Towards reliable hatchery-produced quality blue mussels *Mytilus galloprovincialis*: an integrated approach to optimising supply

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Project No. 2008/202

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1. Non-technical summary

2008/202 : Towards reliable hatchery-produced quality blue mussels *Mytilus galloprovincialis*: an integrated approach to optimising supply

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OBJECTIVES:

1. To identify biological and physical factors that relate to rates of spat settlement, retention, survival, and growth in land-based nursery systems.
2. To assess the use of live algae replacements (algal pastes or microencapsulated diet) to bring broodstock mussels into reproductive condition
3. To determine and identify changes in *Vibrio* number and composition associated with mortality events in mussel spat.
4. Assess and examine performance of additional algal species to improve reliability of live food production for juvenile mussels

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED TO DATE

The key outcomes of this project for the mussel industry are reliable physical and biological data and resources from which current hatchery practices can assessed and modified to improve the reliability of supply of juvenile blue mussels. Reliable supply underpins the ongoing viability of the mussel industry. The project outputs have contributed to the following specific outcomes for the mussel industry :

1. Improved and cost-effective approaches to successfully condition broodstock mussels through manipulation and supplemental feeding with live and artificial diets. These alternatives provide a range of alternatives allow mussel hatcheries to extend production windows closer to year-round supply of eggs/sperm for larval production, leading to supply of mussels throughout the yearly production cycle.
2. Methods to allow monitoring, estimation and management of successful transition of mussel larvae to spat and spat to sea-based nursery phases of hatchery mussel production. The improved rearing outcomes through these critical transitions phases assist and underpin capacity for production growth and the on-going viability of the

domestic and export mussel industry.
3. Improvement of mussel spat production output and efficiency (cost per unit of spat produced) through improved hatchery hygiene and disease management, leading to reduced disease incidence and reduction of the number of catastrophic larval/spat stock loss events during production.
4. Increased surety of the base live algal food supply for juvenile production through expanded range of alternative larval food- the major critical resource supporting hatchery production of blue mussels.

KEYWORDS: Blue mussels, aquaculture, hatchery, production.

Mussel production in Australia is worth over $10 million but remained stable over the three year period 2007-2010. In 2009/10 Australia imported a further 70% of local production of fresh and frozen mussels indicating that increasing consumer demand, however growth in supply has been inhibited by a lack of reliable hatchery production of juveniles (spat) to supply the adult production phase. Maximising production throughout the year relies on managing broodstock, minimising mortality events during larval production, and identifying settlement preferences of spat on the commercial lines.

Suitable live algal and powdered diets were explored to condition broodstock over the summer months, when reproductive condition is less unsynchronised and reliable. Diet did not influence the accumulation of glycogen stores, indicating that the second spawning, even over the summer, is fuelled directly from ingested food not glycogen. Females fed binary diets of *Isochrysis* sp. + *Pavlova lutheri* came into spawning condition and produced larger eggs other live diet combinations. Part or complete replacement of the living algal diet with non-living algal diet formulations improved accumulation of glycogen reserves and gonad growth. Instant Algae Shellfish Diet 1800® (Reed Mariculture) performed well as a non-living algae option to partially replace or supplement the live algal diet.

At the SBS hatchery, settlement of larvae onto 500m continuous dropper lines has suffered from extreme patchiness and subsequent stock loss after transfer the sea. The distribution of settled spat varied between the top and bottom of the settlement tank, and the position of the dropper line on the spool. A sampling approach was developed that would allow reliable estimates of blue mussel spat density settled on lines prior to deployment at the sea-based nursery. Eight replicate short lengths (6 cm) of the dropper line were recommended, with increased replication on the outside and top of the spool- where density variation was greatest.

A disease known as bacillary necrosis is caused primarily by marine bacteria of the genus *Vibrio*, and is a source of unexplained and often catastrophic stock loss during larval and spat nursery phases. Molecular analysis of selective culture (TCBS) isolates showed that cultivable *Vibrio* were overwhelmingly dominated by *Vibrio splendidus* group bacteria. The isolates obtained were highly

diverse with each rearing stage displaying distinct mixtures of *V. splendidus* types. Different *V. splendidius* group isolates also varied significantly in virulence to mussel larvae. The most virulent strain tested was associated with unfiltered hatchery intake seawater, whereas those from healthy larvae rearing systems were of low virulence, suggesting that unfiltered and/or poorly treated intake water presents a greater disease risk to larvae. Mixtures of isolates showed synergistic effects indicating that community interactions also influence virulence to larvae. A diagnostic PCR was developed to detect *V. splendidus* group as a rapid detection method for potentially pathogenic *V. splendidus* bacteria in marine hatcheries. The findings of the study indicate that water management and husbandry methods directly influence diversity and dominance of the *Vibrio* communities and that development of bacillary necrosis is a complex and is not easily predicted or described by concentrating on total bacteria or total *Vibrio* load in rearing systems. Future studies should aim to determine common husbandry-related factors and mechanisms that promote pathogenesis/virulence and detection of genes/proteins associated with virulence of *V. splendidus* group bacteria. The combination of lab and commercial scale experiments in a research hatchery setting would also allow experiments without production-related constraints and run-to-run variation inherent in commercial hatchery production.

For mussels, the early larval phase production relies heavily on live food production of a single diatom strain *Chaetoceros calcitrans* CS-178 whose growth in hatchery culture systems is unreliable. Four purified algal strains were developed and extensively tested in the most hatchery-scale algal culture systems. Lipid profiles and growth performance of two diatom strains, *Chaetoceros* sp. CS-365/01 and CS-365/02 were very similar to *C. calcitrans* CS-178. Feeding experiments found that the two strains are ingested readily by larvae as young as 7 days without significant mortality; indicating that the four strains could be safely fed to mussel larvae. All four purified strains were lodged with Australian National Algal culture Collection (ANACC) and are available for adoption by the hatchery industry.

The project has provided techniques, methods and knowledge to address critical hatchery production bottlenecks associated with broodstock management, high and unpredictable larval and spat mortality, and suitable and reliable live feeds for both the larval and spat phases of mussel production. The outcome of the research assist the future commercial viability of hatchery-based mussel production allowing the industry to move from environmentally unsustainable reliance on collecting wild spat and realize the market potential of Australian mussels.

2. ACKNOWLEDGMENTS

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Dr Brett Ingram (Victorian DPI) and Mr Ian Mercer provided advice and input to algal strain selection, and research priorities from a Victorian mussel industry perspective.

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3. BACKGROUND

In 2009/10, Australia produced 3,462 t of mussels worth $10.1 million with 70% of production dominated by South Australia and Tasmania, followed by Victoria and Western Australia producing approximately 15% of the total each. Australia imported an additional 70% of local consumption mainly from NZ, indicating that increased local production is warranted. Mussel aquaculture production world-wide is largely supported by collection of wild juveniles with small numbers of juvenile mussels produced in hatcheries to supplement wild collections. However, collection of mussel spat from the wild imposes critical limits to the capacity of the mussel aquaculture industry to increase production and to control product quality and timing of supply to markets. Reliance on wild spat leaves the industry vulnerable to recruitment failure, restricts production to seasonal availability, and limits capacity to develop selective breeding programs.

In recent years there has been insufficient wild spat settlement to meet the demands of the expanding aquaculture mussel industry. The only way that the mussel industry can compete against imported products and allow Australian consumers access to Australian product is through reliable hatchery production of quality-assured spat. Until recently the demand and value of mussels has been too small to warrant large scale hatchery production, and most shellfish hatcheries focus on higher value species, or species for which wild collection of juveniles is not possible, (e.g. introduced oysters).

There is considerable potential for growth in the mussel aquaculture industry, which has been recognised in a number of states in Australia. In 2009/10, Australian mussel production was 505t, worth $1.9 million (ABARE, 2007), and imported approximately 2,430t, suggesting that there is potential to increase production by almost 400%. This has resulted in the development of existing waters for mussel aquaculture in Tasmania and the opening of new waters in Victoria support the vision of growth in mussel aquaculture. However during 2000-2010 there was a drop of production by 36% in Victoria (2005/06) and 30% in Tasmania (predicted 2006/07) that was attributed to the industry's reliance on unreliable wild mussel spat fall which was limiting production and market growth.

Commercial hatcheries in Tasmania and Victoria have undertaken to address this reliance on wild spat collection through the development of hatchery production facilities for mussel spat. Both have invested substantial infrastructure to spat production and are highly committed to delivering spat to mussel growers in Victoria and Tasmania. Furthermore, Spring Bay Seafoods has developed processing facilities for mussels based on market demand with the development of packaging to deliver ready-to-eat mussels to the Australian consumer via supermarkets based in strong potential for market growth. However, production bottlenecks in the hatchery include broodstock management, suitable live feeds for the larval and spat phases, and high and unpredictable larval and spat mortality. These bottlenecks will prevent development of commercially viable hatcheries and maintain reliance on collecting wild spat; a situation considered to be environmentally unsustainable. Therefore, it is critical that techniques and methods for reliable hatchery production are developed to realize the market potential of Australian mussels.

This project directly addressed two of the five strategic National challenges; "Natural resources sustainability" and "Response to demand: profitability". Against the Tasmanian Strategic Plan (2005-2008) this project supported growth across the mussel aquaculture sector, and in the process increase capacity and employment in rural and regional communities and increase the economy contribution of mussel aquaculture. With reliable production and development of processed mussels encouraging increased consumption rates there is strong potential for the market to continue growth by a further 30%.

While it is possible to apply the suite of generic shellfish hatchery methods to larval rearing of the Australian blue mussel, *Mytilus galloprovincialis*, the main problems are associated with managing the reproductive cycle of the adults, the production of suitable live feeds for the larvae, and spat (settled juveniles) who need to attach to a substrate using a byssus thread. Unlike oyster spat, mussel hatcheries need to supply a complex settlement substrate into the water with the spat. This large settlement structure creates a significantly more complex system in which to manage spat health and stocking density compared with the single-seed management used in oyster hatcheries.

4. NEED

Mussel production in Australia has remained stable over the 3 year period 2007-2010, and in 2009/10 Australia produced 3,462 t of mussels worth $10.1 million. South Australia and Tasmania produced almost 70% of Australia mussels in 2009/10, with Victoria and Western Australia producing approximately 15% of the total each. In 2009/10 Australia imported a further 70% of local production of fresh and frozen mussels, mainly from NZ, providing evidence that consumer demand for mussels is high and there is scope for the market to support increased local production. In response to this demand considerable capital investment in mussel production and processing has been invested throughout Australia which needs to be supported by reliable hatchery production.

This project addressed high and unpredictable mortality rates of blue mussel seed during the larval rearing and early nursery phase. There is a need in the hatcheries to develop techniques and approaches that maximise production of quality mussel seed, through informed decisions about how physical and biological conditions in the hatchery affect health and growth of larvae and spat. In consultation with the commercial mussel hatcheries we identified gaps in understanding and knowledge of mussel spat production that contributes to unexplained mortality of recently settled spat and highly variable success during transfer of spat to grow-out sites.

1. Providing the right biological and physical environment will maximise rates of settlement and then the retention, growth, and survival of juveniles following settlement. Much of what we know about mussel spat is derived from wild collection of settled animals and observing what these individuals do in the laboratory (eg Cáceres-Martínez et al. 1994, Alfaro 2006, Carton et al. 2007). Once settlement and metamorphosis has taken place, mussels do not form a permanent attachment if the site is unsuitable individuals will detach the byssus thread to go in search of new sites. Once close to the substrate individuals will also use mucous threads to “capture” substrates, particularly when being moved through the water in high energy environments (Cáceres-Martínez et al. 1994). Both byssus thread and mucous production are energetically expensive, and excessive production may compromise the energy stores needed for metamorphosis and during the early juvenile period. As a result newly settled mussel spat need to be managed attached and not as unattached single seed individuals, as is done in the oyster industry.

   In the mussel hatchery the set mussel larvae are transferred into settlement tanks which contain pre-conditioned settlement rope in a static water system with aeration provided. Following a hatchery-based nursery phase the settlement ropes are transferred directly to the grow-out area where the spat grow until they can be removed, graded, and densities adjusted for the next phase of grow-out. To date, no methods or sampling protocol had been developed to determine the distribution, growth and survival of the newly settled spat prior to transfer to the farm-based nursery site. As a result little was known about where on the settlement ropes the larvae preferred to set or if the location on the settlement ropes affected the growth rates of the spat. In this phase of the project we developed sampling

protocols that would provide an accurate picture of the density, distribution patterns and size of spat. By describing the pattern of settlement of spat on the settlement rope in different areas of the settlement tank it was possible to identify the preferences of larvae when settling and where in the nursery tank the growth rates were greatest.

2. The condition of parental stock, particularly the female, can influence rates of growth and mortality during the early life stages of the offspring. The capacity of the females to produce eggs depends on food available to her when she is producing eggs. While it is possible to collect mature broodstock from the farms to trigger spawning, the spawning season typically is limited to spring, with some evidence of a smaller summer spawning peak (Fearman 2010). To ensure that larval production can be carried out over an extended period and not be reliant on natural reproductive cycles it is necessary to determine the nutritional needs of broodstock.

3. The use of appropriate live feeds and temperature conditions to get broodstock *M. galloprovincialis* into spawning condition has been established (Fearman et al. 2009). However, production of live feeds is a major bottleneck in bivalve hatcheries and nurseries as algal production accounts up to 30% of the hatchery operational costs (Coutteau and Sorgeloos, 1993). To also require production of live feeds for broodstock is an additional cost that limits the number of broodstock that can be held and the time over which they can be held.

   While microalgae of high quality is not essential as a food for adult bivalves and bulk culture in ponds or tanks can reduce the costs of production, controlling the proximate composition of algae grown in these conditions is more challenging. Lipid content in microalgae is often highly variable and a function of environmental conditions. To reduce costs associated with algal production and to have access to feeds of known quality and composition alternatives have been explored, in particular formulated diets have been explored to supplement conventional microalgae diets. *Mytilus edulis* broodstock fed My Stock to supplement live microalgae diet resulted in animals obtaining greater amounts of PUFA in their diet and resulted in a greater hatching rate compared with the use of an unsupplemented live microalgae diet (Nevejan et al., 2008). *Macoma bathica* broodstock fed a diet supplemented with PUFAs also resulted in greater fecundity and larger eggs production compared with diets with no supplements (Hendriks et al., 2003). *Crassostrea* gigas females produced more eggs and a greater percentage of larvae when broodstock were supplemented with lipid microspheres (Robinson, 1992). M1 bivalve food (Aquasonic Pty Ltd) is a commercially produced dried algal powder that was considered to have potential to replace live algal feeds to get adult mussels into reproductive condition.

4. Unexplained mortalities in shellfish hatcheries can also be caused by bacterial disease. Determining how and why such mortalities occur is hampered by limited understanding of conditions that facilitate disease and determining the appropriate management response to the disease. Shellfish hatchery mortalities occur very rapidly (24-36 hr), and often before

staff realise there is a serious problem. As a result, assessment of the bacterial community associated with mortality is typically undertaken following the event. The bacteria present are then potentially a mix of those associated with the original infection and opportunist bacteria that colonise and degrade necrotic tissue. Under these circumstances it is very difficult to establish the disease-causing organism, or the source/conditions that led to the disease. By monitoring the bacterial communities in the hatchery we aim to identify bacterial causes of mortality and improve understanding of the links between the biological and physical characteristics of the larval/nursery rearing system and potentially harmful changes in the bacterial flora. While Vibriosis is an episodic problem throughout larval rearing, currently the critical point suffering mortalities is settlement-the 5 days including metamorphosis (to pediveliger) and transition to a settlement substrate. Therefore we will concentrate our effort on characterising potential sources (including larval rearing) of pathogenic bacterial flora developing during this early nursery phase. The most commonly reported bacterial shellfish and other marine hatchery species is Vibriosis, caused by various marine or halotolerant bacteria belonging to the family Vibrionaceae. Hatcheries are well aware of the role of *Vibrio* species in hatchery disease and routinely monitor live feeds (eg algae cultures) for *Vibrio*. However, due to the considerable staff effort and lengthy testing time (approx 48 h minimum), systematic monitoring is difficult for hatcheries to achieve and typically carried out after the event to establish the cause of a mortality. Therefore, we focused our efforts on *Vibrio* species (through use of the *Vibrio* selective medium, TCBS), but also carried out total cultivable bacterial community assessment using a broad-based marine agar medium, Zobell's marine agar (ZM1, ZM10).

5. OBJECTIVES

Original project objectives

1. To identify and assess maternal traits that affect quality and survival of mussel spat
2. To assess the effect of the nutritional status of pre-settlement mussels on rates of spat settlement, retention, growth, and survival
3. To identify biological and physical factors that affect rates of spat settlement, retention, survival, and growth in land-based nursery systems
4. To adapt and assess the value of a stress test as a tool to assess quality of spat at the end of the land-based nursery phase.
5. To determine and identify changes in *Vibrio* composition and numbers associated with mortality events in mussel spat.

Reporting format

The report is structured according to standard FRDC formatting requirements. Due to changes in industry need and priority during the course of the project, the original objectives above were modified and agreed with the industry partners and FRDC. The changes to objectives were: 1) replacement of objective 1 with a new objective (revised objective 4); 2) removal of objective 4; 3). Addition of revised objective 2; and 4) combination of objectives 2 and 3 into a single revised objective 1.

The revised project objectives were:

5. To identify biological and physical factors that relate to rates of spat settlement, retention, survival, and growth in land-based nursery systems.
6. To assess the use of live algae replacements (algal pastes or microencapsulated diet) to bring broodstock mussels into reproductive condition
7. To determine and identify changes in *Vibrio* number and composition associated with mortality events in mussel spat.
8. Assess and examine performance of algal species to improve reliability of live food production for juvenile mussels

The report consists of four experimental sections whose outcomes are described in sections 6.1 to 6.4, with the corresponding results and outcomes presented in sections 7.1 to 7.4. Sections 1 and 2 address revised objectives 1 and 2 concurrently; sections 3 and 4 address revised objective 3 and 4 respectively.

6. METHODS

6.1 Factors affecting settlement, retention, survival, & growth in land-based nursery systems

Estimating Density of Spat on Spools Prior to Deployment

At Spring Bay Seafoods, mussels are on-grown on 500 m continuous droppers attached in a series of loops (5-6 m long) to a sub-surface backbone. Therefore the pediveligers are set onto a continuous dropper (multi-filament braided polypropylene) in the land-based nursery tanks before the droppers, with the mussel spat, are relocated onto the farm lease for the sea-based nursery phase. The settlement rope is wound onto a large spool with three layers to the spool. As a result some sections of the dropper are on the outer most wind of the spool and are fully exposed, to water movement, while sections of the dropper that are wound in the middle and inside layers of the spool are consider to be is less exposed and more sheltered. Spools are placed into a tank approximately 1.7 m tall and the ready to set larvae are added to the water from above the spool.

Estimating the number of spat on settlement ropes prior to deployment on the farms is critical to managing and quantifying retention rates of spat through the first few months post-settlement. However, the size and aggregative behaviour of spat means that determining how to sample the ropes to obtain a precision estimate of spat number has been problematic for the hatchery. Estimating the density and size of spat on the dropper requires removing a small section of the dropper which is then examined under a microscope to enumerate the number of spat on the section. The hatchery had been removing a single random 10cm section of dropper to estimate numbers of spat on the dropper. We used a pilot study to determine the best section length of dropper and the number of sections to count to determine number of size of spat on the total dropper. To achieve this we trialled five different section length (2 cm, 4cm, 6cm, 8cm, and 10 cm) with 30 replicates for each of the five section lengths.

As this experiment required taking sections of commercial dropper that contained a commercial production of spat and was to go out to the farm nursery, it was only possible to take three 1 m sections of the dropper from different sections of the dropper. Each 1 m section was then cut into different lengths to obtain multiple sections that were 2 cm, 4cm, 6cm, 8cm, or 10 cm in length. Each section of the dropper was fixed in 80% ethanol, then at a later date examined under the microscope to find, remove, and count each spat. To locate all the spat the rope of the dropper was unravelled and the fibres separated to allow all spat to be located and counted.

Patterns of settlement and mortality in relation to physical and biological environment

To determine settlement preferences on the spool of the continuous dropper within a tank (1 m tall and 2 m in diameter) environment, 52g of pediveliger mussels (approx. 6.5 million individuals) were placed in the tank with a spool of 500 metres of black leaded dropper line to set. The distribution of live spat on the spool of continuous dropper was estimated 16 days later on 31 March 2010. On this day the continuous dropper was sampled from nine different locations within the spool; at

three heights (top, mid, and bottom of the spool) and at two different positions in the spool (outer wind of spool and innermost wind of spool). From each of the nine combinations of height and position eight 6 cm sections were randomly removed from the dropper and stored in 80% ethanol for examination in the laboratory. Each section of dropper was examined under a microscope for the presence of spat, this included teasing apart the rope. All spat were scored as dead (no fixed soft body tissues was seen within the valves) or alive (fixed soft tissues was seen within the values), and the length of each spat recorded.

6.2 Use of live microalgae & a live algae replacement to broodstock condition mussels

Living diets to facilitate broodstock conditioning in blue mussels

The experiment was conducted at Spring Bay Seafoods mussel hatchery facility using 516 adult (80-100 mm shell length) little blue mussels (*Mytilus galloprovincialis*) randomly selected from the grow-out site. The mussel shells were scrubbed clean of biofouling and 36 individuals were randomly selected for initial sampling and live weight (g) was recorded; remaining mussels were randomly allocated to twelve 40 L fiberglass circular tanks (40 mussels per tank). Mussels were held indoors under ambient temperature at 12.1 ± 0.1 °C (mean ± S.E), filtered seawater (40 µm) flow-through system at a flow of 1.2 L min^{-1}. Aeration was provided and no feed provided two days prior to the start of the experiment. Tanks were cleaned weekly, but the mussels were not moved during cleaning to avoid stress.

Each of the 12 tanks were randomly allocated to one of four microalgal diets; three diets using combinations of two species of microalgae and one diet using a combination of three species (Table 6.2.1). Diets were designed to modify the percentage of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) lipids based on published data (Table 6.2.2). Microalgae were drip-fed into tanks (over a period of twenty hours) and each tank was provided with 10% dry algae per grams of dry meat weight of mussel (Fearman and Moltschaniwskyj 2010).

Table 6.2.1: The ratio of microalgae used in each of the four diets and the estimated percentage of fatty acids composition in each diet.

Diet	Ratio*	Total Lipid (%)	DHA (%)	EPA (%)
Isochrysis sp. + *Chaetoceros muelleri*	70:30	16.25	6.30	1.50
Isochrysis sp. + *Pavlova lutheri*	60:40	16.80	9.00	8.00
Chaetoceros muelleri + *Pavlova lutheri*	80:20	8.40	1.80	8.00
Isochrysis sp. + *Chaetoceros muelleri* + *Pavlova lutheri*	33:33:33	13.00	5.90	8.30

* Ratio based on dry weight of microalgae species for calculating cell density.

