both the budget as well as methods of future studies.
V. The identification and quantification of the composition of the bacterial communities colonising settlement plates should be considered as a topic for future studies. While some species of bacteria may act as settlement cues and be beneficial to the growth of post-larval *Haliotis sp.*, other are known to cause mass mortalities.
VI. In light of the fact that mucous biosecurity concerns are still valid even when mucous has been handled in such a way as to reduce this risk, alternative cues like *Ulvella lens* and GABA should be investigated despite their challenges, as they are less likely to facilitate the transmission of pathogenic bacteria to newly settled animals.

The importance of points IV and V should be of primary consideration for future studies. The significance of these two aspects of settlement biofilms has been brought to light recently at the farm where these studies were conducted.

During early 2021 sporadic settlement, reduced growth rates and abnormally high mortality rates were observed in the settlement department of HIK Abalone Farm (Pty) Ltd. Histology reports by two veterinarians and microbiology analyses of affected settlement tanks and plates at both hatchery sites indicated that while biofilm coverage and food ingestion appeared normal, species composition may be of nutritionally inadequate diatoms.

Despite evidence of adequate grazing efficiency, as indicated by ruptured frustules within the guts, signs of gut stress consistent with nutritional stress were evident in spat sampled from plates and tanks. The diatom community, identified from scrapings taken around animals on sampled plates, showed a community dominated by *Baccilaria sp., Grammatophora sp*. and *Licmophora sp*. The microbiological analyses revealed that bacteria of the genus *Vibrio* were growing on settlement plates in affected tanks. *Vibrio* species identified by PCR included: *Vibrio splendidus* and *V. atlanticus*; with *V. splendidus* strains being associated with larval mortality in *Crassostrea gigas* (Gay et al. 2004).

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