Table 6.2.2: Estimated percent of total lipid, DHA (Docosahexaenoic acid), EPA (Eicosapentaenoic acid), n3 (double bond from 3rd carbon atom) and n6 (double bond from 6th carbon atom) for microalgae species used in experiment.

Microalgae species	Dry wt (pg/cell)	Total lipid (%)	Proportion of total lipid dry weight (%)			
			DHA	EPA	Total n3	Total n6
Isochrysis sp. (T.ISO)	30	20	9	1×10^{-4}	30	6
Pavlova lutheri	100	12	9	20	35	3
Chaetoceros muelleri	75	7.5	1×10^{-4}	5	5	7

The microalgae were grown in 500 L polyethylene bag cultures containing 450 L of F/2 medium, to late logarithmic phase (approximately 5 to 9 days) under 24:0 h L:D regime and harvested (20 % by volume) for feeding every fourth day. All bags received aeration (+ 3 % carbon dioxide). Bags were checked regularly for bacteria and presumptive *Vibrio* by streak plating 10 µL of microalgae culture on Zobell's Marine Agar (ZM1) and TCBS agar respectively. Bag cultures containing detectable *Vibrio* sp. were discarded. Prior to determining the quantity of microalgae to provide to the tanks each day, a visual for mortalities was undertaken. Mortalities were removed from the tank and the quantity of microalgae was calculated for the biomass of mussels in the tank on the day.

Broodstock mussels were destructively sampled on Day 14, Day 28 and Day 49 of the experiment. Three randomly selected mussels from each tank were opened and the meat weight recorded and the meat from each mussel divided into two along the dorso-ventral axis. One half was preserved in FAACC solution (formalin, acetic acid, calcium chloride and distilled water) for histological processing and the other half stored in -20 °C freezer for estimation of glycogen concentration.

On Day 49 of the experiment, all remaining mussels from each treatment were placed into a 200 L black spawning tray and thermal cycling was used to trigger spawning by holding mussels in 22 °C and 12 °C seawater alternately at 30 minutes intervals with an air exposure of 5 minutes between cycles. Spawning individuals were removed from the spawning tray and placed into individual containers containing 1 L of 22 °C of seawater to continue spawning and the duration of spawning recorded. After approximately 1.5 hours of spawning female mussels were removed and the waters containing the eggs was gently poured through a 75 µm screen to remove faeces and debris and the eggs were collected on a 43 µm screen. The collected eggs were place into a 500 mL container of 1 µm filtered seawater and mixed before two 0.5 mL samples was pipetted and placed into 0.5 mL of fixative (10 % formalin in seawater) for enumeration later. Fecundity of each female mussel was determined by counting the oocytes averaging the mean of the two samples.

All the eggs from females in the same diet treatment were pooled and placed into a 10 L bucket with 1 µm filtered seawater and fertilized using sperm from males from the same diet treatment. Fertilised eggs from each diet treatment were divided equally and placed into one of eight (two tanks per treatment) 200 L commercial larval rearing tanks filled with 100 L of 40 µm filtered, UV treated seawater), and temperature regulated at 23 °C by an electric aquarium heater with gentle aeration provided.

Approximately 48 hours post-fertilisation, larval rearing tanks were drained and larvae collected on 43 µm screens. Collected larvae from an upwelling tank were placed into a 10 L measuring jug topped up with 10 L of seawater, a plunger was used to thoroughly mix the larvae. Three homogeneous subsamples of 0.5 mL were pipetted out and placed into three 1.5 mL centrifuge tubes containing 0.5 mL of fixative each (10 % formalin in seawater). D-larvae from same treatment were determined by multiplying count of D larvae by 10,000 and the mean of the six subsamples (from a treatment) multiplied by two to attain the sum of D-larvae in a treatment.

Mussel tissue preserved in the fixative was dissected (approximately 5 mm thick) adjacent at the middle section to expose middle section area of the gonads. Dissected mussel tissues were histologically processed and were sectioned at 5 µm (Heidelberg Model: HM 340). Sectioned tissues were stained with Haematoxylin and Eosin and mounted under DPX. Histological images were captured under 10x magnification using a Leica DC300F digital microscope camera and Leica IM50 image capture software. For female mussels, a measuring grid drawn on an A4 sized transparency slide consisting of 1 cm squares with indicators on the four corners of a 3 cm square, was laid over the screen for counting of mature, vitellogenic, atretic oocytes, and adipogranular cells lying on the indicators of the 3x3 cm squares. Smaller cell types; previtelogenic oocyte and oogonia were counted using 1x1 cm squares. Similarly, for male mussels, the exact measuring grid was used. Counting of adipogranular cells lying on the indicators of the 3x3 cm squares and smaller cell types; spermatozoid, spermatogonia, spermatocyte and spermatide were counted using 1x1 cm squares. The counts were conducted over the entire sectioned gonad with the cell density calculated using stereological techniques (Weibel, 1979). Identification of oocyte and cell types was based on described gonadal stages found in Suarez et al. (2005). Total number of individual cell and oocyte types from a sectioned gonad was categorized into five different gonadal stages (Table 6.2.3 and 6.2.4).

Table 6.2.3: Determination of gonadal stage in female mussel by cell and oocyte proportion (<=indicates less and >=greater).

Stage	Oogonia	Previtellogenic	Vitellogenic	Mature	Adipogranular	Atretic
			Cells/oocytes (values in %)			
0	<10	<10	<5	<5	80>	-
1	<20	<20	<20	-	60>	-
2	<20	<40	<40	10	40>	-
3	<5	<20	60>	20>	<10	-
4	-	<10	10	<10	70>	<10
5	<5	5	<5	<5	30>	50>

Stage; 0=Proliferation of cells, 1=Bridge, 2=Proliferation of oocyte, 3=Maturing of oocyte, 4=Spent and 5=Lysis of oocyte

Table 6.2.4: Determination of gonadal stage in male mussel by cell proportion.

Stage	Spermatogonia	Spermatocyte	Spermatozoid	Spermatide	Adipogranular
			Cells (values in %)		
1	10>	10>	20>	-	<40
2	10>	10>	40>	5>	60
3	10>	10>	60>	<5	<30
4	<5	<5	<10	<5	50>
5	5>	5>	20>	-	60>

Stage; 1=Bridge, 2=Proliferation of cell, 3=Maturing of cell, 4=Spent and 5=Lysis of cell

Mussel tissue stored in -20 °C was freeze dried with a commercial freeze drying machine until no further reduction in weight or 95 % dehydration was achieved from the initial wet weight. The tissue was grounded to fine powder using a mortar and pestle and homogenised in 5 mL of 100 mM

sodium chloride solution and 8 mL of distilled water. Homogenate was pipetted into two 1.5 mL centrifuge tubes (Eppendorf) and kept at -80 °C until analysis. For the determination of glycogen concentration, thawed homogenate was centrifuged for 1 minute at 3,000 rpm and 50 µL of supernatant was pipetted into four 1.5 mL centrifuge tubes and 25 µL of potassium carbonate solution was added. Five hundred µL of amyglucosidase solution pH 4.5 (Sigma-Aldrich) was added into two of the four 1.5 mL centrifuge tubes for determining glucose reaction. Tubes with amyglucosidase solution were incubated at 55 °C for 90 minutes. Control tubes were incubated at 4 °C for 90 minutes. Samples tubes (incubated at 55 °C) were placed immediately on ice (after 90 minutes) and 250 µL of cold perchloric acid (0.6 M) were added to both sample and control tubes to stop further amyloglucosidase activity. All tubes were centrifuged for 3 minutes at 13,000 rpm and stored at 4 °C until analysis. Glucose concentration was analysed using a GM7 Micro-Stat (Analox Instruments). Glucose readings were converted to derive stored glucose levels and the two replicates averaged to provide a mean value.

Data Analysis

An orthogonal ANOVA was used to analyse the effect of treatment and week on somatic condition index of broodstock mussels over sampling period and the effect of treatment and time on glucose level. A Nested ANOVA was used to analyse variation of egg size produced by females within a diet and among treatment. A One-way ANOVA was used to analyse; fecundity and length of D-larvae among treatments. If significant difference was found (P <0.05), a Tukey's Post-Hoc test was used to analyse the data. The assumption of equal variance was examined using residual plots and data transformed if necessary. A $\chi 2$ test of independence was used to analyse; the count frequency distribution between spawners and non-spawners in each treatment, occurring frequency of gonadal stages in sampled mussels within treatments and the occurring frequency of gonadal stages in sampled mussels in sampled weeks. A regression analysis was performed to determine the relationship between somatic growth index and glucose level. If a curvilinear relationship was found in the scatterplot, the data was log-transformed to linearize the relationship.

Non-living diets to facilitate broodstock conditioning in blue mussels

To examine the value of using non-living diets to facilitate broodstock conditioning in blue mussels, two artificial algal diets were trialled as complete and partial replacements for live microalgal to broodstock condition blue mussels at Spring Bay Seafoods mussel hatchery facility. M1 bivalve food (Aquasonic Pty Ltd) is suitable for a range of bivalves and is an ultra-fine powder with a main particle size of 10-15 microns with one gram considered equivalent to 30 billion cells of microalgae; it was prepared by blending the powder for 30 sec with distilled water. Each week M1 was weighed into aliquots for desired cell densities for each treatment (Table 6.2.5) and stored in the refrigerator until fed to the broodstock. The second artificial algal diet was Instant Algae Shellfish Diet 1800® (SD) (Reed Mariculture). Instant Algae Shellfish Diet 1800® is a mix of four microalgae species (provided *Isochrysis*, *Pavlova*, *Tetraselmis*, and *Thalassiosira weissflogii*); 0.95 L of Instant Algae Shellfish Diet is equivalent to 1,800 L of dense algae culture at approximately 2 billion cells

per ml. Instant Algae Shellfish Diet is provided in a liquid form and at the start of each week aliquots for the cell densities for each treatment (Table 6.2.5) were stored in the refrigerator until fed to the broodstock. The non-living algal diets were provided as a partial to full replacement of the live algae added to the raw seawater. The percentage replacement of live algae was 30%, 40%, 50%, 60%, 70%, 80% and 100% replacement. The use of 100% live algae, SD or M1 diet was set as 5% per g of dry weight/day, which is considered sufficient food to facilitate broodstock conditioning; this was actually a supplement as raw seawater was provided to the mussels over the entire experiment (Table 6.2.5). Feed was delivered to each tank of mussels by a dose pump set at 2.08 L h^{-1} over 24 hours. The amount of supplementary feed (live or non-live algae) was adjusted weekly based on mortalities within each treatment.

Table 6.2.5: Algal cell densities (living and non-living) added for each treatment in the broodstock conditioning experiment. A binary living algal diet composed of *Pavlova lutheri* and *Isochrysis* sp. (provided in equal concentrations) and two artificial algal feeds M1 bivalve feed (M1) and Shellfish Diet (SD) used as either 100% replacement or supplement (30-80%) to living algal diet.

Treatment	Litres of *Pavlova lutheri* added = value in this column divided by cell density	Litres of *Isochrysis* sp. added = value in this column divided by cell density	Grams of M1 added	Millilitres of SD added
100% Live algae	29,000	29,000	0.00	0.00
30% SD	21,000	21,000	0.00	1.18
40% SD	17,000	17,000	0.00	3.93
50% SD	14,000	14,000	0.00	9.83
60% SD	12,000	12,000	0.00	19.66
70% SD	8,700	8,700	0.00	32.76
80% SD	6,000	6,000	0.00	46.80
100% SD	0	0	0.00	58.50
30% M1	21,000	21,000	0.09	0.00
40% M1	17,000	17,000	0.31	0.00
50% M1	14,000	14,000	0.79	0.00
60% M1	12,000	12,000	1.57	0.00
70% M1	8,700	8,700	2.62	0.00
80% M1	6,000	6,000	3.74	0.00
100% M1	0	0	4.68	0.00

The average wet weight of mussels was measured (46.31 ± 1.95 g), and individuals were randomly allocated to each of the 15 x 100L experimental tanks and allowed to acclimate overnight. An attempt was made to use females that were spent; this was achieved by obtaining animals from the farm close to the end of the spring spawning season and by Experimental tanks each held 30 adult mussels in a raw seawater flow-through system using ambient water temperature (11.7 – 15.6 °C). Each experimental tank was randomly allocated to one of 14 dietary treatments or the 100% live algae treatment (control) with one tank per treatment. In additional males were put into a separate tank to be used for fertilisation of oocytes at spawning and fed only live algae.

The algal species used were two flagellates *Pavlova lutheri* and *Isochrysis* sp. (T-Iso) in a 1:1 cell density mix. Algae were grown in batch culture under continuous light with the addition of Walnes nutrient medium and CO_2 added to the incoming air supply. *Pavlova lutheri* and T-Iso were grown in 500 L polypropylene bags and harvested in stationary phase. Algal densities were determined daily by algal cell counts.

Mussels were held in the experimental system for eight weeks, with surface water temperature measured and mortalities were removed and recorded daily. Broodstock tanks, feed bins and feed lines were cleaned weekly. Triplicate 1000mL water samples were collected weekly from experimental tanks, tank outlets and the 100% algal feed bin to determine dry algal weights. Prior to sampling Whatman GF/C 47mm filters were labelled with permanent marker and dried in an incubator until the weight did not alter. Filter paper weights were measured prior to filtering the samples through a Millipore filtration apparatus and returned to the incubator. Filter weights were measured until the weight remained constant and the final weight of the sample was recorded. Dry algal weight determination was calculated as follows: final – initial filter weight/volume of filtered water=dry weight of algae entering and leaving the tanks. An average of the replicates was recorded.

At the end of week 4, 6, and 8 spawning was induced to determine the fecundity of females in the different dietary treatments. Mussels were placed in spawning trays for 15 minutes aerial exposure and then covered with ambient (~20°C) filtered seawater for a two hour period with aerial exposure for 10 minutes every half an hour. Mussels were then returned to the broodstock system for feeding to resume.

Females that spawned were removed from the spawning tray and allowed to spawn for two hours individually in a beaker containing seawater. The female was then removed from the beaker and returned to the broodstock tank. The volume of water within the beaker was recorded and a well mixed 20 mL sample of the oocytes was collected and stored in seawater formalin. From the 20 mL sample 2 x 25 µL subsamples were collected to determine the fecundity of the female. From those samples 10 randomly chosen oocytes/ subsample were measured in diameter using a compound microscope and eyepiece graticule at 10x magnification to get an average oocyte diameter. Any spawning males were removed from the treatment and placed in tank for use during fertilisation.

At the end of the last spawning run the number of mussels remaining in each treatment was recorded, the animals were weighed and placed on ice for return to the University of Tasmania. All mussels were opened and the gonad removed and fixed in FAACC (formalin, acetic acid, calcium chloride) for histological processing. A piece of fixed tissue from the gonad of each mussel was embedded in paraffin, sectioned to 5 µm, and sections stained with Haemotoxylin and Eosin and mounted with DPX. Sections were assessed to determine which of four gonad developmental stages each female was in at the conclusion of the experiment (Table 6.2.6), and assessed for fullness of the vesicular connective tissue (VCT), which is a measure of glycogen store and fullness of the adipogranular tissue (ADG) which is a measure protein and lipid stores (Table 6.2.6)

Table 6.2.6: Classification of gonad development stage (adapted from Gabbott, 1976; Pipe, 1987b; Mathieu and Lubet, 1993)

Stage	Description of gonad development stage
Stage 0 (empty)	Mantle dominated by storage tissue and has little gonadal tissue. Sex may not be determined. Female acini that are recognized have oocytes in the previtellogenic stages, and male acini have only early stages of sperm cells and no spermatids or spermatozoa
Stage 1 (early filling)	Acini easily recognized and sex can be determined. Most oocytes have started vitellogenesis and some are already mature. In male acini immature sperm cells make half of the acinus radius or more. Mature residual gametes may be seen in individuals recovering from spawning.
Stage 2 (filling)	Most oocytes are in late vitellogenesis, with visible yolk granules and vitelline envelope, but still attached to the acinus walls. A minority of the oocytes are detached and fully mature. The males have more spermatids but still abundant immature sperm cells, and the latter make at least one-quarter of the acinus radius, typically five cells or more.
Stage 3 (mature)	Morphologically similar to stage 2, but proportion of mature gametes in acini is greater and proportion of gonad tissue relative to storage tissue is usually greater. Some individuals have acini with empty lumens and gametes along acinus walls. These were considered as recovering from spawning and given a stage of gonad development according to the development of the gametes present.
Stage 4 (partial spawn)	Combination of areas of mature gametes packed closely together, and large areas of empty mantle dominated by storage tissue

Table 6.2.7. Description of the vesicular connective tissue (VCT) glycogen store stage and the adipogranular tissue (ADG) stage with protein and lipid stores (Gabbott, 1976; Pipe, 1987b; Mathieu and Lubet, 1993)

Stage	VCT	ADG
Stage 0	no filled VCT cells	no filled ADG cells
Stage 1	filled VCT cells are accumulating between the acini and towards the mantle epithelium, but not yet surrounding the acini forming a continuous web	some filled ADG cells are seen among the VCT cells. Alternatively, less filled ADG cells form a web between the VCT cells
Stage 2	the VCT cells are filled and almost completely surround the acini with a layer of more than one cell in thickness.	filled ADG cells form a web between the VCT cells

6.3 Diversity & pathogenicity of *Vibrio* in mussel hatcheries

Vibrio *sampling*

Samples for genetic diversity analyses were collected from Spring Bay Seafood (SBS) marine hatchery (Triabunna, Australia) during May 2008 and May 2009 (autumn).

A preliminary assessment was undertaken May 2008 to assess the diversity of *Vibrio* genotypes and concentrations associated with different operational areas and surfaces in the hatchery, and different stages of the juvenile mussels during the production cycle: 1) sperm and eggs suspensions used for production of D-larvae, 2). Seven-day old larvae reared in static rearing tanks, 3). Recently-settled spat from flow-through spat settlement tanks, and 4) dried settlement ropes used for in spool tanks. Sperm/egg suspensions were sub-sampled using a sterile pipette, suspended in McCartney bottle with 9 mL of sterile filtered seawater (FSW), serially diluted to 10^{-5} and all dilutions spread-plated on TCBS agar. Seven day old (from D-larvae), larvae were sampled by collection of subsamples from commercial rearing runs at the hatchery. Recently settled (24-48hr) spat were collected from rope surfaces in settlement tanks by sterile pipette. Larvae/spat samples were then resuspended in a known volume of FSW and a volume equivalent to 500 larvae/spat filtered through clean (bleached, freshwater rinsed and dried according to standard hatchery practice) 20 µm plankton mesh screens by addition of sterile FSW. The washing steps were repeated several times, and the larvae/spat were transferred to a sterile 1.5 mL centrifuge tube, crushed with a sterile plastic mini-pestle, suspended in a sterile bottle with 10 mL of sterile seawater, serially diluted to 10^{-5}, and all dilutions spread-plated on TCBS agar.

Spat settlement ropes are typically reused after 3-4 weeks of drying outdoors and were considered a potential inoculation source for *Vibrio*, especially during "tank conditioning"- a period of 5 days during which dried rope spools are immersed in flow-through spat tanks prior to addition of pediveligers. Three separate dry ropes were sampled by cutting a 5 cm length from the dry spooled rope. The rope sub-sample was pulled apart on a clean surface with sterile tweezers and the strands collected and placed in a sterile bottle containing 10 mL of sterile FSW, shaken vigorously, the suspension serially diluted to 10^{-5}, and dilutions plated onto TCBS medium as previously described.

Surface samples were collected from a range of wet/damp surface areas in the hatchery. A standard sterile foil template (5 cm^2) was placed in the surface and the exposed area of the template was brushed carefully with a sterile- cotton swab and the swab placed in a sterile bottle containing 10 mL of sterile filtered seawater. The sample was shaken vigorously and the sample suspension serially diluted to 10^{-5} and all dilutions plated onto TCBS medium.

Isolates for preliminary DNA sequencing analysis were gathered using targeted selection. Plates were scanned for colonies of differing morphology from a selection of TCBS dilution plates from the different hatchery water and surface samples.

A second assessment in May 2009 aimed to assess and compare *Vibrio* genotype diversity associated with the SBS mussel hatchery seawater and rearing systems (Fig. 6.3.1) Samples were collected from untreated seawater (pumped from 30m depth) from Spring Bay (BSW), and 20µm filtered header tank seawater (HTSW). Samples were also collected from three stages of the hatchery production cycle receiving header tank seawater after UV irradiation: 1) broodstock tank seawater (BTSW), 2) static larval tank seawater (LTSW), and flow-through spat settlement tank seawater (STSW).

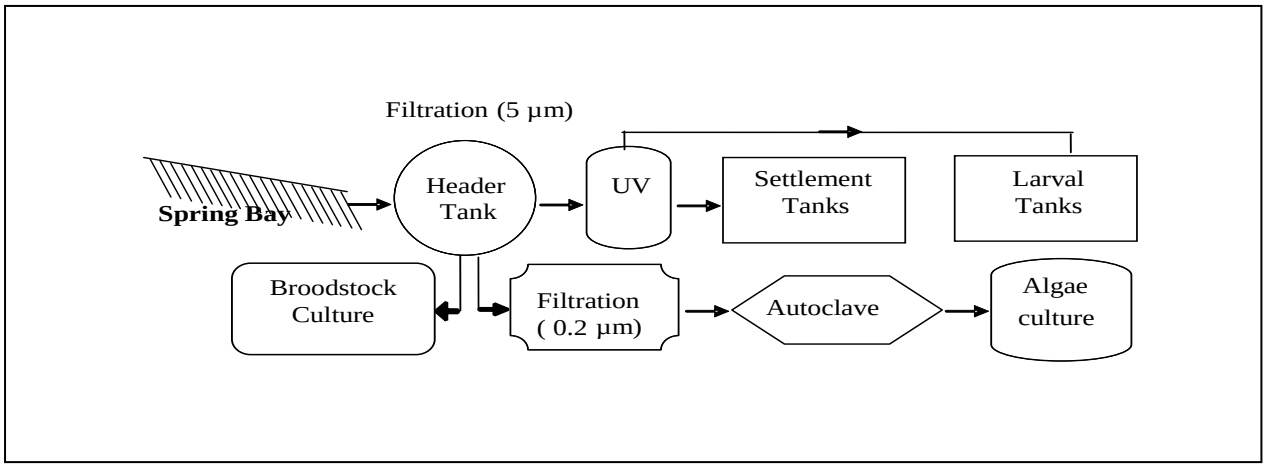

Figure 6.3.1: Outline of Spring Bay Seafoods seawater system. Raw seawater is pumped to header tanks for short term storage and settlement of solids. Water is filtered to 5µm (nominal) and UV-treated prior to use in static larval rearing tanks and flow through spat settlement tanks. Flow-through broodstock tanks receive unfiltered header-tank water. For algal culture, header tank water is filtered to 0.2 µm and autoclaved prior to use for larval-rearing.

Triplicate 10 mL water samples were collected from each source using a sterile 20 mL syringe, transferred into sterile 30 mL McCartney bottles, vigorously shaken, and ten-fold serial dilutions prepared to 10^{-5} dilution. Cultivable *Vibrio* were isolated by spread-plating 200 µL of each sample dilution onto Thiosulfate Citrate Bile Sucrose (TCBS) agar (Oxoid, Australia) and the plates incubated at 21±2°C for 48 hours. Presumptive *Vibrio* colonies were enumerated from at least 2 dilution plates.

For *atpA* gene sequencing studies, a library of 40 *Vibrio* isolates from each water source was established by random selection of colonies from dilution plates followed by streak-plate purification. For long-term storage, *Vibrio* strains were archived at -80°C in cryovials containing 10% glycerol and Protect Beads® (Oxoid, UK).

DNA sequencing of the atpA *and 16S rDNA genes of* Vibrio *isolates*

DNA was extracted using a standard CTAB protocol (Ausubel et al. 1999) or colony-pick, heat lysis protocol (von Canstein et al. 2001). Single bacterial colony using a sterile wooden probe and the cell mass suspended in tube with 567 µL of 10 Mm Tris-HCl, 1 Mm EDTA, pH 7, 4 (TE buffer) and vortexed until dispersed thoroughly. A volume of 30 µL of 10% sodium dodecyl sulfate (SDS) was

added and mixed thoroughly by shaking, 3 µL of 20 mg mL^{-1} proteinase K (AMRESCO® Inc., Ohio USA) was added and the tube mixed thoroughly by shaking, followed by incubation for 60 min at 56°C. Subsequently, 100 µL of 5 M sodium chloride (NaCl) and 80 µL cetyl trimethyl ammonium bromide (CTAB)/NaCl (10% CTAB, 0.7 M NaCl) was added, mixed thoroughly by shaking and incubated for 30 min at 65°C. An equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed thoroughly by repeated inversion, and centrifuged for 5 min at 13 000 rpm. The aqueous phase was removed to a new tube and equal volume of TRIS-buffered phenol was added, mixed thoroughly, and centrifuged for 5 min at 13 000 rpm. The aqueous phase was removed to a new tube and chloroform: isoamyl alcohol extraction repeated again. The aqueous phase was removed to a new tube and equal volume of cold isopropanol alcohol was added, mixed thoroughly by repeated inversion, and centrifuged for 15 min at 13 000 rpm. The supernatant was removed and the DNA pellet washed in 400 µL of 70% ethanol and then centrifuged for 5 min at 13 000 rpm. The supernatant was removed and DNA re-suspended in 50-200 µL of TRIS-EDTA (TE) buffer.

Preliminary isolates collected and purified during the May 2008 collections were characterised by PCR amplification and sequencing of the 16S ribosomal RNA gene (rDNA). Approximately 1450 bp of the 16S rRNA gene was amplified using PCR primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Weisberg et al. 1991). The reaction mixtures were prepared in a total volume of 50 µL containing 38.5 µL of sterile MilliQ water, 2 µL of 2.5 µM mix of the four dNTPs, 2 µL of 50 mM $MgCl_2$, 1 µL of each 10 µM PCR primer, 1U of BioTaq DNA Polymerase (Bioline, UK), and 20 ng of genomic DNA. PCR thermal cycling consisted of 5 min at 96°C, followed by 30 cycles of amplification. Each cycle consisted of denaturation at 95°C for 15 sec, annealing for 30 sec at 49°C, and primer extension at 72°C for 1 min, followed by a final extension for 5 min at 72°C and a temperature hold at 15°C until reactions were removed from the cycler. PCR-amplified DNA fragments (1450 bp) were checked and purified as described above, and sequenced in both directions using the forward or reverse amplification primers 27F or 1492R. Short (<900bp) and/or low quality sequences obtained from isolates were omitted from further analysis. A consensus sequence for each strain was determined by alignment of forward and reverse chromatogram traces, and comparison with published *Vibrio* 16S rDNA sequences to establish their affinity with the Gamma-proteobacteria and genus *Vibrio*. Sequences were then aligned with representative *Vibrionaceae* sequences (129 sequences in total) using the multiple sequence alignment program CLUSTAL X (Thompson et al. 1997) and Geneious 4.0.3. Phylogenetic analyses were constructed using the neighbour-joining method (Saitou and Nei, 1994) and support for clusters assessed using the bootstrap random resampling (200 replicates). The 16SrDNA trees were rooted using *Vibrio fischeri* (X74702.1) as an outgroup sequence. Phylogenetic trees were drawn using the software NJPlot (Perrière and Gouy, 1996) and Geneious 4.6.3 (Biomatters Ltd, NZ).

A total of one hundred presumptive *Vibrio* isolates collected in May 2009 were characterized by DNA sequencing of the ATP synthase A subunit gene (*atpA* PCR conditions and thermal cycling parameters for the gene were modified from Thompson et al. (2007), and used degenerative

primers designed to anneal to position 37 (atpA37F, 5'-CTDAATTCHACNGAAATYAGYG-'3) and 1554 (atpA1554R, 5'-TTACCARGWYTGGGTTGC-'3) of the ATP synthase A subunit gene (Fig. 6.3.2).

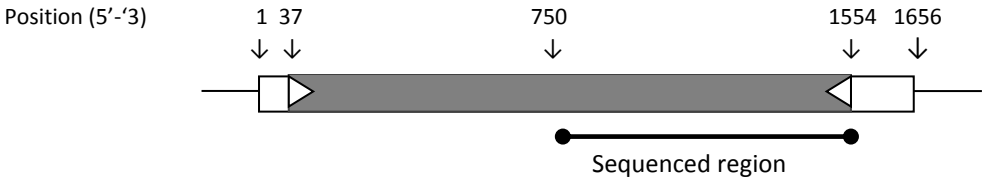

Figure 6.3.2: The region of *atp*A gene amplified using PCR primers atpA37F and atpA1554R (grey shading) producing a PCR amplicon size of approx. 1500 bp. White triangles indicate the relative position of the amplification primers. The 800bp region sequenced using the reverse amplification primer is indicated.

Reactions were carried out using an Eppendorf Master Cycler Gradient (Eppendorf, USA) in 50 µL volumes and contained the following: 1 x ammonium buffer (160mM $(NH_4)_2SO_4$, 670mM Tris-HCl, pH 8.8 at 25°C, 0.1% Tween-20), 0.25mM of each dNTP, 1.5mM $MgCl_2$, 0.3µM of atpA37F and *atpA1*554R primers, 2U of BioTaq DNA polymerase (Bioline, UK), and 5 µL of *Vibrio* colony suspension. The thermal cycling program consisted of (i) 5 min at 95°C, (ii) 3 cycles of 1 min at 95°C, 2 min 58°C and 1 min at 72°C, (iii) 25 cycles of 35 s at 95°C, 1 min at 58°C and 1 min at 72°C, and (iv) a final extension of 10 min at 72°C. Successful PCR products were visualised by electrophoresis through 1% (w/v) agarose/TBE gels stained with ethidium bromide. Amplicons of the expected size (1500 bp) were confirmed by comparison to DNA size standards (Hyperladder II; 100-2000 bp; Bioline, UK) and purified using Montage™ PCR ultra-filtration spin columns (Millipore Corp., USA) according to manufacturer's instructions. DNA concentration estimated using a Turner TBS-380 DNA fluorometer (Turner Designs, USA).

The distal portion of the *atp*A gene of each isolate was sequenced using the reverse amplification primer (atpA1554R) used in the primary PCR (Fig. 6.3.2). Sequence reactions and electrophoresis were prepared and carried out by the Australian Genome Research Facility (AGRF, Brisbane, Australia) using an ABI 3730 DNA sequencer and ABI Big-dye terminator chemistry (Applied Biosystems, USA). Resulting chromatograms were checked manually for base-calling accuracy, aligned for preliminary comparisons, and all base variations verified by comparison of chromatogram traces using Geneious Pro 4.5 to 5.6 (Biomatters Ltd, NZ). Remaining ambiguous base calls were denoted by the appropriate IUB codes for mixed bases.

Phylogenetic and diversity analyses

All partial *atpA* gene sequences obtained from all isolates were compared by sequence alignment using Geneious Pro software (versions 4.6.3 to 5.6.3; Biomatters, NZ), and strains with identical *atpA* sequences determined by pair-wise comparison. The 40 corrected unique partial *atpA* gene sequences were then aligned with 119 *Vibrio atpA* gene reference sequences from the dataset of Thompson et al. (2007), accessed from the GenBank database (National Center for Biotechnology Information, NCBI).

Phylogenetic analyses of the 159 sequences were constructed using PAUP* version 4.0b10 (Swofford, 1993) using the neighbour-joining (NJ) algorithm (Saitou and Nei, 1987), implemented via the Geneious Pro software package. Analyses were rooted 16 using two *V. fischeri* (LMG 4414, ATCC 700601) *atpA* sequences from Thompson et al. (2007). Consistency of major clusters was determined by comparison with NJ-trees constructed using both Tamura-Nei and maximum-likelihood distances. Consistent *V. splendidus* clusters identified from all-taxa trees were identified and within- and between-cluster genetic distances calculated. As most sequences were allied with the *V. splendidus* group (*sensu* Thompson et al. 2007) a second analysis was carried out including only mussel hatchery sequences and *V. splendidus* references strains, using *V. tapetis atpA* sequences (LMG19704, LMG19705) as an out-group taxa.

Vibrio splendidus group-specific PCR

The feasibility of developing *V. splendidus* specific PCR detection was also examined. A set of *V. splendidus*-specific *atpA* PCR primers were designed for use as a non-quantitative diagnostic tool (products of 300-600bp), and also for conversion to quantitative PCR format (PCR products of 100-250bp). All *atpA* gene sequences obtained during the study were aligned with 5 published *V. splendidus* group sequences (GenBank Accessions: EF601244, EF601289,EF601323, EF601257, and EF601258) and a consensus *V. splendidus* group sequence (75% threshold) aligned with a selection of *Vibrio* and non-*Vibrio* sequences (Fig. 6.3.3): *V. tapetis* strain LMG19705 (EF601367.1), *V. pomeroyi* strain LMG20537 (EF601318.1), *V. fortis* strain LMG21557 (EF601322.1). *V. penaeicida* strain LMG19663 (EF601263.1), *V. chagasii* strain LMG13237 (EF601256.1), *V. aestuarianus* strain LMG7909 (EF601288.1), *Aliivibrio salmonicida* strain LF1238(NC011316.1), *Aliivibrio wodanis* strain K26 (EF601362.1), *Pseudomonas aeruginosa* PA7(NC009656), *Listonella pelagia* LMG19995 (EF716865), *Alteromonas macleodii* 'Deep ecotype' (CP001103) and *Aeromonas hydrophila* subsp. *hydrophila* ATCC7966 (AF059494.1). The alignment was examined manually for regions of *atpA* conserved among *V. splendidus* that were sufficiently different from non-*V. splendidus* group representatives and these regions selected as targets for PCR primer design. All primers were designed manually and primer characteristics calculated using OligoCalc online (Kibbe, 2007) to ensure optimal PCR characteristic (Invitrogen, 2009). An example of the PCR primer design process is shown in Figure 6.3.3 (*V. splendidus* primer set 2F715 and 5R944).

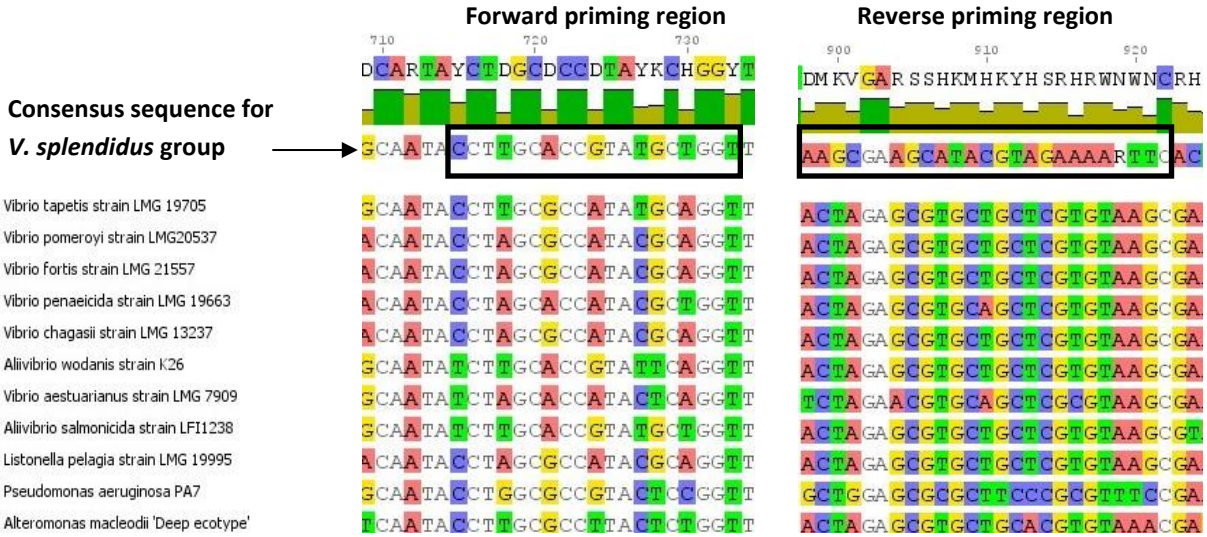

Figure 6.3.3: Example alignment of partial sequences of the *atpA* gene of *Vibrio* species. Black boxes indicate the variable regions (indicated by different base colours) and base mismatches to the 75% threshold consensus sequence for *V. splendidus* group strains from which forward and reverse primers were designed.

In the example above the forward primer has a maximum mismatch of five bases and a minimum of one base mismatch with *Aliivibrio salmonicida* LFI1238 whereas the reverse primer has a minimum mismatch of one base with *Vibrio fortis* LMG21557. Preliminary specificity validation of all primer designs was conducted by comparison with 93 *V. splendidus* group *atpA* sequences. Primer sequences were subjected to primer BLAST (NCBI) to assess potential cross-hybridization with Genbank database sequences. Matched primer pairs were selected according to annealing temperatures (<5°C difference) and product sizes between 100 and 300 bp for efficient annealing and extension. Complementary annealing was examined using NetPrimer (Abd-Elsalam, 2003) prior to PCR testing and validation.

Optimisation and validation of V. splendidus*-specific PCR primers*

Three of the developed primer sets were selected for testing and validation using five *Vibrio splendidus* group strains selected to represent the phylogenetically distinct clusters identified in phylogenetic analyses. Additional strains isolated from phyllosoma cultures of Southern rock lobster (*Jasus edwardsii*) previously classified by biochemical testing were also included: two *V. splendidus* strains *V. harveyi*, *V. calviensis*, *V. anguillarum*, *V. proteolyticus* and *V. penaeicida*. An additional 18 randomly selected bacterial colonies from spread-plates (seawater Vogel-Johnson medium) of mussel spat tank seawater and a bacterized culture of the live algal species *Tetraselmis suecica* (a common hatchery food species) were also included in PCR specificity screening. DNA from the random isolates was prepared as described earlier.

Optimal annealing temperatures for each primer set was determined by using thermal gradient PCR cycling with primer annealing varying from 50 to 55°C. The thermal cycling consisted of: 95°C for 5

min followed by 25 cycles of: i) 95°C for 35 seconds; ii) annealing at 50.4, 51.8, 53, 54 or 55°C for 1 min; iii) 72°C for 50 seconds; followed by a final extension at 72°C for 5 min. All reactions included 16S rRNA gene primers (27F and 1492R; Weisburg et al. 1991) as an internal DNA quality control; absence of the 1450bp product was used to indicate false negatives due to potentially poor DNA quality. Reactions were carried out in 50 µL volumes and contained the following: 1 x ammonium buffer, 0.25mM of each dNTP, 2.0mM $MgCL_2$, 0.5µM of each designed *atpA* forward and reverse primer, 0.2µM of each 16S control primer, 2U of BioTaq DNA polymerase (Bioline, UK) and 5.0uL of DNA from the *Vibrio* test strain DNA.

Pediveliger pathogenicity assays

Three *Vibrio* strains collected in May 2008 (strains 86, 125 and 169) representative of three distinct genetic clusters (based on 16S rDNA sequencing) were selected for comparison of pathogenicity to mussel pediveligers. Healthy mussel pediveligers (17-20 days old, average shell length of 150-180 µm) were supplied by Spring Bay Sea Foods and maintained in FSW at 22°C with aeration, and fed daily with 1.5 x 10^5 cells mL^{-1} of *Chaetoceros calcitrans* until used in pathogenicity experiments. The selected *Vibrio* strains were grown at 27°C for 24h in Tryptone Soy Broth (Oxoid, Australia) supplemented with 2% NaCl. After incubation broths were centrifuged at 17800 *g* for 10 minutes, the cell pellets washed with sterile FSW, resuspended in FSW and the bacterial cell concentration estimated by direct counting using a Nebauer haemocytometer and adjusted to 10^8 cells mL^{-1} by addition of sterile FSW.

Pathogenicity assays were carried out in triplicate in sterile 12-well multiwell plates (Iwaki, Japan) with each well containing 5 mL of FSW with a mussel pediveliger density of 10 mL^{-1} (Fig. 6.3.4). The *Vibrio* strains were added either individually or in 2-and 3-way equal combinations at starting at a total *Vibrio* concentration ranging from 10^2–10^7 CFU mL^{-1}. Controls contained pediveligers but not the test *Vibrio* bacterial strains. No algal food was added during the 48h assay period. The proportion of live mussel pediveligers was determined at 24h and 48h post-challenge by observation and counting using a dissecting microscope. Pediveligers sinking to the bottom of the wells with no intravalvular movement were counted as dead.

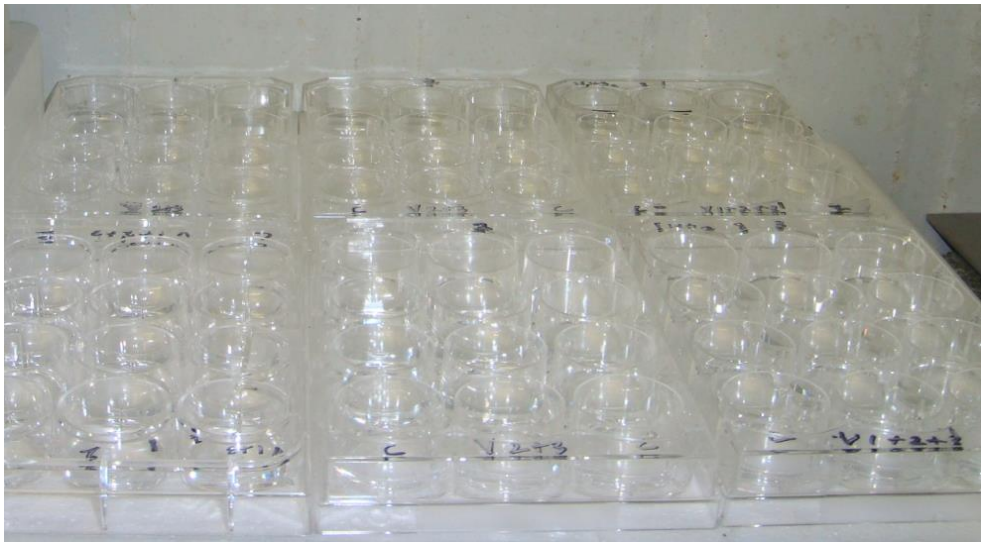

Figure 6.3.4. Multi-well plate pediveliger assay plates used for *Vibrio* pathogenicity assays.

Larval mortality was determined 24h and 48h after exposure and plotted against bacterial concentration for each treatment. The 50% lethal concentration (LC_{50}) was calculated by the method of Reed and Meunch (1938).

$$\text{Log } LC_{50} = \alpha \log b + c$$

Where α=(The mortality higher than 50%-50%)/(The mortality higher than 50%-The mortality lower than 50%); b=dilution rate, in current experiment $b=10^{-1}$; c=the log of minimum dilution rate, when the mortality higher than 50%).

Prior to analysis, data were transformed logarithmically to satisfy homogeneity of variances. Differences in estimated LC_{50} between *Vibrio* strains and combinations at 24h and 48h were compared using one-way ANOVA followed by Tukey's post-hoc test (α=0.05) using SPSS version 16.0.

6.4 Replacement live foods for larval culture

Strain selection and screening

A range of small temperate *Chaetoceros* strains and other potentially viable replacements strains with potential for larval bivalve culture were considered and assessed based on size and morphology (Table 6.4.1).

Table 6.4.1: Algal strains considered as replacement for live food species for larval mussel culture.

Strain	Species	Cell size (µm)	Morphology
Currently used			
CS-178	*Chaetoceros calcitrans*	3-6	Single cells, short spines
CS-176	*Chaetoceros muelleri*	6-8	Long spines
Replacement species considered			
CS-20	*Thalassiosira pseudonana*	3-5	Mostly single cells, no spines
CS-173	*Thalassiosira pseudonana* (3H)	3-5	Mostly single cells, no spines
CS-365/01	*Chaetoceros* sp.	5	Single cells, short spines
CS-365/02	*Chaetoceros* sp.	5	Single cells, short spines
UPMAAHU10*	*Chaetoceros calcitrans* (Malay)	3-6	Single cells, short spines
CCMP1015*	*Thalassiosira psuedonana*	3-5	Mostly single cells, no spines
CS-70	*Chaetoceros* cf. *mitra*	5	Irregular shaped cells, spines visible
CS-343	*Chaetoceros* sp.	6-8	rectangular to roundish, some spines
CS-256	*Chaetoceros* sp.	6	Rounded, mis-shapen cells, some spines
CS-251	*Chaetoceros simplex*	5-6	Short chains, some spines
CS-425	*Attheya septentrionalis*	5	Single cells, spines
CS-426	*Entomoneis punctulata*	5	Single cells

*unlikely to be viable candidates due to AQIS/biosecurity restrictions on importation and restriction on uncontrolled release of non-native material to the environment.

After consultation with industry representatives, four strains were selected based on preferred industry priority. The presence and relative proportion of the essential fatty acids (EFA) arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) was not considered. Comparative strains *C. calcitrans* CS-178 and *C. muelleri* CS-176 were used for comparative purposes as both are widely used in mussel and bivalve oyster culture and performance in standard hatchery algal culture systems was well known.

Hatchery bag culture

The growth dynamics of the four candidate strains selected by industry were compared against industry standard strains *Thalassiosira pseudonana* CS-20, *C. muelleri* CS-176 and *C. calcitrans* CS-178. All culture strains examined were purchased from the Australian National Algal Collection (CSIRO Marine and Atmospheric Research, Hobart) and sub-cultured into 200 mL erlenmyer flasks containing 150 mL of Walnes medium prepared using 35ppt filtered seawater. Stock cultures for

experiments with hatchery culture systems were maintained by serial sub-cultured at 20 ^{o}C and a light intensity of 120 µmoles photons PAR m^{-2} s^{-1}.

All six strains were first grown to late stationary phase in replicate 10 L carboys, 500 L bags except for *C. calcitrans* which showed poor/unreliable growth in 500 L bags. All strains were also grown in 40L hanging bags on a semi-continuous basis (Fig. 6.4.1) for 2-4 exponential-phase growth cycles, with 90% harvest and medium replacement on a 3 day cycle. Cell concentration of cultures was estimated daily (twice daily in 40 L bag experiments) by *in-vivo* fluorescence (Turner Designs 10AU), from triplicate sub-samples and the mean value plotted against time. Triplicate sub-samples for cell counts were taken daily and fixed using Lugol's iodine for later analysis. Cell concentrations were estimated using a Nebauer haemocytometer from samples representing mid-log and early stationary phases of each culture bag. Exponential growth rates (divisions day^{-1}) were calculated over a minimum of three data points using the equation: $k=log_2 (N_2 / N_1) / (t_2 - t_1)$; where N_1 and N_2=cell concentration at time t_1 and t_2 respectively (Levasseur et al. 1993).

After antibiotic purification, the unpurified parent strains were also compared to representative purified isolates for 3 exponential-phase cycles as described above. Differences in strain growth rate and harvest cell concentration were analysed using one- or two-way analysis of variance (ANOVA) with or without replication over time (dependent on experiment design). Experiments comparing growth rate and harvest concentration of antibiotic-purified strains with unpurified strains used two-way analysis of variance (ANOVA), with strain (*strain*) and antibiotic treatment (*ABtreat*) as factors, and replication over time. All analyses were carried out using the software program SPSS (Ver 19.0).

Fatty acid analysis

The presence and relative proportions of the essential fatty acids (EFA) ARA, EPA and DHA, Pettersen et al. 2010) was determined from published data for *T. pseudonana* strains CS-173 and CS-20, *C. muelleri* CS-176 and *C. calcitrans* CS-178. For both *Chaetoceros* sp. CS-365 strains, fatty acid analyses were carried out as follows. Replicate 250 mL cultures of each strain were grown in 500 mL erlenmyer flasks at 20 degrees and a light intensity of 100 µmoles photons PAR m^{-2} s^{-1}. Cell biomass was harvested at late-logarithmic phase (approx. 7 days after inoculation), by gravity/vacuum filtration onto glass fibre filters (GFC, Whatman Co, USA), the filters immediately frozen at -80 C and transported on ice to the analytical laboratory. Lipid analysis was carried out by Analytical Services Tasmania (AST, Hobart) according to standard methods (FAME, fatty-acid methyl-ester).

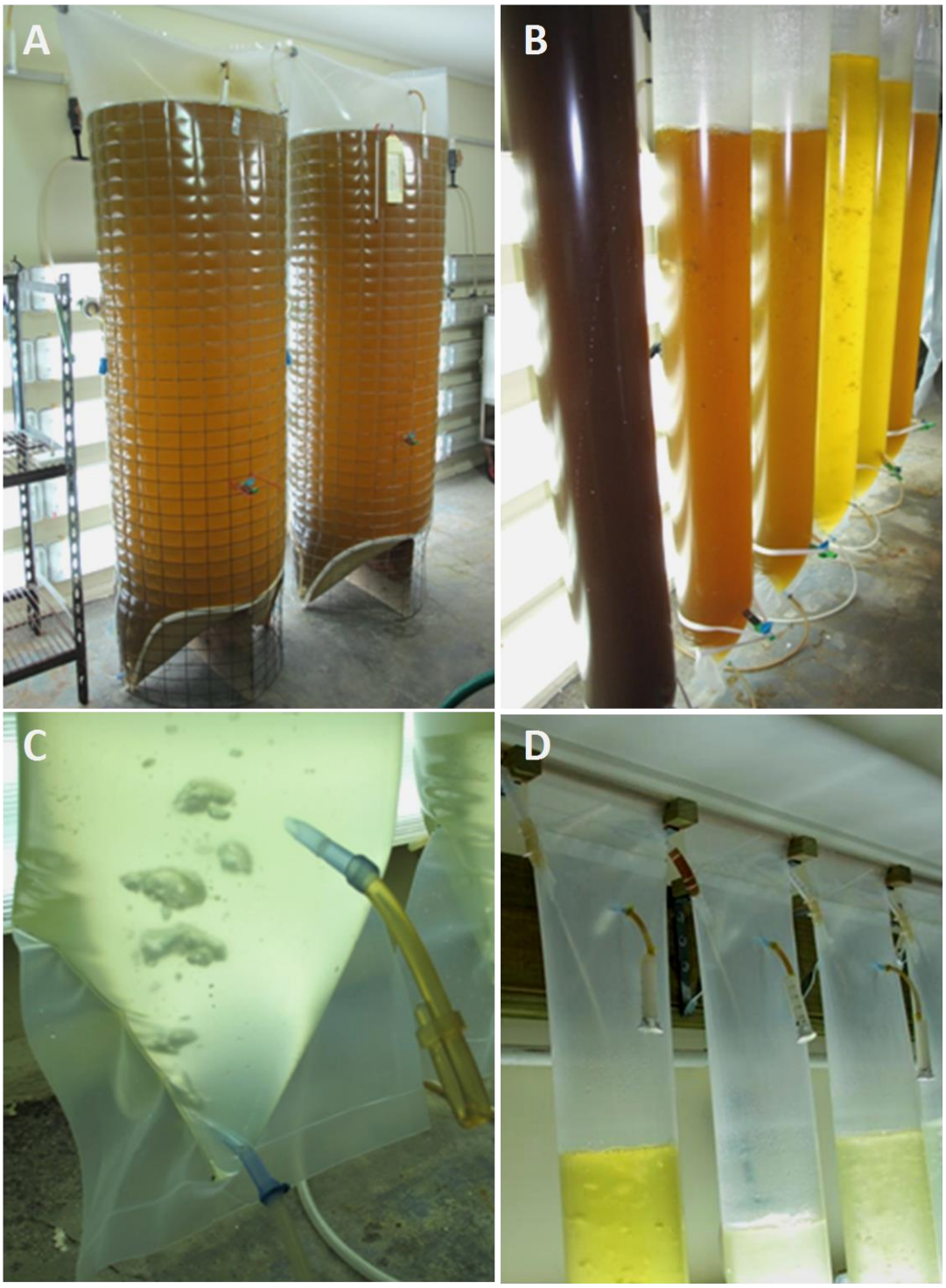

Figure 6.4.1: Hatchery bag culture systems used in the study. A. Batch/semi-continuous 500L polyethylene bag cultures with airline fittings and harvest tap. B-D. Semi-continuous 40L hanging bag system. B. Several 40L bags at mid-late-exponential and stationary phase. C. Modified harvest port and airline for 40L bags. D. Air outlet and refilling port of 40L bags.

Purification and bacterial screening of candidate strains

All four strains were subjected to antibiotic treatment using a combination of liquid and solid purification approaches. Three rounds of treatment were undertaken with liquid medium cultures grown 150 mL erleneyer flasks containing 100 mL of F/2 medium at laboratory stock culture conditions (described earlier). All cultures were screened for antibiotic sensitivity of culture-associated bacteria. Stationary phase stock cultures of the four candidate algae were spread-plated on to solid Zobell's marine agar (ZM medium), test discs of 10 different antibiotics (Becton-Dickinson, Australia) applied to the surface and the plates incubated at 20-20°C in the dark for 7 days. Plates were examined for clear zones and those showing zones of no bacterial growth were selected for further use in purification experiments.

Purification of liquid cultures was carried out by addition of cocktails of the antibiotics ampicillin gentamycin, cefotaxime, and neomycin to early stationary phase cultures (Table 6.4.2). After antibiotic addition, cultures were incubated for three days under stock culture conditions. After three days, 10 mL sub-samples of culture were removed aseptically, washed by repeated centrifugation/re-suspension in sterile growth medium to remove residual antibiotics, and the cultures allowed to recover grown to late stationary phase before bacterial screening. Low concentrations were unsuccessful at removing all cultivable bacteria (see below) therefore additional rounds of purification were carried out higher concentration (referred to herein as "*med-AB*" and "*high-AB*").

Table 6.4.2: Antibiotic cocktail concentrations used for culture purification experiments.

Antibiotic	Antibiotic concentration (µg mL^{-1})				
	Low 1	Low 2	Low 3	Med-AB	High-AB
Ampicillin	100	150	200	500	1000
Cefotaxime	100	125	150	250	500
Neomycin	100	125	150	250	500
Gentamycin	50	100	150	250	500

Antibiotic-treated liquid cultures of each strain were also subjected to an additional round of purification using a solid medium approach. Antibiotic purified strains were grown to late log phase in 50 mL erlenmyer flasks containing 25 mL of Walne's medium (medium used by Spring Bay Seafoods) and 200 µL of each culture spread-plated onto Walne's medium solidified with 0.8% Bacto agar. The most effective antibiotics identified in pre-screening that were not used in liquid treatments were used. Test discs of imipenen (10ug), carbenicillin (100ug) and cefotaxime (30ug) were placed in a triangular arrangement at 30mm from each other on the plate surface, the plates wrapped in parafilm, and incubated at stock culture conditions for 1 week. Six individual colonies were aseptically removed from the clear zone between the three discs, and either transferred on to sterile solid Walne's medium or directly to a 50 mL flask of sterile Walne's medium. Plates and flasks were then incubated for several weeks to establish a viable culture and cultures subjected to bacterial screening.

Screening of cultures for cultivable bacteria

Presence of cultivable bacteria in post-purified cultures was determined from liquid cultures grown to stationary phase in Walne's medium. A volume of 200 µL of culture was spread onto Zobell's marine agar plates prepared at 1x concentration (ZM1) and 1/10 concentration (ZM10) of added nutrients. Screening was also carried out on 0.8% agar medium prepared from culture medium filtrate of log-phase cultures of each of the respective candidate cultures (herein called Algal Organic Carbon medium, AOC). All bacterial detection plates were wrapped in parafilm and incubated at 22-25°C for seven days prior to examination for bacterial colonies. After incubation, surfaces of spread plates were examined for presence of bacterial colonies by eye. All plates without macroscopic colonies were also examined for microscopic (and clear) colonies at 60x magnification using a Leica MZ9 binocular stereomicroscope equipped with brightfield and darkfield trans-illumination. The presence of distinct colonies was taken as evidence on non-axenic status.

PCR-based bacterial screening of cultures

Total DNA was extracted from stationary-phase cultures of the 24 plate-purified using a cetylmethylammonium bromide purification (CTAB) method (Ausubel et al. 1999). Two positive control samples (original non-axenic culture, and DNA from a bacterial source) were included with all algal culture extractions to assess relative extraction quality and efficiency. Negative control samples (extractions from sterile culture medium) were included to detect the level of false positive amplification arising from casual contamination during extraction or extraction reagent contamination. The presence of bacterial DNA in extracted samples was then determined by PCR amplification of approximately 500 bp of the bacterial 16SrDNA gene, using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 518R (5'-ATTACCGCGGGCTGCTGG-3') (Weisburg et al. 1991). The PCR was performed using a MJ Research PTC-200 Thermal Cycler (MJ Research, USA). Reactions were carried out in 50 µL reaction volumes containing 10 *p*moles of each primer, 1.25 U of Taq polymerase (BioTaq, Bioline, UK), 3 mM $MgCl_2$, 200 mM of each dNTP and Bioline ammonium PCR buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris HCl (pH 8.8), 0.1% Tween -20). The thermal cycling consisted of an initial denaturation at 96°C for 5 mins, followed by 30 cycles of: denaturation at 95 °C for 15 s, annealing at 50°C for 30 s, extension at 72°C for 1 min; and a final extension at 72°C for 5 mins. Aliquots of 10-20ul of each PCR reaction were electrophoresed through 1% agarose/TBE gels and examined for 16SrDNA products of expected length; presence of a visible PCR product of expected length was considered to indicate the presence of bacteria.

Purified strains of all four candidate strains were supplied to research partner (Spring Bay Seafoods) on July 3rd 2012 for independent testing in the hatchery. Representative purified strains and algal culture conditions were lodged with the Australian National Algal Culture Collection (CSIRO Marine and Atmospheric Research, Hobart) to be made available for purchase by other marine hatcheries.

Larval feeding experiments

One representative strain of each plate-purified candidate strains was selected for feeding experiments with 7 day old mussel larvae. Strains of *Chaetoceros muelleri* CS-176, and *Chaetoceros calcitrans* CS-178 were included as comparisons. Six day old mussel larvae produced as part of a SBS Hatchery commercial run were collected from the tank by gentle filtration onto nylon mesh screens, packed in damp absorbent material, transported to the laboratory, and maintained at 6 °C overnight. The cooled, packed larvae were removed from cool storage, allowed to equilibrate to 22°C over a period of an hour, and sub-samples resuspended in 100 mL of 0.2 µm FSW. Larvae were observed for normal motility and swimming activity, the density (larvae mL^{-1}) estimated using a Sedgwick-Rafter counting chamber, and larvae transferred at a concentration of 15 larvae mL^{-1} to 100 mL of 0.2 µm filtered seawater sterile in 120 mL polypropylene jars (Technoplas, Australia).

Replicate jars were fed a single species diet consisting of one of the four candidate species, delivered as a daily ration of $3.5x10^6$ cells (100 mL at $3.5x10^4$ cells mL^{-1}) at the beginning of each 24hr period for three days. Comparative feeding experiments with single species diets of *Chaetoceros muelleri* and *Chaetoceros calcitrans* CS-178 were fed at the same level. Due to the smaller cell size of *C. calcitrans* CS-178, an additional replicate treatment was included, fed at a daily ration of 10^7 cells (100 mL at 10^5 cells mL^{-1}). Water exchanges were not carried out over the period of the experiment. Replicate residual algal cell counts carried using a Neubauer haemocytometer, prior to feeding at 24 and 48 h, and additional algal cells added at 24 h and 48 h to restore the cell concentration to the starting density of each treatment (3.5×10^4 cells mL^{-1} or 10^5 cells mL^{-1}). The proportion of algal diet ration cleared by the mussel larvae during 24 hr feeding periods was estimated by direct calculation from initial and residual algal cell counts, and concentrations/volume of additional food added. At 72 h, 1 mL subsamples were loaded into a Sedgewick-Rafter cell and larvae observed for evidence of ingestion (dark green/brown colour within gut region of larvae). Larval mortality was estimated from replicate sub-samples; larvae were scored as dead if there was no evidence of motility (not swimming) and no evidence of perivalvular/cilial movement.

7. RESULTS AND DISCUSSION

7.1 Factors affecting settlement, retention, survival, & growth in land-based nursery systems

Estimating density of spat on spools prior to deployment

The precision of the estimate spat density is a function of the variability of the density and the average density, and is related to confidence that we have in the estimated density. Variability in the density of spat increased with the size of the sampling unit and was relatively large (Fig. 7.1.1). It was difficult to cut the 2 cm and 4 cm length of rope and the 8 cm and 10 cm length rope took approximately equal time to inspect and locate the spat. Therefore, it appeared that the 6cm lengths of rope were the best sampling unit size to estimate the density of spat on the continuous dropper. Given that it took 13-18 mins per 2 cm section to extract and count spat, the 6 cm length was a good compromise between time and the difficulty of cutting sections of the dropper >6 cm.

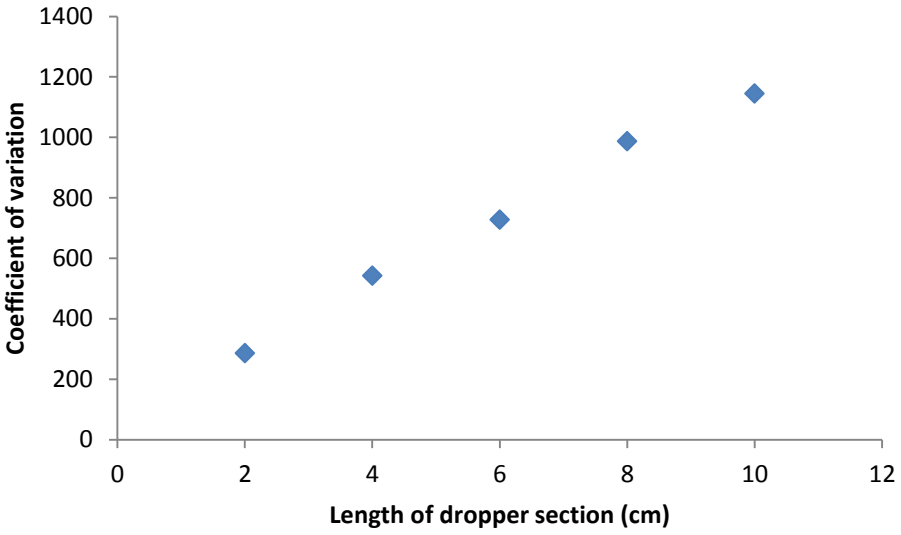

Figure 7.1.1: Changes in the coefficient of variation in estimates of density of spat for the different sampling unit size (ie 2 cm, 4 cm, 6 cm, 8 cm, and 10 cm rope lengths).

The relationship between precision and sample size followed a negative exponential relationship, with precision decreasing to <0.4 as we approach a sample size of 30 for all five of the sampling unit sizes (Fig. 7.1.2). To obtain a precision of < 0.3 it would be necessary to take approximately 28 replicates. Such replication is not going to be logistically feasible in a commercial setting given the time required to process each sample. Taking eight 6 cm replicates will provide a precision of approximately 0.57.

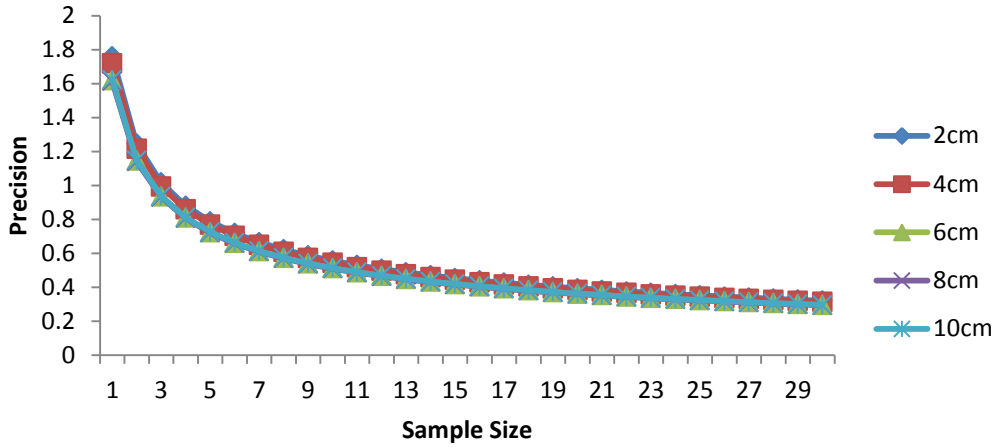

Figure 7.1.2: The change in the precision of density of spat estimates for the different sampling unit size (ie 2 cm, 4 cm, 6 cm, 8 cm, and 10 cm rope lengths) as sample size (ie replicates) increases.

Patterns of settlement and mortality in relation to physical and biological factors

Of the estimated 6.5 million pediveligers added to the settlement tank, it was estimated that 2.84 million spat, 95% confidence limits [2.43 million, 3.25 million]) were present on the dropper two weeks later and immediately prior to transfer to the farm nursery site. Based on these two estimates approximately 44% (37-50% based on 95% confidence limits) of the pediveligers added to the settlement tank successfully settled and survived for 16 days on the dropper line. It was likely that spat could have settled on surfaces other than the rope eg tank wall or floor, or the stainless steel spool. The number of dead spat still attached to the rope at the time of sampling was estimated to be approximately 21% of the 6.5 million added pediveligers (1.39 million dead spat, 95% CL [1.12 million, 1.66 million]). Pediveligers have a preference for settling on complex surfaces like rope, than smooth surfaces (Carl et al. 2012), therefore we are assuming that potentially by 16 days that 50% of the pediveligers would have settled on the ropes (Carl et al. 2012).

Approximately 2.68 million spat were unaccounted for, ie those not located dead or alive on the rope, and this may be due a number of factors. Not all the pediveligers added to the tank may have been fully competent to settle, particularly slower growing individuals, and they may have been unable to continue growing and developing in the settlement tank. Even if competent to settle, if *Mytilus* pediveligers are able to delay settlement for several weeks like many other bivalves (eg Gribben et al. 2011), by staying in the water column or detaching from a settlement surface to go back into the water column these individuals would be lost to commercial production. Estimates of the dead pediveligers we counted were only those animals that had attached to the rope and were still attached to the rope, although dead, at the time of sampling.

A strong positive correlation between the density of living and dead spat ($r=0.80$, $n=181$, $P<0.001$), suggests that either mortality is density dependent or that factors causing mortality are specific to certain regions of the spool.

There was little evidence that the density of live spat on the rope differed between the outer and inner spool (F= 0.45, df 2, 121, *P*=0.832), but they did show a preference for the height in the tank that they settled and survived post-settlement (F=2.20, df 2, 121, *P*<0.001). The density of spat in the top half a meter of the tank was almost three times greater than in the lower two-thirds of the tank (Fig. 7.1.3).

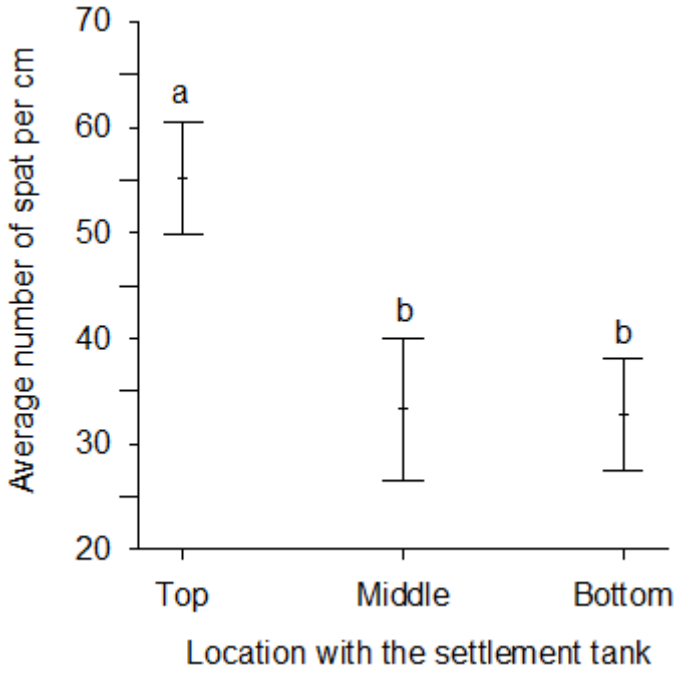

Figure 7.1.3: The mean density of live spat (per cm piece of rope) that settled and survived in each of three depths in the settlement tank. Means with different letters are significantly different from one another.

There were differences in the density of dead spat among the different locations on the dropper (F=23.12, df 3, 172, *P*<0.001). The outer wind of the spool had the greatest density of dead spat, particularly in the top half of the tank, where the densities of dead spat were 3-4 times greater than any other place on the spool (Fig. 7.1.4).

Sixteen days after the spat settled there were significant differences in the average size of spat that were a function of where on the dropper the spat had attached themselves ($F_{depth*location}$=3.106, df 4,441, *P*=0.015). The largest spat were those that had attached themselves on the section of rope that were near the top of the tank and on the outside wind of the spool (Fig. 7.1.5). These spat were approximately 3-4 times larger than spat found in the other locations in the tank (Fig. 7.1.5). As mussel spat have the capacity to move after they have settled it is not clear if these larger spat had spent the first 16 days of their post-settlement life in these locations and grown faster, or if larger faster growing individuals had relocated from other locations on the spool to these locations.

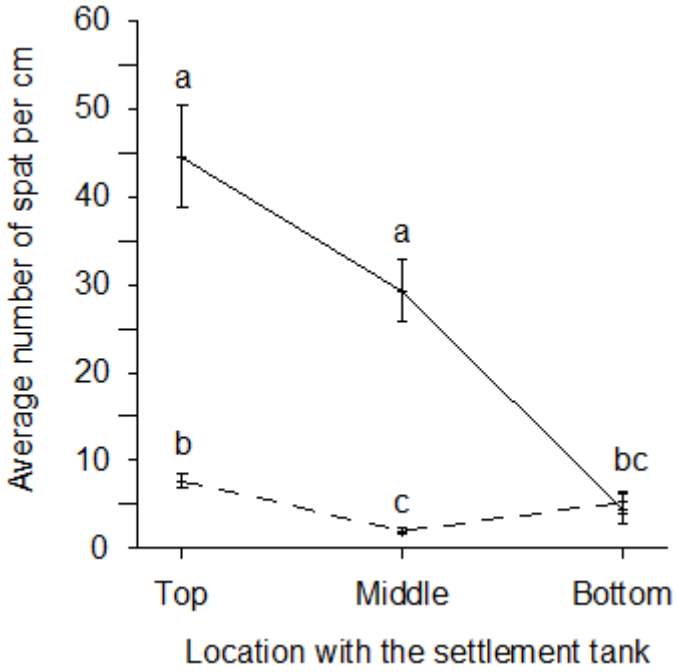

Figure 7.1.4: The mean density of dead spat (per 2 cm piece of rope) on the rope in each combination of the three depths in the settlement tank and positions in the spool. Dashed line = inside spool, solid line = outside of spool. Means with different letters are significantly different from one another.

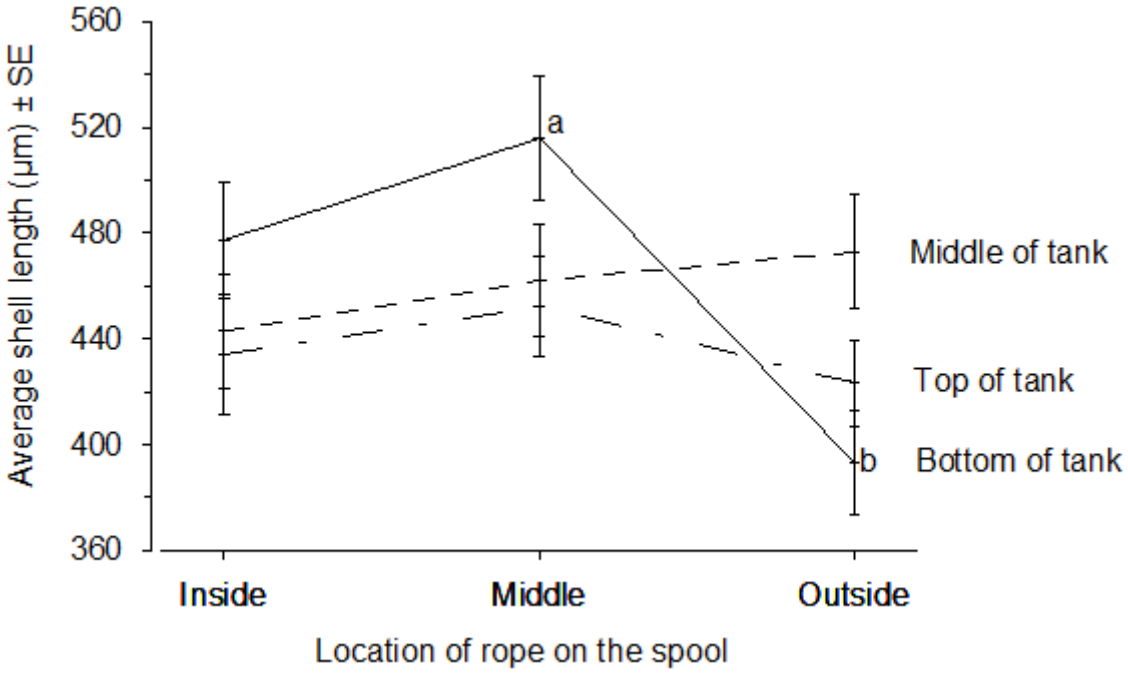

Figure 7.1.5: The mean shell length of spat collected from in each combination of the three depths in the settlement tank and positions in the spool. Means with different letters are significantly different from one another; means with no letter provided are not significantly different from any of the means.

7.2 Use of live & artificial diets for broodstock conditioning

Living diets to facilitate broodstock conditioning in Mytilus galloprovincialis

There was no significant difference in mean stored glucose concentration in broodstock mussels among the four sampling weeks (F=0.71, df 3, 24, *P*=0.554), diet (F=0.65, df 3, 8, *P*=0.604), or sampling week with diet (F=0.93, df 9, 24, *P*=0.516). Large standard error values present in mean stored glucose values of broodstock mussels in sampling week revealed wide distribution of means across individual diets (Fig. 7.2.1). High variation of the stored glucose level found among individuals made it impossible to detect any differences among the diets fed to the broodstock. While stored glucose in the form of glycogen is one energy source used to fuel reproduction, variability among females in not unusual in *M. galloprovincialis* (Fearman 2100). Some of this variability is thought to due to the capacity of females to use either capital (ie glycogen) or income (ie directly from food) sources of energy to fuel reproduction (Fearman 2010). This variability is substantially greater after the spring spawning event, with some females continue production of new eggs using food as an energy source, while others begin the process of glycogen storage (Fearman 2010). In either case the value of measuring glycogen concentration to assess reproductive capacity may have little value in this species.

The ratio of spawning and non-spawning broodstock mussels differed among the four diets at the end of the conditioning period (χ^2=9.12, df 3, *P*=0.028). Mussels fed either the binary diet *Isochrysis* sp. + *Pavlova lutheri* or *Isochrysis* sp. + *Chaetoceros muelleri* had a greater number of spawning individuals than was expected, although the latter gave the greatest proportion of spawning individuals (Fig. 7.2.2). The ratio of spawning to non-spawning individuals was independent of diet for the other binary diet and the ternary diet. This outcome was similar to that found by Fearman et al. (2009), who found that this binary diet did better than the single algal species by themselves. Curiously the three microalgal species when in binary combinations did demonstrate some capacity to increase the capacity to fuel reproduction over the experiment. However, the ternary combination ie all three species, did not appear to increase the proportion of spawning individuals and was poorer that two of the binary combinations.

There was no significant difference in mean number of eggs produced by females among the four diets (F=0.78, df 3, 44, *P*=0.514). The variation in egg production was greatest in binary diet *Isochrysis* sp. + *Pavlova lutheri* compared to others (ratio of 3:1) because a single female produced three times as many eggs than the mean value in the diet (Fig. 7.2.3). However, the mean number of eggs among the diets was not significantly different even with this female removed from the analysis (F=0.32, df 3, 43, *P*=0.812). However, the average egg diameter did differ among females fed the different diets (F=4.71, df 16,180, *P*<0.001). With females fed the ternary diet of *Isochrysis* sp. + *Chaetoceros muelleri* + *Pavlova lutheri* producing significantly smaller eggs (Fig. 7.2.4). Eggs produced by females in this study were 10 µm larger than the eggs found in the same species by Sedano et al. (1995). Egg size is considered to be an indicator of condition; therefore eggs produced from broodstock under nutritional stress would have been smaller and contained less

nutritive matter (Bayne et al. 1978). In this study, females fed the diet on all three microalgae species produced the smallest eggs. *Mytilus galloprovincialis* eggs have high proportions in protein (45 % of total dry weight) followed by lipid (22 %) and small amounts of carbohydrate (3 %) (Sedano et al. 1995). However, the diet *Isochrysis sp*. + *Chaetoceros muelleri* + *Pavlova lutheri* has a high protein and lipid concentration, therefore may the greater protein content of these species may have been used for somatic growth, rather than egg production

The survival of D-larvae 48 hours after fertilisation produced by females fed on the binary diet *Isochrysis* sp. + *Chaetoceros muelleri* was four times greater than for females fed on any other diet (Fig. 7.2.5), although this difference of D-larvae production was not linked to the size of the larvae. There was a significant difference in the average D-larvae shell length among the four diets ($F=26.85$, df 3, 236, $P<0.001$), however, the largest larvae produced was by females fed the ternary diet (Fig. 7.2.6). The D-larvae from females fed on the binary diet *Isochrysis* sp. + *Chaetoceros muelleri* while on average were smaller than those produced by females fed the ternary diet, it was only a matter of approximate 4μm (Fig. 7.2.6). The success of fertilised egg to hatch and metamorphose into D larvae stage is associated with the amount of endogenous lipid reserves allocated to the egg during vitellogenesis (Devauchelle and Mingnant 1991; Caers et al., 2002). Specifically, eicosapentaenoic and docosahexaenoic acid have an important role in the hatching success of a range of bivalve species including *M. edulis* (Nevejan et al., 2008), *Crassostrea gigas* (Caers et al., 2002), and *Pecten maximus* (Soudant et al., 1996). The *Chaetoceros muelleri* + *Pavlova lutheri* diet used in this experiment contained the lowest proportion of DHA and EPA and these females produced eggs with the poorest hatching rate. Additionally, poor survival to D-larvae was exhibited by broodstock fed *Isochrysis* sp. + *Pavlova lutheri* which has low omega-6 fatty acid content, indicating that these lipids are also important for early larval development. Both omega-3 and omega-6 fatty acids are considered to be essential source of energy in embryogenesis for *Crassostrea gigas* (Caers et al., 2002) and this is also supported by the present study.

Female mussels fed diets high in total lipids i.e. *Isochrysis* sp. + *Pavlova lutheri* and *Isochrysis* sp. + *Chaetoceros muelleri* produced the greatest number of eggs compared with the other diets. Female bivalves fed mixed microalgae diets have greater reproductive output (Utting and Millican, 1997) and better larval viability to spat phase, however, specific lipid components in the diet determines the reproductive outcome. In *M. edulis*, the average fecundity of females fed a cholesterol-rich mixed diet was 5.0 x 106 eggs and a maximum fecundity of 11.2 x 10^6 eggs was attained by an individual from a mean dry meat weight of 2.35 g per mussel. Although the mean dry weight of mussel used in this experiment was 2.53 g, the average fecundity of diet *Isochrysis* sp. + *Pavlova lutheri* was 4.47 x 106 eggs and a maximum fecundity of 13.4 x 106 from an individual of the same diet is relatively similar. Pacific oyster, *Crassostrea gigas*, broodstock fed a diet rich in omega-3 fatty acids produce a greater number of eggs than broodstock fed without lipid enrichment (Caers et al., 2002). In this experiment, mussels fed diet lower in total lipid produced fewer eggs when fed a diet of *Isochrysis* sp. + *Chaetoceros muelleri* + *Pavlova lutheri*. Therefore, lipid levels within a mixed diet could have contributed a crucial role on fecundity in broodstock *M. galloprovincialis* conditioning.

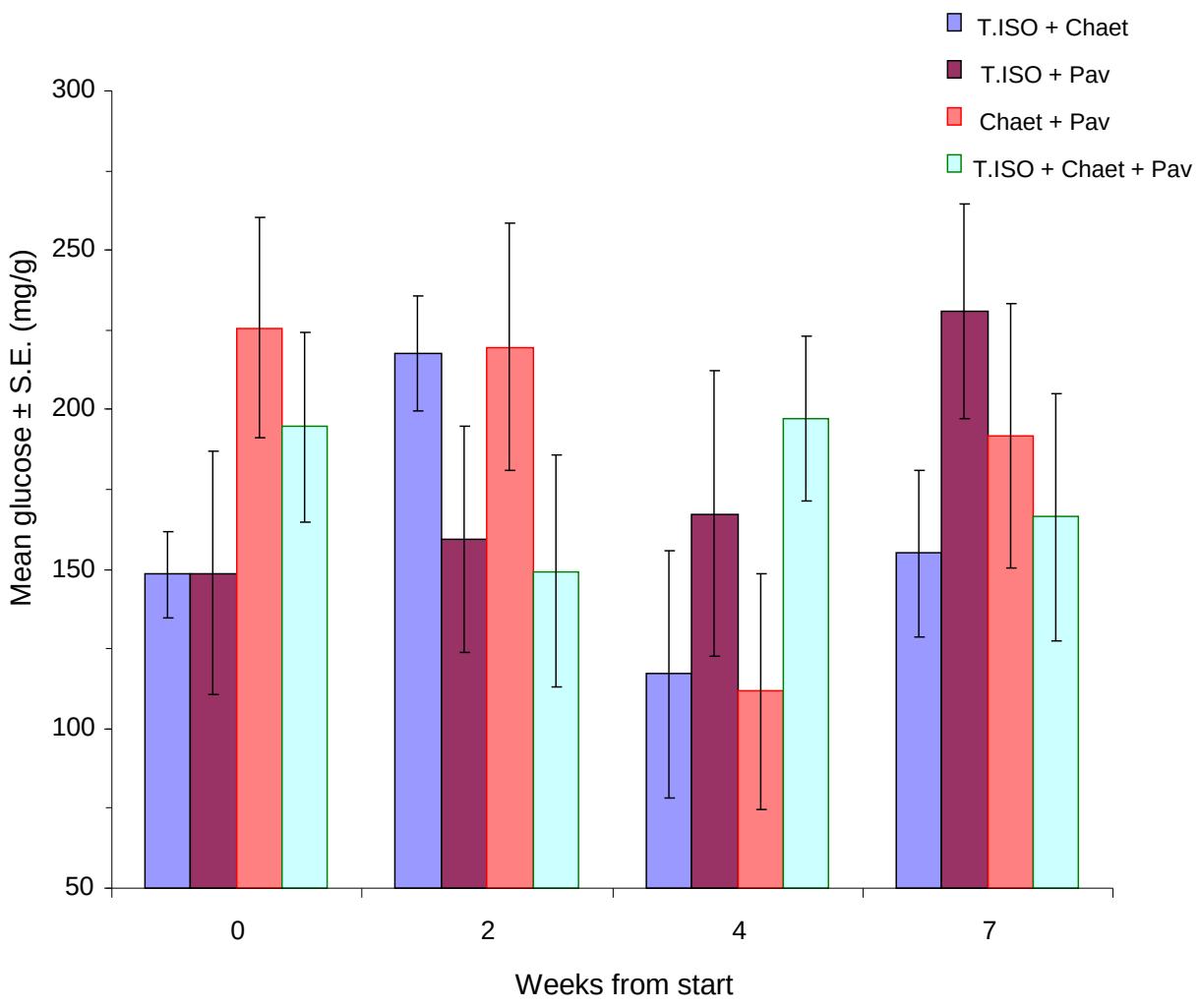

Figure 7.2.1: Glucose concentration of broodstock mussel (*M. galloprovincialis*) from the four diet diets sampled over time.

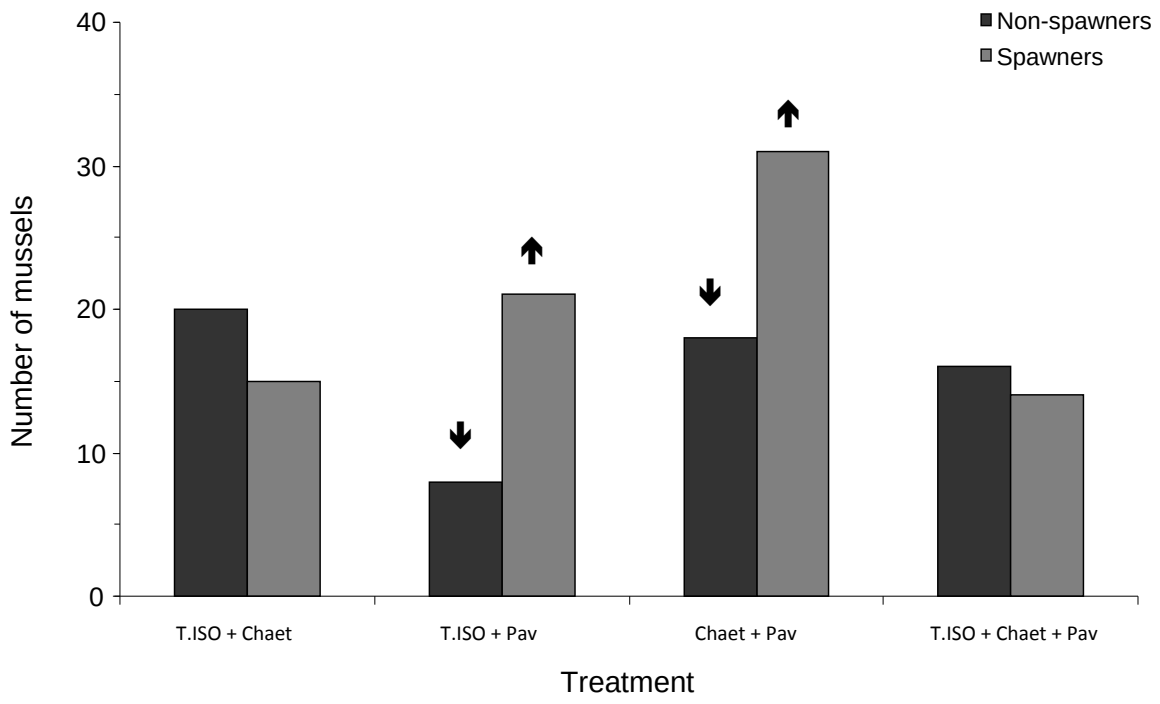

Figure 7.2.2: Numbers of broodstock mussel (males and females) spawned in respective diets. Arrow ↓ indicates fewer mussels observed than expected and ↑ indicates more mussels observed than expected.

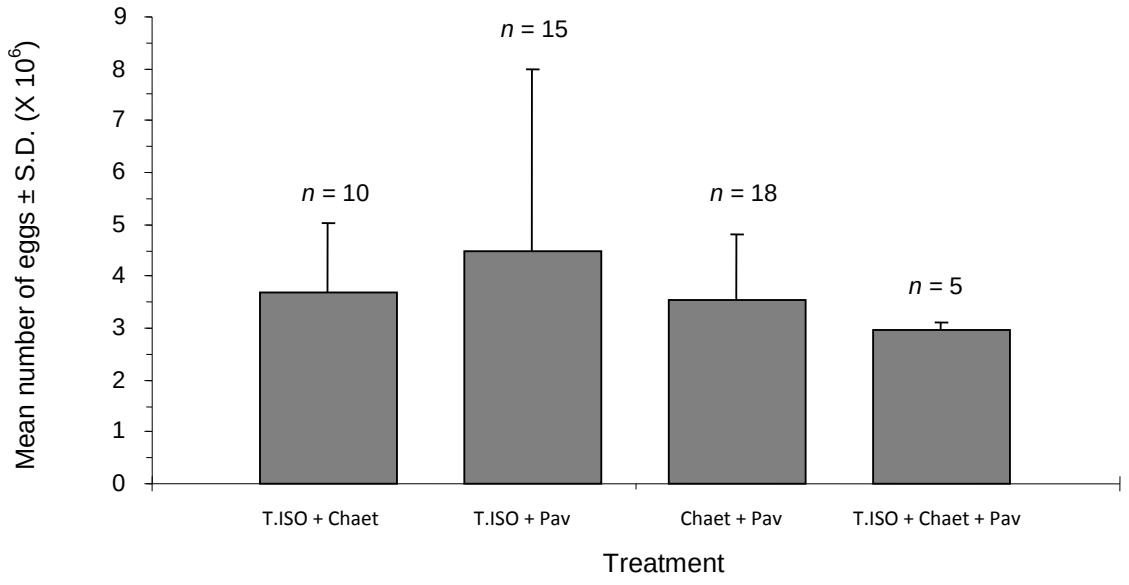

Figure 7.2.3: Mean number of eggs produced by females from the four diets. n=number of females spawned in the respective diet.

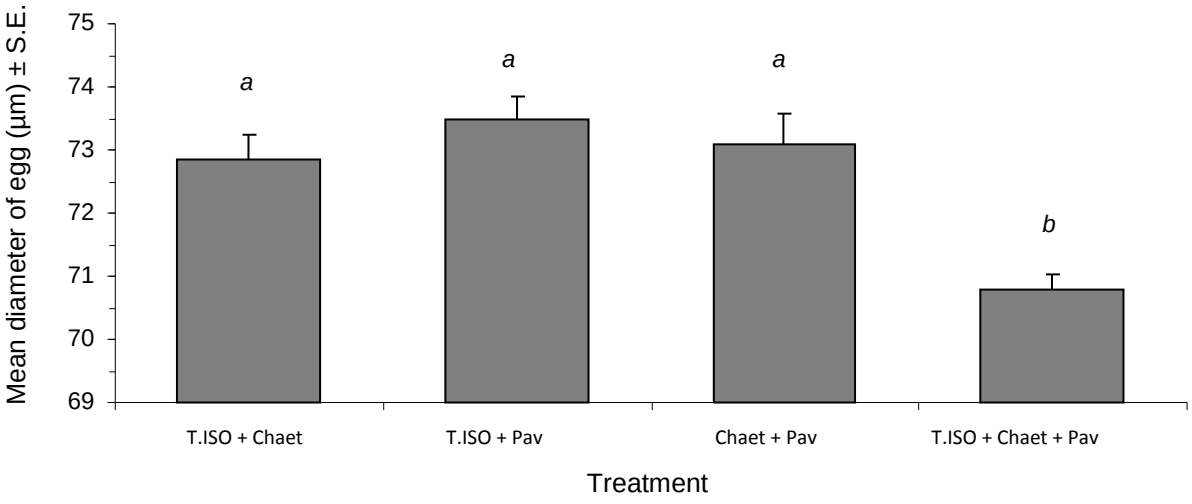

Figure 7.2.4: Mean diameter of eggs from the four diets. Means with different letters are significantly different from one another. n=60 for each diet.

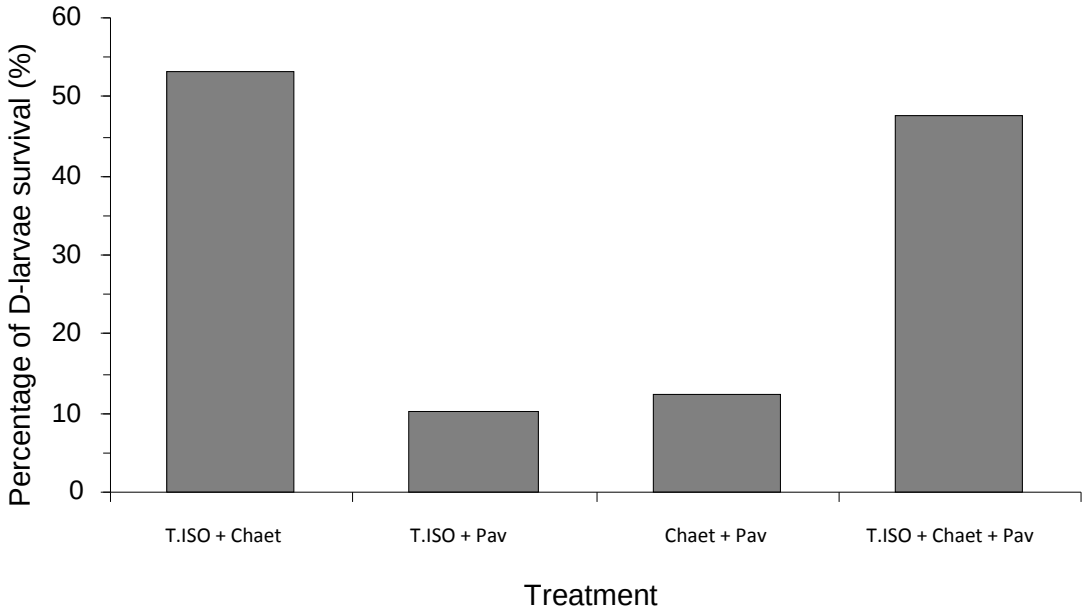

Figure 7.2.5: Percentage of D-larvae produced by females fed on each of the four diets.

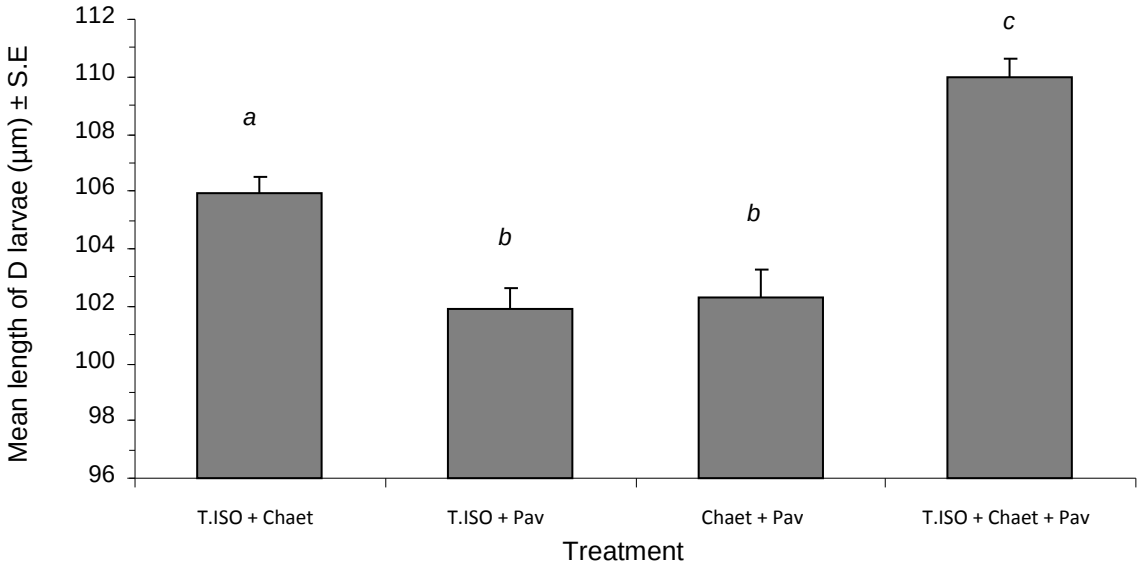

Figure 7.2.6: Mean length of D-larvae produced by females fed on each of the four diets; n=60 for each diet. Means with different letters are significant in difference from one another

Non-living diets to facilitate broodstock conditioning in blue mussels

The average total mass of animals included in the experiment was 46.3 g at the start of the experiment and 53.4 g by the end of the experiment. Of the 450 animals a total of 342 animals survived, with mortalities in the diets ranging from 63% in the group animals who had 100% replacement of live feeds with M1 diet to 10% in the group of mussels fed 60% replacement with SD diet. The control group who were fed 100% live algae had a mortality rate of 13%, a rate which was exceed by 11 of the 14 diets. There was no evidence of a correlation between percent replacement and mortality for either of the non-living diets (M1 $r=0.45$, $n=8$, $P>0.05$; SD $r=0.09$, $n=8$, $P>0.05$).

Water temperatures in the experiment ranged from 11.7-15.6^{o}C, with increases and decreases of 3-4^{o}C occurring over two weeks on several occasions during the experiment (Fig. 7.2.9). The timing and magnitude of mortalities in the experiment appeared to occur during both peaks and troughs in the water temperature (Fig. 7.2.9). While it was difficult to conclude definitively that temperature contributed to the death of animals in the experiment, controlling the temperature would assist in controlling physiological processes that relate to the use of energy. Ideally in the broodstock conditioning process maximum energy should be being allocated to reproduction and least to basal metabolism and growth. For *M. galloprovincialis* the best temperature for gametogenesis and broodstock conditioning from direct energy allocation, and not from stored energy production, is 7-10^{o}C (Fearman & Moltschaniwskyj 2010).

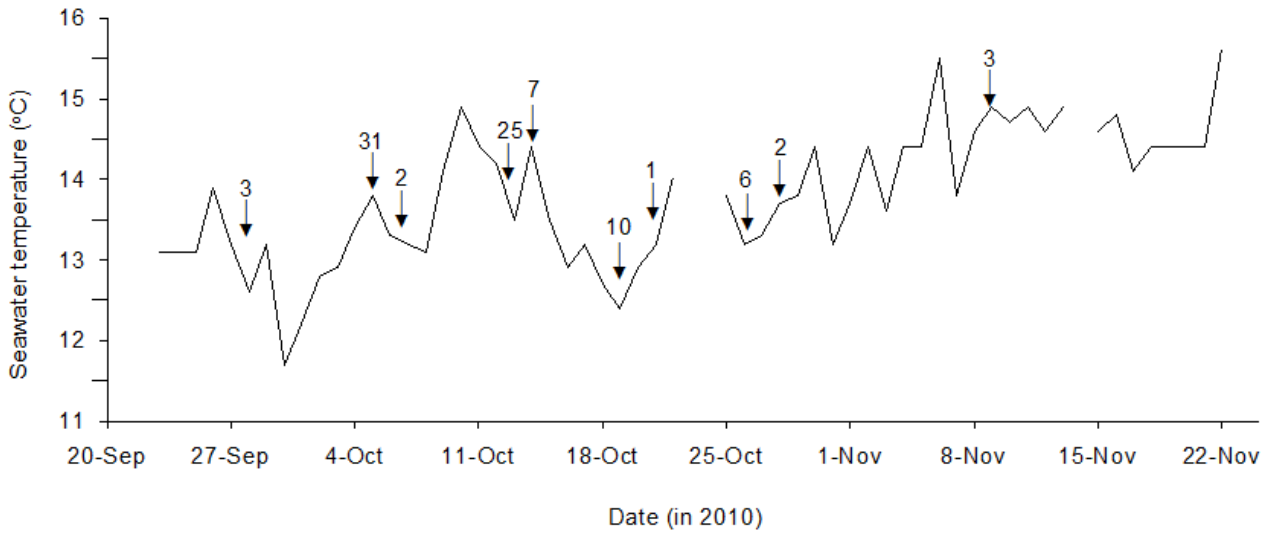

Figure 7.2.9: Daily water temperature (°C) in a full seawater recirculation system for broodstock conditioning from 23rd September to 22nd November 2010. The arrows and numbers indicate both the time and number of individuals that during the experiment.

The relative frequency of females that started the process of gonad development was greatest for individuals supplemented with the M1 diet than the SD diet ($\chi^2=10.01$, df 4, $P=0.04$); with almost

20% fewer females supplemented with M1 with empty gonads (Fig. 7.2.10). While 34% of the females supplemented with M1 had some degree of gonad development, only 16% the SD supplemented females had some gonad development (Fig. 7.2.10). Given that most females has gonad empty of eggs, it was not possible to examine the association between amount of supplementation and amount of gonad development, so Stage 1, 2 and 3 were collapsed into a single category. There was no evidence that the amount of supplementation affected gonadal development (χ^2=16.66, df 12, *P*=0.16). Overall, approximately 8% of the females across the experiment appeared to have spawned at some stage during the experiment (Fig. 7.2.10). This provided evidence that greater gonadal growth was supported by dietary elements supplied by the M1 diet.

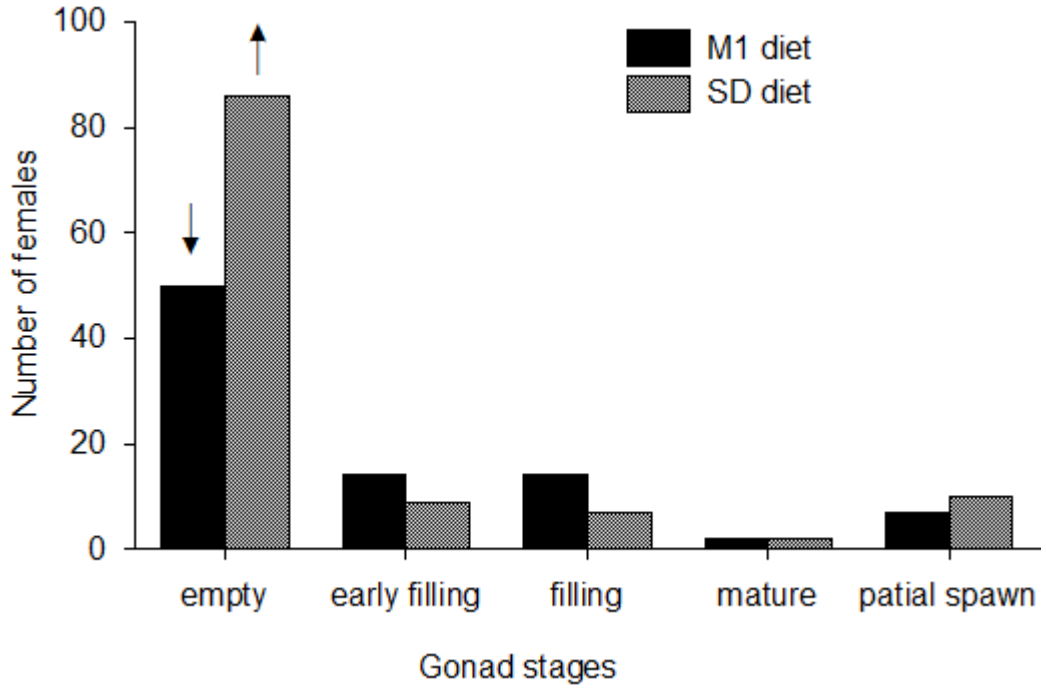

Figure 7.2.10: Percent of females in five gonad developmental stages fed two different diets, M1 Bivalve Feed (n=87) or Shellfish Diet (n=114). The arrows indicate those gonad stages where the observed frequencies were less than (↓) or more than (↑) expected under the hypothesis that gonad stage was independent of diet.

The type of supplemental food affected the extent of glycogen stores in the vesticular connective tissue (VCT) that the females accumulated during the experiment (χ^2=13.13, df 2, *P*=0.001); with more that 20% of females across the experiment accumulating glycogen stores. The proportion of M1 diets fed females that had VCT full of glycogen was double that of females fed the SD supplement (Fig. 7.2.11). There was evidence of an association between the amount of supplementation with a non-living diet and the extent of glycogen stores (χ^2=44.65, df 12, *P*<0.001), with greater frequencies of females with extensive glycogen stores seen when 30% or 100%

supplementation was provided (Fig. 7.2.12). Almost 30% fewer females full of glycogen (VCT stage 2) were present when 60 and 70% supplementation was provided (Fig. 7.2.12). While accumulation of glycogen stores will be important for fuelling of gonadal growth over the winter; the focus on immediately gonadal growth was essential for the holding time of broodstock to be a short as possible. So while supplementation provided enough energy to allow the females to accumulate glycogen stores it will necessary to experiment with environmental factors, like temperature (Fearman & Moltschaniwskyj 2010), to facilitate allocation of excess energy to gonadal growth rather than to storage.

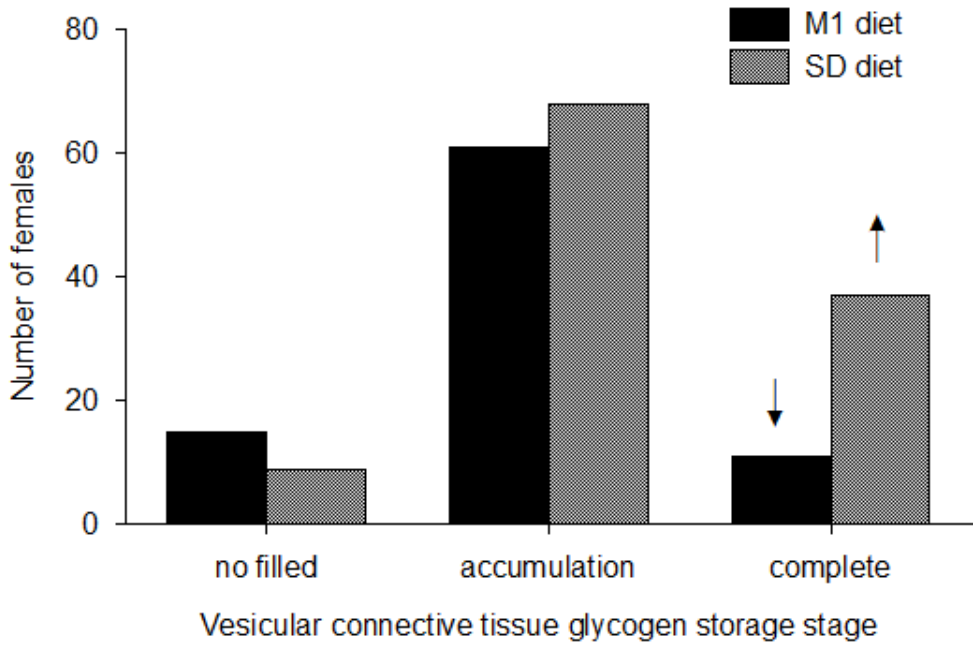

Figure 7.2.11: Percent of females in 3 developmental stages of vesicular connective tissue (VCT) fed two different diets of M1 Bivalve Feed (n=87) or Shellfish Diet (n=114). The arrows indicate those VCT stages where the observed frequencies were less than (↓) or more than (↑) expected under the hypothesis that glycogen storage was independent of diet.

The extent of protein and lipid stores in the adipogranular (ADG) tissue was no different between the two diet supplements (χ^2=2.12, df 2, *P*=0.347). Approximately 43% of females had no lipid or protein stores, while another 42% had some filling of the cells with lipid and protein. There was an association between the percentage of diet supplementation and the extent of protein and lipid stores in the ADG, but this was due to fewer females in some categories of ADG fullness rather than increase in frequencies in some categories (χ^2=26.71, df 12, *P*=0.008). When little supplementation occurred (30%) most females (>75) has some lipid and protein deposits, with only 16 having no lipid or protein accumulation which is half that seen in the great supplement levels (Fig. 7.2.13).

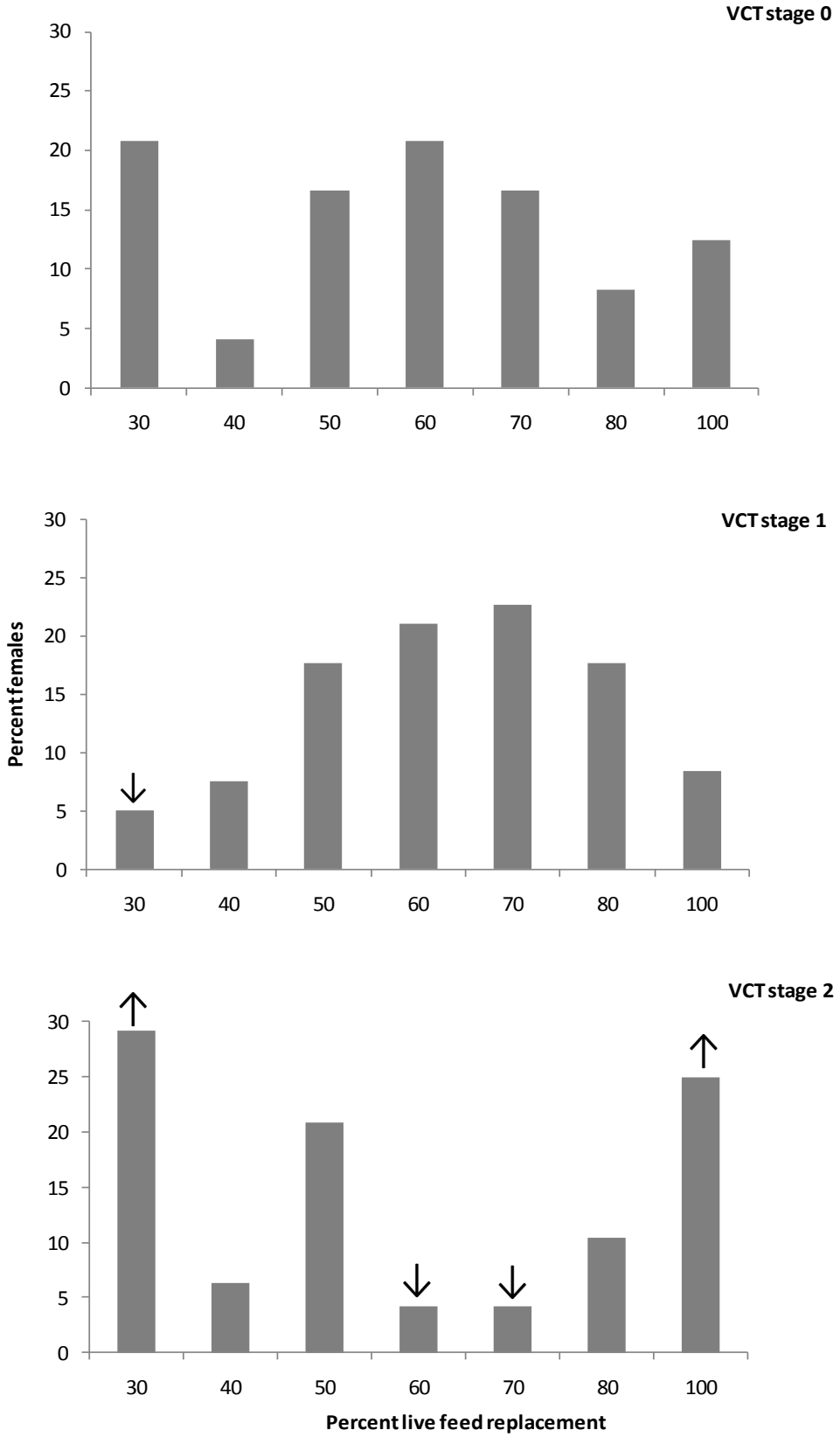

Figure 7.2.12: Percent of female broodstock in VCT developmental stages 0, 1 and 2, when fed one of seven different percent replacement diets of live feed with artificial feed. The arrows indicate those cells where the observed frequencies were less than (↓) or more than (↑) expected under the hypothesis that extent of glycogen storage was independent of diet.

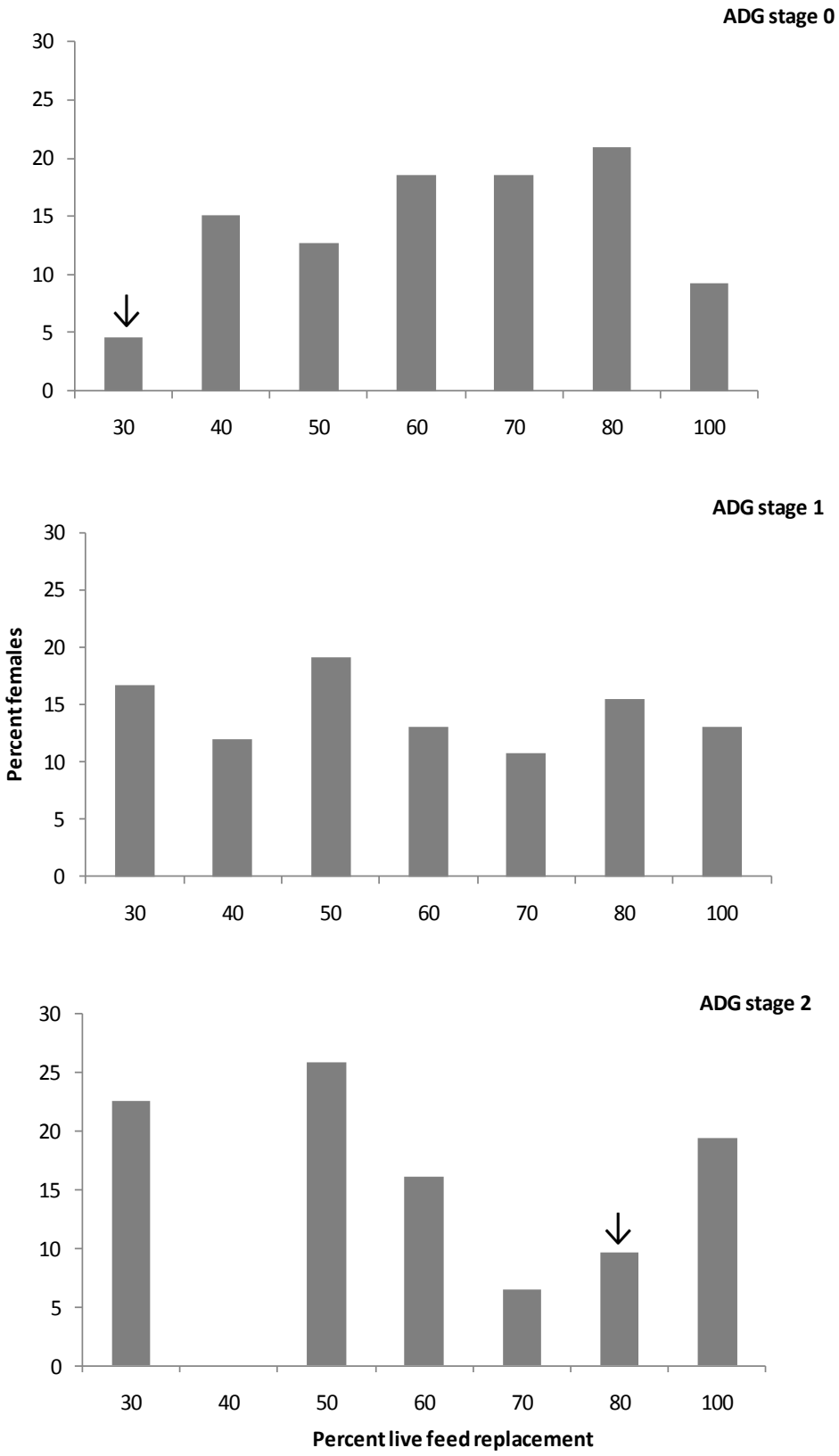

Figure 7.2.13: Percent of female broodstock in ADG developmental stages 0, 1 and 2, when fed one of seven different percent replacement diets of live feed with artificial feed. The arrows indicate those cells where the observed frequencies were less than (↓) or more than (↑) expected under the hypothesis that extent of lipid and storage was independent of the percentage replacement.

The only week that spawning occurred was five weeks into the experiment; when a total of 19 females spawned out of a potential 217 females in the experiment across the 15 diets including the control with 100% algae. This represents just over 8% of the females, which is what was predicted to have spawned based on the histological data. This suggests that the only females that spawned during the experiment were those that were exposed to thermal cycling to trigger spawning. Of those females that did spawn 79% were being fed a diet with SD as a partial replacement, while 21% were being fed M1 as a partial replacement. None of the females being fed 100% algae spawned during the experiment.

Fecundity of females that spawned ranged from 348,000-8,433,600 eggs in the first 90 minutes of spawning. The highly variable fecundity among females did not appear to be a function of the type of non-living diet fed to the females ($t=1.79$, df 4, $P=0.132$). Only for the SD non-living diet was there sufficient females across the range of replacement to explore the correlation between the percent of supplementation and fecundity; and there was no evidence of a correlation ($r=-0.45$, $n=5$, $P=0.447$). Therefore, supplementation or replacement with non-living algal diet was not a useful way to increase fecundity of female blue mussels in the conditions that the females were held. However, there is a possibility that the water temperatures used in this experiment were not the best to facilitate egg production; which may occur at cooler water temperatures.

Average egg size ranged from 75-83 µm, with a strong positive correlation between the percent replacement using SD and the size of the eggs produced by the female ($r=0.93$, $n=5$, $P=0.021$), although there was no correlation between fecundity and egg size ($r=-0.26$, $n=5$, $P=0.679$).

7.3 Diversity & pathogenicity of *Vibrio* spp associated with mussel hatchery culture

Total Vibrio counts from hatchery seawater and surfaces

Total counts from hatchery water samples ranged over 1.5 orders of magnitude from (6.4 ± 0.3 x 10^2) to (1.0 ± 0.2 x 10^4) CFU mL^{-1}. The mean presumptive *Vibrio* concentration was significantly different among all water system samples (F= 332.274, df 3, 8, *P*<0.001). Header tank water contained a lower concentration than raw seawater, and larval or broodstock culture water. The *Vibrio* population density was significantly greater in larval culture water and broodstock culture water (Fig. 7.3.1). The *Vibrio* concentration of water leaving the UV treatment system routinely below detection limits of the sampling (<0.1 CFU mL^{-1}).

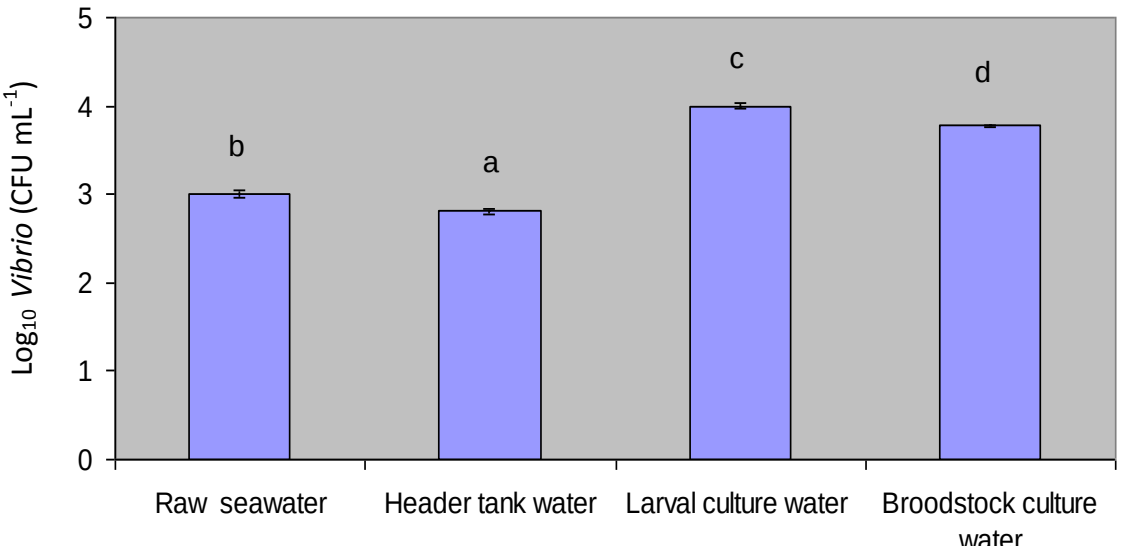

Figure 7.3.1: The average (± SE) total counts of presumptive *Vibrio* (CFU mL^{-1}) collected from water samples at SBS hatchery. Means with different letters are significantly different from one another.

At the SBS hatchery the raw seawater drawn from Spring Bay passes through course water filtration system (5 µm pore size) prior to entering the header tanks (Fig. 6.3.1). *Vibrio* are able to attach to various natural and artificial particle substrates (Hood and Winter, 1997), therefore filtration may be partially responsible for the observed reduction in header tank water compared to the raw seawater.

Substantial variation in total presumptive *Vibrio* was observed among samples taken from hatchery surfaces and equipment (Table 7.3.1). Floor and drainage samples exhibited counts of 10^2 - 10^3 CFU cm^{-1}. This indicates that surfaces and drains are a sizeable *Vibrio* reservoir (circa 10^{10} CFU across entire tank floor at SBS). Contamination of tanks by as much as 1% of the floor community (likely much lower) would contribute very little to initial tank inoculum (<10^2 CFU per tank). However, depending on the level of filtration/treatment of wash water, the hatchery floor could act as significant sources of pathogenic *Vibrio* strains entering hatchery water systems via splashing and seawater aerosols generated during hosing/cleaning activities.

Table 7.3.1: Mean (± SE) presumptive *Vibrio* associated with hatchery surfaces, equipment and biological samples

Source	Presumptive *Vibrio* (mean ± SE)
Hatchery surfaces and equipment	
Floor	$1.3 \pm 0.3 \times 10^3$ CFU cm^{-2}
General drainage	$1.2 \pm 0.1 \times 10^2$ CFU mL^{-1}
Spat tank drainage	$2.2 \pm 0.4 \times 10^3$ CFU mL^{-1}
Dry settlement rope	$3.0 \pm 0.8 \times 10^6$ CFU cm^{-1}
Biological Samples	
Larvae tank (larvae + water)	$1.2 \pm 0.4 \times 10^5$ CFU mL^{-1}
Egg Suspension	$3.4 \pm 0.7 \times 10^4$ CFU mL^{-1}
Sperm Suspension	$4.9 \pm 0.6 \times 10^4$ CFU mL^{-1}
Spat tank (water + larvae)	$1.1 \pm 0.8 \times 10^4$ CFU mL^{-1}

Anecdotal information from SBS hatchery suggested that a number of previous larval mortalities were associated with UV treatment of header tank water either failing or being inefficient. This is certainly possible because UV sterilization may not eliminate all bacteria in seawater (Tubiash, 1975) including pathogenic *Vibrio* (Brown, 1981). Cultivable *Vibrio* associated with raw and hatchery header tank seawater was in the order of 10^3 CFU mL^{-1}, however, none (<0.1 CFU mL^{-1}) were detected in UV treated seawater exiting the SBS treatment system, and *Vibrio* have never been detected exiting the UV system during routine hatchery testing with TCBS medium (I. Duthie, SBS Hatchery Manager, pers. comm.). An operational and well maintained UV unit should produce treated seawater that is not a significant source of *Vibrio* in the SBS hatchery. Another potential source for *Vibrio* are biofilm communities established on PVC seawater reticulation and/or tank surfaces (Karunasagar et al. 1996, Otta et al. 2001), however, regular maintenance and decontamination of seawater reticulation systems should limit this source under most circumstances.

Many *Vibrio* are capable of entering a so-called viable but non-cultivable (VBNC) state if presented with an unfavourable environment that causes physical and chemical stress (Oliver and Bockian, 1995). In this state they retain pathogenic potential (Colwell et al. 1985; Oliver and Bockian, 1995; Baffone et al. 2003). Bacteria in this state can potentially recover and are fully cultivable under favourable conditions (Roth et al. 1988; Nilsson et al. 1991; Whitesides and Oliver, 1997; Mizunoe et al. 2000). It is possible that UV treatment might cause VBNC state of some *Vibrio* that subsequently, recover under favourable conditions offered during larval and spat culture.

Total cultivable *Vibrio* associated with larvae, sperm and eggs was generally an order of magnitude greater (approx. 10^4 CFU mL^{-1}) than either raw or header tank seawater, indicating that the food sources (cultured algae) or the stock themselves carry a substantial *Vibrio* community under typical

commercial culture conditions. Axenic algal cultures are used to feed the larvae/spat and any algal food where *Vibrio* is detected by TCBS plating (>0.1 CFU mL^{-1}) is not used for larval culture.

Internal colonization of male and female gonads by *Vibrio* prior to artificial spawning can lead to pathogenic *Vibrio* invading the gonad and contaminating the larval culture (Song et al. 1997, Sugumar et al. 1998). Such vertical transmission of *Vibrio* has been suggested previously in studies of the European oyster *Ostrea edulis* (Lodeiros et al. 1987). The significant concentrations of *Vibrio* detected in samples of broodstock water, sperm, eggs, larvae and spat, indicates the dominant source of *Vibrio* is the larvae themselves, perhaps established from communities associated with eggs and sperm at fertilization and concentrated to higher concentrations in gut through feeding (Lane and Birkbeck, 2000).

The samples examined in this study were not associated with outbreaks of Vibriosis or significant larval mortality and thus represent a baseline estimate of *Vibrio* flora under typical commercial operating conditions in the absence of disease. There is limited published comparative data from mussel hatcheries, however, the values observed here are very similar to those from the same types of source material (e.g. rearing seawater, larvae, broodstock) reported from marine hatcheries of other molluscs such as oysters, scallops (*Argopecten* spp.; Riquelme et al. 1995, Sainz-Hernandez and Maeda-Hernandez 2005), the clam *Ruditapes* spp. Mechri et al. 2012), tropical (*Panulirus ornatus*) and European (*Homarus gammarus*) lobsters (Bourne et al. 2004, Scolding et al. 2012), and even freshwater prawns *Macrobrachium rosenbergii* (Kennedy et al. 2006). The consistency in the level of *Vibrio* across a range of different species and hatcheries indicates a common underlying factor that controls *Vibrio* abundance in hatchery systems. The design, water treatment, flow management, stocking densities and husbandry practices of most marine hatcheries are generally very similar and operated with parameters and flow rates adopted or slightly modified from those developed for oyster hatchery culture. Concentration and distribution of organic carbon (used for growth by marine heterotrophic bacteria) in the systems would also be expected to be very similar, and the likely to be the major factor controlling *Vibrio* abundance.

Unexplained mortality observed during the settlement process has typically occurred after transfer to flow-through settlement tanks containing aged and conditioned settlement ropes. At SBS, settlement tanks are surface sterilized and filled with UV-sterilized seawater (approx 1000 L) prior to the addition of spools of settlement rope (up to 500 m). Tank conditioning of the settlement rope occurs for several days prior to the addition of mussel pediveligers, during which time a microbial community develops which is thought to promote and enhance pediveliger settlement onto the rope substrate. Our investigations showed that used and dried settlement rope contain a high number of presumptive *Vibrio* ($3.0 \pm 0.8 \times 10^6$ CFU cm^{-1}, see Table 7.3.1). Therefore, a 500 m length of settlement rope in a tank represents a total inoculum *Vibrio* community of approximately 10^{11} CFU, equivalent to an initial population of 10^5 CFU mL^{-1} in a 1,000L tank. Dried ropes clearly retain a substantial viable bacterial community including *Vibrio* and represent the major inoculum source from which conditioned settlement tank/spool communities establish.

The development of total cultivable *Vibrio* populations in settlement tank water during conditioning and settlement is shown in Figure 7.3.2. Total cultivable *Vibrio* increased from < 10^2 CFU mL^{-1} at the start of tank conditioning and reached approximately 10^5 CFU mL^{-1} by day 6 (Fig. 7.3.2). A similar pattern was observed after the tank was drained, refilled and pediveligers added, beginning at 10^2 CFU mL^{-1} but increasing rapidly over the first three days to 10^4 CFU mL^{-1} by day 3, and approaching 10^5 CFU mL^{-1} by day 6.

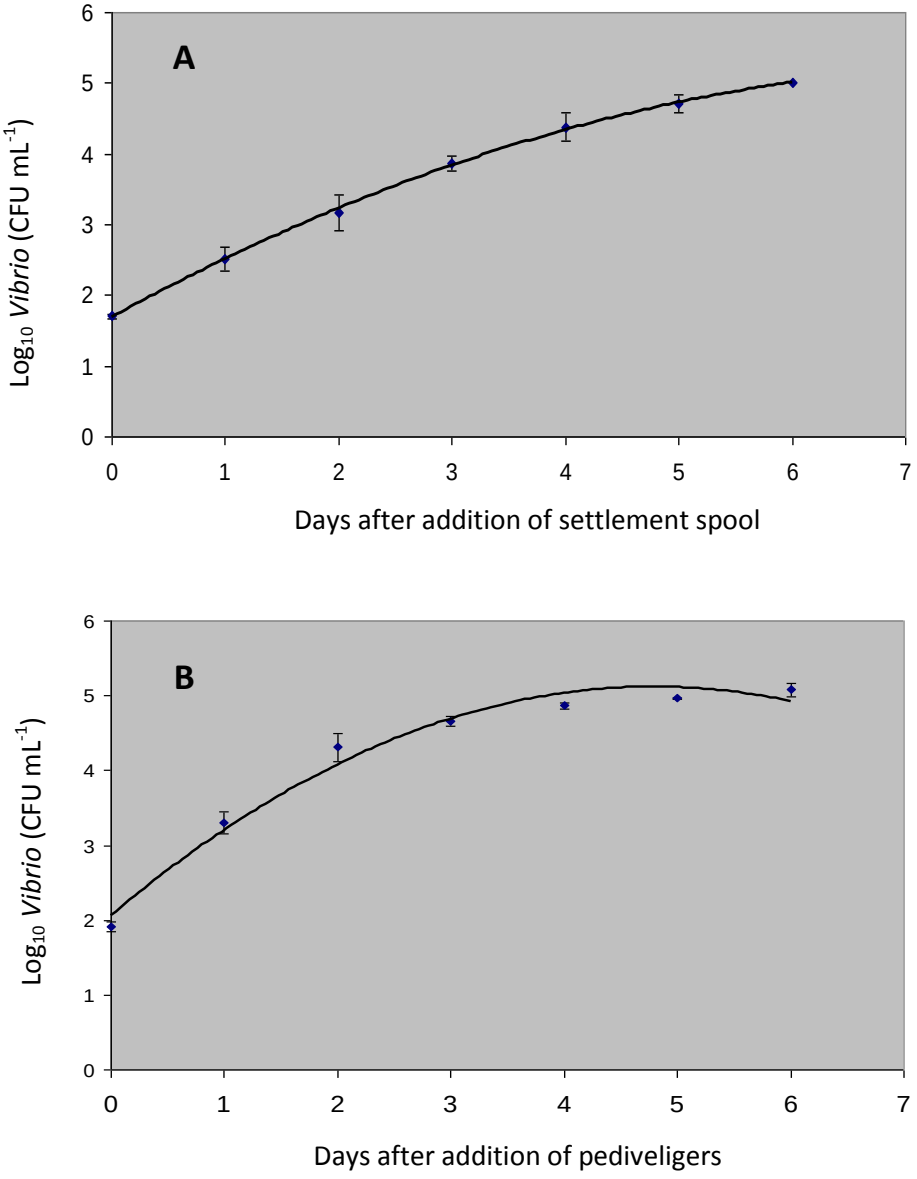

Figure 7.3.2: Concentration of presumptive *Vibrio* in mussel settlement tank water. A) During tank conditioning, prior to addition of larvae; B) After addition of pediveligers. Note that the settlement tank is drained and refilled with UV-treated FSW prior to addition pediveligers to the conditioned tank.

Despite the relatively high *Vibrio* counts encountered during settlement, no significant settlement or post-settlement mortality was encountered during the sampling period. Despite the substantial *Vibrio* community associated with production, mortalities at this point of production have generally

been minimal since the introduction of UV-sterilization to the hatchery water system (Ian Duthie, Hatchery manager; pers. comm.). Our data suggest that the *Vibrio* associated with mussel larvae, tanks and rope are either non-pathogenic or considerably less virulent than those associated with raw seawater contaminated sources such as untreated seawater, or broodstock culture water, and sperm/egg suspensions. Free living and particle-associated *Vibrio* are ubiquitous in marine environment (Bidle and Fletcher 1995) and coastal seawater may harbour more than 1000 *V. splendidus* genotypes (Thompson et al. 2005), each varying considerably in their pathogenicity to larval molluscs and potentially interacting synergistically in causing shellfish mortality (e.g. Gay et al. 2004). Establishment of an inhibitory or of highly virulence community during spat tank conditioning, perhaps in combination with increased susceptibility during metamorphosis, may explain the highly variable mortality at this phase of juvenile production.

Hatchery Vibrio 16S rDNA gene sequencing

Sequencing of 16S rRNA was attempted for 23 isolates from a range of hatchery sources. A total of 19 isolates returned interpretable sequence data of which 5 resulted in short partial forward sequences only (Table 7.3.2). The remaining four isolates returned mixed or poorly resolved sequence that could not be reliably corrected and interpreted.

The relatively low success rate of PCR product consensus sequencing of this gene has been previously noted for *Vibrio* species, and is thought to result from extensive heterogeneity (up to 2% divergence) among multiple rDNA loci on *Vibrio* genomes (Moreno et al. 2002). For this reason, subsequent molecular characterisation (May 2009) focussed on an alternative single-copy gene in *Vibrio* species, the *atpA* gene.

Phylogenetic analysis of the 16S rDNA gene sequences is shown in Figure 7.3.3. Thirteen of the 19 isolates sequenced were allied with the genus *Vibrio*, predominantly with the *V. splendidus* group of species (10 isolates) - *V. splendidus* (7), *V. lentus* (1) and *V. cyclotrophicus* (2). Phylogenetic analysis of the nine longest higher quality *Vibrio* sequences showed that hatchery isolates clustered primarily within the large, poorly resolved "*V. splendidus*-group". In our analysis the "splendidus-group" formed a well-supported cluster occupying the crown of the *Vibrio* tree. The group contained five distinct clusters with weak to moderate support (50-80%), interspersed with a range of more divergent 16S rDNA genotypes. Mussel hatchery isolates clustered among three *V. splendidus*/*V. cyclotrophicus* clusters with the exception of isolates 162 and 169 which were more distantly allied to a large cluster of marine *Vibrio* species, including *V. mediterranei, V. shiloni, V. tubiashi, V. pectenicida, V. tapetis, V. nereis,* and *V. corallilyticus* and reflects the relatively few reference taxa included in the 16S rDNA analysis.

Surprisingly, five isolates were non-*Vibrio* clustering outside the Vibrionaceae. One isolate was loosely allied to the Gamma-proteobacterial genus *Shewanella* (Alteromonadales). The other four were related to *Balneola alkaliphila* (Phylum Bacteroidetes) a genus that appears to be able to grow on highly alkaline media including TCBS which is considered highly selective for *Vibrio* and close relatives (Urios et al. 2006). There is no published evidence to indicate that these organisms are

pathogenic to molluscs. They are likely to be relatively uncommon members of the total community detected due to their ability to grow on highly selective TCBS medium. Further study would be required to determine whether these bacteria can be associated with larval and or spat mortality.

Table 7.3.2. The relationship of 16S rRNA sequences from *Vibrio* strains isolated from SBS hatchery to published *Vibrio* sequences (Genbank). Shorter forward-only sequences are indicated by grey shading.

Strain	Source	length (bp)	Nearest neighbour Species	Genbank	Identity (%)[a]
12	intake seawater	1421	*V. splendidus*	AJ874367	99
13	intake seawater	1420	*V. splendidus*	EU091334	99
29	intake seawater	353	*V. splendidus*	DQ874365	86
31	header tank water	1412	*V. cyclitrophicus*	AM422804	99
37	header tank water	1347	*V. lentus*	AM162659	99
47	header tank water	1359	*Balneola alkaliphila*	EU008564	96
50	header tank water	731			98
64	broodstock tank water	1356			94
77	mussel eggs	411	*V.splendidus*	AF025329	84
83	mussel eggs	1360	*B. alkaliphila*	AM990877	97
86	mussel sperm	1190	*V. splendidus*	EU091329	99
89	sperm	944	*Shewanella sairae*	AB081762	99
100	spool tank water	1448	*B. alkaliphila*	EU008564	93
108	mussel larvae	653	*V. splendidus*	EU091332	100
125	mussel spat	1412	*V. cyclitrophicus*	DQ481608	99
133	mussel spat	933	*V. splendidus*	EU091328	99
150	mussel spat	559	*V. tubiashii*	GQ454987	92
162	mussel spat	1398	*V. orientalis*	X74719	98
169	mussel spat	1412	*V. orientalis*	X74719	98

a=16S rRNA sequences were aligned to the nearest neighbour based upon BLAST searches in the GenBank database.

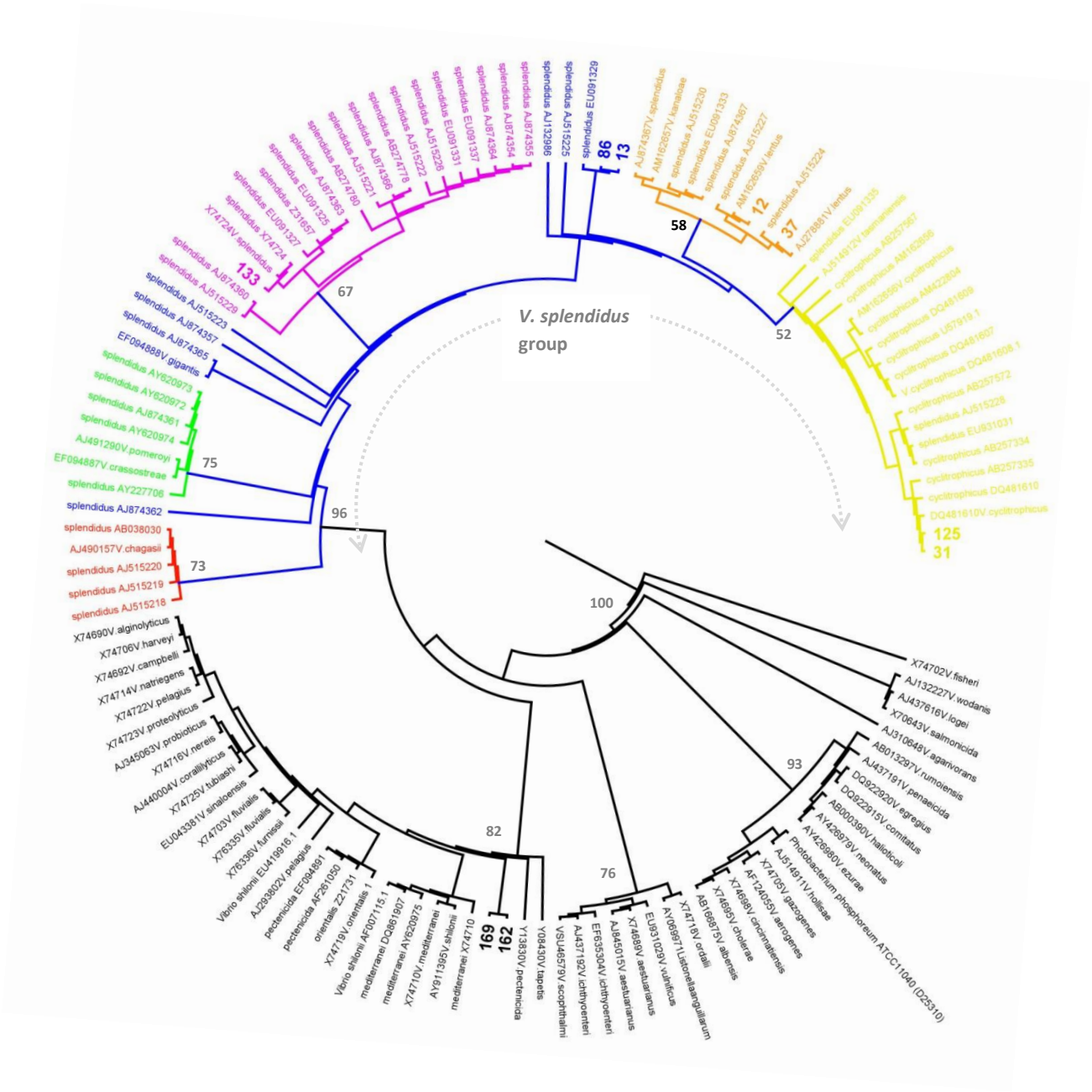

Figure 7.3.3: Genetic affinities of *Vibrio* strains isolated from SBS mussel hatchery based on 16S rRNA gene sequences. Blue branches indicate the *V. splendidus* group (sensu Thompson et al. 2007) with distinct clusters of *V. splendidus* genotypes outlined by coloured branches. Mussel hatchery isolates indicated by isolate number in bold text. Figures at nodes of main clusters indicate bootstrap support (200 replicates). Neighbour joining analysis of 129 *Vibrio* partial 16S rDNA sequences constructed from Tamura-Nei genetic distance and rooted using *Vibrio fischeri* (accession X74702) as the out-group taxon. Branches are proportionally transformed for clarity.

Hatchery Vibrio atpA *genotype diversity*

Of the 112 *Vibrio* isolates collected, the targeted 1500 bp *atpA* amplicon was successfully amplified from 92 isolates (81%, Fig. 7.3.4). Co-amplified artefactual products (size 800 bp and >3000 bp) were observed for some isolates but did not interfere excessively with direct consensus DNA sequencing of the product. A total of 91 isolates produced high quality sequence with low noise resulting in reverse sequence reads of approximately 800-900 bp for most isolates; three products failed to produce readable sequence.

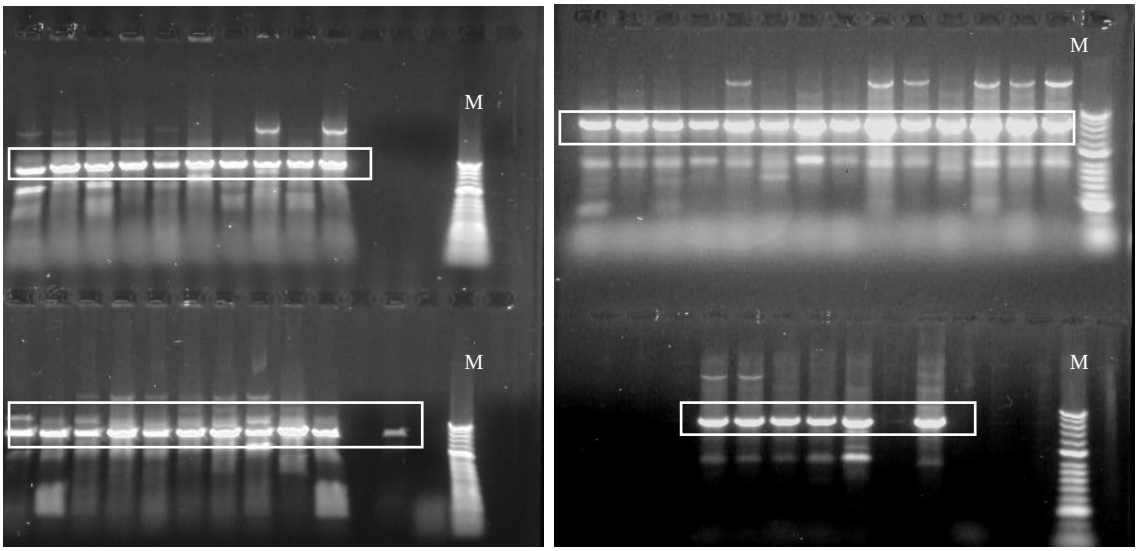

Figure 7.3.4: Amplified *atpA* gene fragments from 40 *Vibrio* isolates from (a) bay seawater (BSW) and (b) spat settlement tank seawater (STSW). The target product of 1500 bp is shown in the white-line boxes. M: DNA size marker.

The comparison and analyses of *atpA* sequences largely confirmed the assessment from preliminary work using the 16S rDNA sequences. After alignment and comparison with reference *Vibrio atpA* sequences, 85 strains (97%) were found to be allied with the *V. splendidus* group, and two isolates were allied the *Vibrio harveyi* group (Fig. 7.3.5). One sequence recovered was closely related to *Shewanella livingstonensis* and was not included in further analyses. The 87 *Vibrio* isolates, yielded 40 unique *Vibrio atpA* sequences, comprised of two identical *V. harveyi* group genotypes, and 88 *V. splendidus* group genotypes with extensive sequence variation ranging from 1 to 39 bp (up to 4.8% sequence divergence).

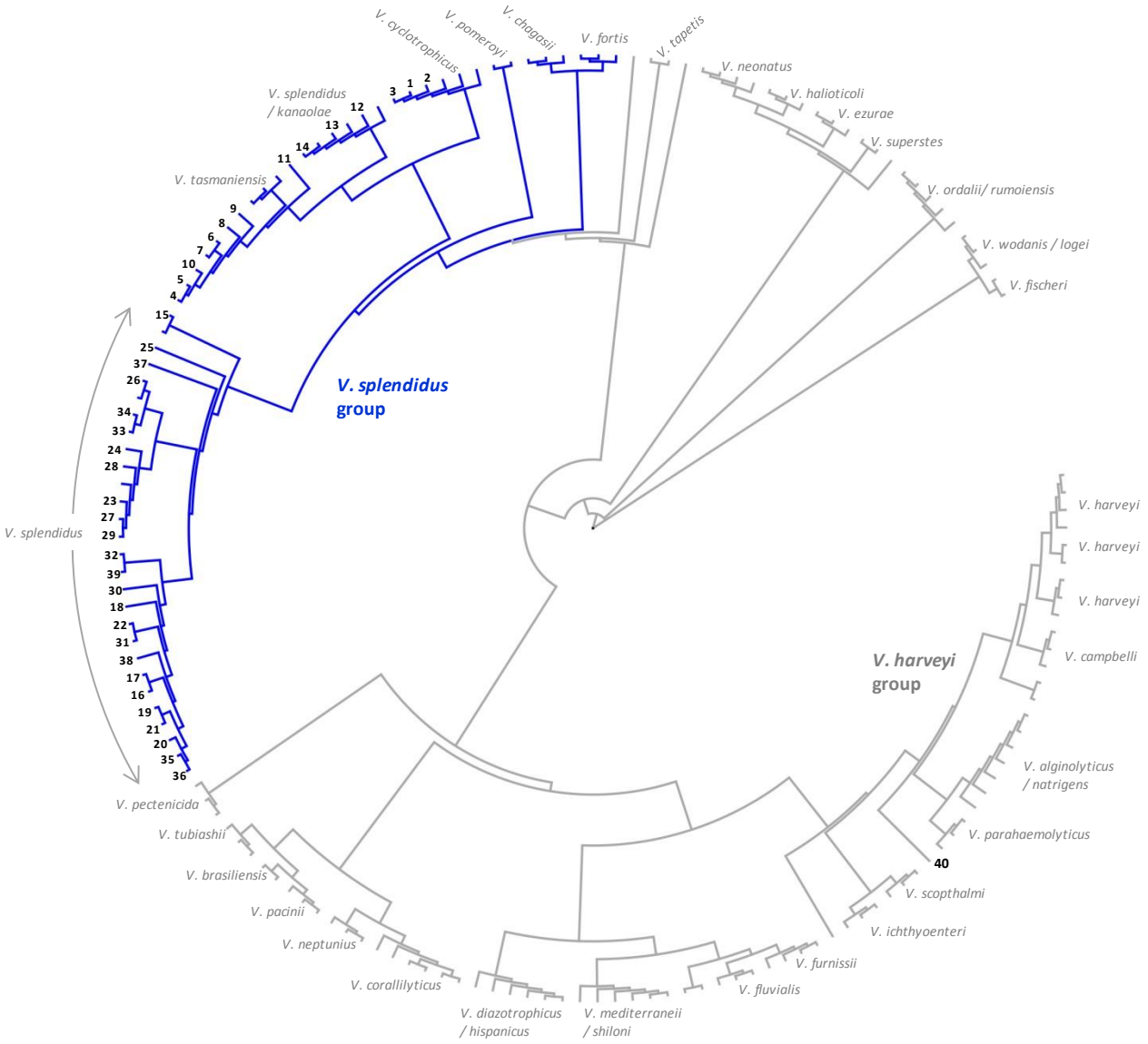

Figure 7.3.5: Overview of phylogenetic affinities of 40 unique *atpA* gene partial sequences recovered from 98 mussel hatchery isolates (sequence numbers shown in black text) relative to 119 *atpA* sequences from reference *Vibrio* species (Thompson et al. 2007). Major species clusters of Thompson et al. (2007) are indicated in grey text. Neighbour joining tree constructed from Tamura-Nei genetic distances. Branches transformed proportionally for clarity.

The *V. splendidus* group sequences clustered five moderate to weakly supported sub-groups (Fig. 7.3.6) allied with reference sequences of *V. splendidus*, *V. tasmaniensis* or *V. cyclotrophicus* (Table 7.3.3). The majority of isolates (>60%) fell within one sub-group, *V. splendidus* subgroup D, which could be divided into two weakly supported clusters (cluster D1 and D2). Subgroups B C and E (9-12.6%) were related to *V. splendidus* and *V. tasmaniensis* and subgroup A (5.7%) related to *V. cyclotrophicus* (Table 7.3.3). Two *V. harveyi* related isolates (subgroup F), were found only in the spat tank sample.

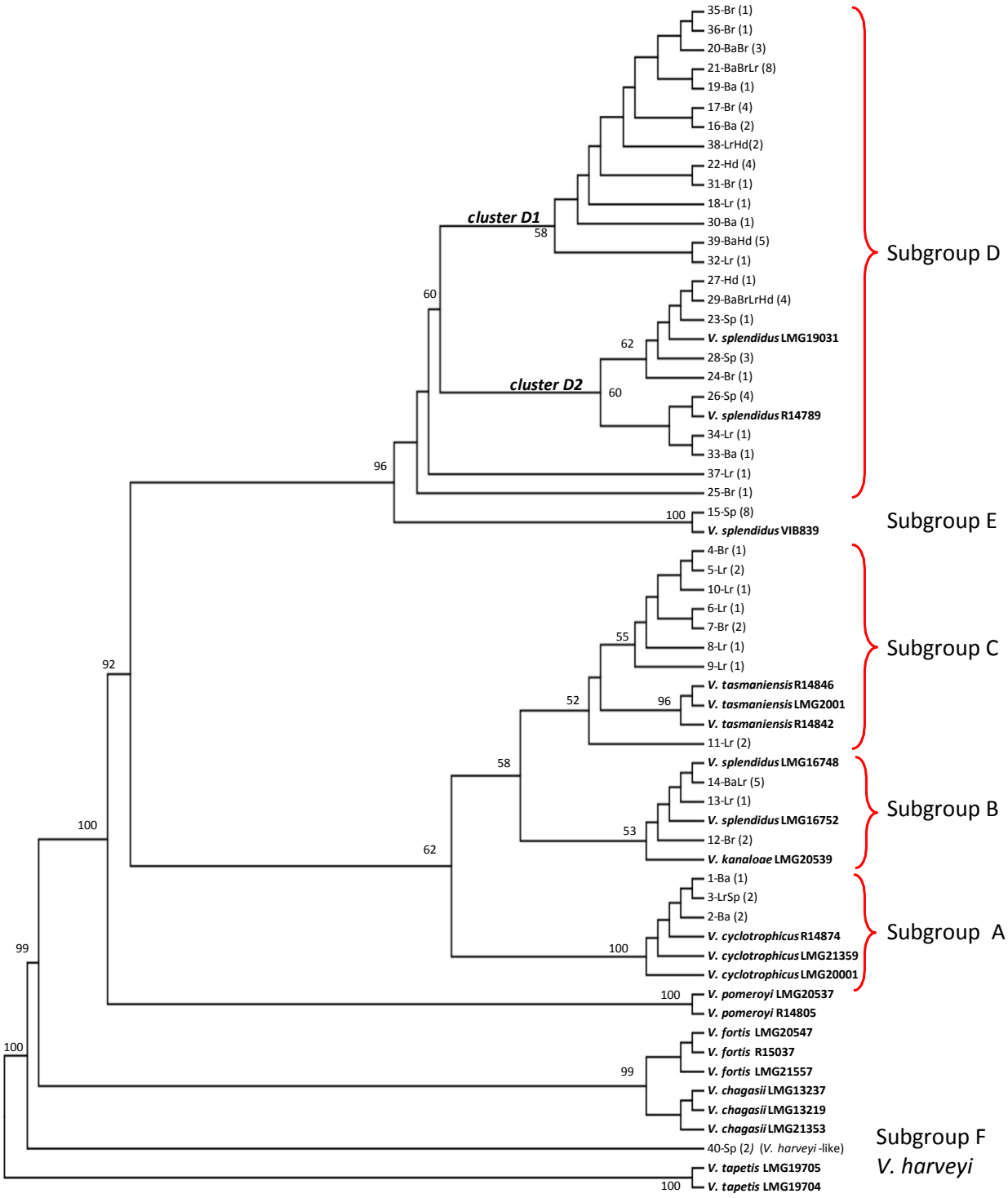

Figure 7.3.6: Phylogenetic relationships among the 40 distinct partial *atpA* genotypes of cultivable *Vibrio* from Spring Bay mussel hatchery. Isolates indicated by isolate number, source/s (Ba=bay seawater, Br= Broodstock culture seawater= Br, Lr= Larval culture seawater, Sp= Spat culture seawater= Sp, Hd=Header tank seawater), and frequency encountered (brackets) among the 98 isolates sequenced during the study. Reference *V. splendidus* group strains from Thompson et al. (2007) indicated by bold text. Genotype subgroups (A-F) among hatchery isolates are indicated. Neighbour-joining tree constructed from Tamura-Nei distances. Figures at branch points indicate bootstrap support (200 replicates) for clusters of isolates.

Table 7.3.3: Frequency of *Vibrio atpA* genotype sub-groups associated with mussel hatchery culture. Sub-clusters of group D are shown in grey text; Note that not all Group D genotypes were allied with cluster D1 or D2.

Subgroup	Species alliance	Distinct *atpA* genotypes	Count (n)	Frequency (%)
A	*V. cyclotrophicus*	3	5	5.7
B	*V. splendidus/kanaloae*	3	8	9.2
C	*V. tasmaniensis*	8	11	12.6
D	*V. splendidus*	24	53	60.9
cluster D1		*14*	*33*	*37.9*
cluster D2		*8*	*16*	*18.4*
E	*V. splendidus* VIB839	1	8	9.2
F	*V. harveyi* group	1	2	2.3

Group D genotypes dominated the cultured *Vibrio* community of Bay seawater, the hatchery header tank, and the broodstock tank (Fig. 7.3.7), although the dominant genotype differed in all three samples, indicating that the *Vibrio* community composition differs among water sources despite being derived from stored bay seawater. When pooled by phylogenetic subgroup frequencies, differences were not significant due to high *Vibrio* genotype diversity and comparatively low sampling frequency. However, after pooling *V. splendidus* groups by higher order phylogenetic clusters (Groups D/E versus Groups A/B/C), the proportion of Group D/E genotypes was significantly less (and Group A/B/C significantly more) in the larval water sample compared to other water samples (χ^2=17.78, df 4, *P*=0.0014).

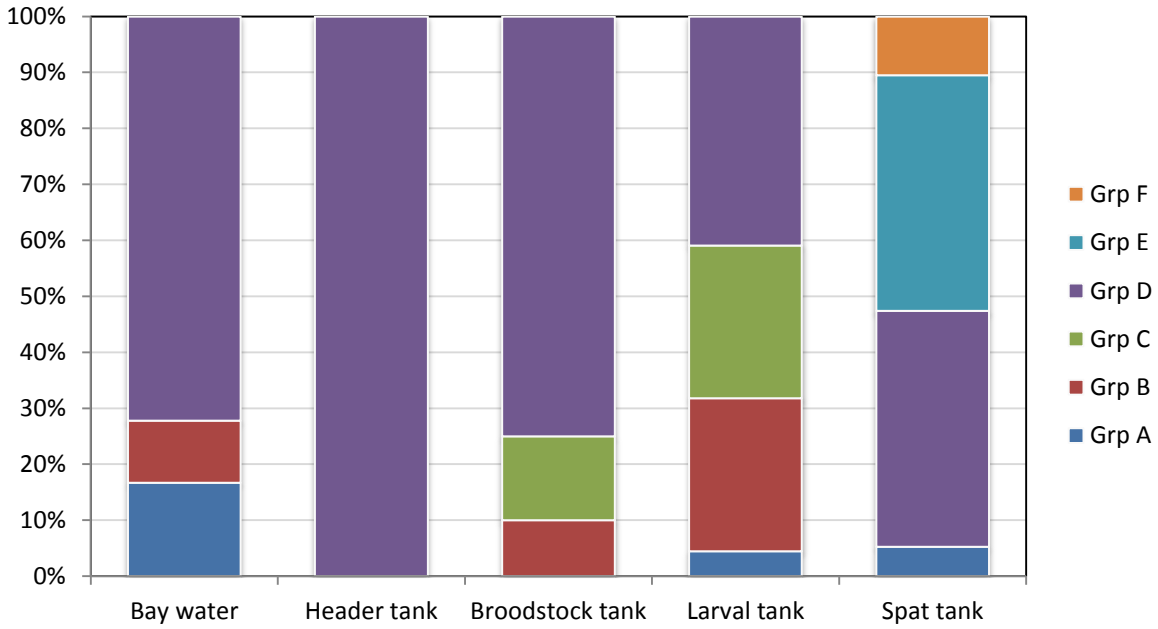

Figure 7.3.7: Proportion of six *Vibrio atpA* genotype subgroups associated with each hatchery water source sample.

The diversity *Vibrio atpA* genotypes associated with each source was similar the exception the header tank and spat tank samples (Table 7.3.4). These samples exhibited relatively fewer *atpA* genotypes and lower Shannon's diversity (*H'*), due to the samples being dominated (40-50%) by single but different *V. splendidus atpA* genotypes (Group D= type 22Hd, Group E= type 15sp) neither of which were detected in other water samples (see Fig. 7.3.7).

Table 7.3.4: Abundance and diversity of cultivable *Vibrio atpA* genotypes associated with each seawater sample.

Sample/Source	Cultivable *Vibrio* (CFU mL^{-1})		Isolates (n)	*atpA* types (n)	Proportion unique genotypes (%)	Diversity (*H'*)
	Mean	±SE				
Bay seawater	2.04×10^5	4.6×10^4	18	11	61.1	2.274
Broodstock tank	1.58×10^7	1.0×10^6	20	11	55.0	2.346
Larval tank	2.80×10^5	1.0×10^4	22	16	72.7	2.665
Spat tank	2.14×10^4	1.5×10^4	19	6	31.6	1.531
Header tank	8.10×10^5	3.8×10^5	8	5	62.5	1.386

Comparisons of the phylogenetic relatedness of the cultivable Vibrio communities (UniFrac PCO analysis) indicated that bay, broodstock and larval tank water communities were relatively similar but that spat and header tank water samples were relatively distinct from the other *Vibrio* communities examined (Fig. 7.3.8).

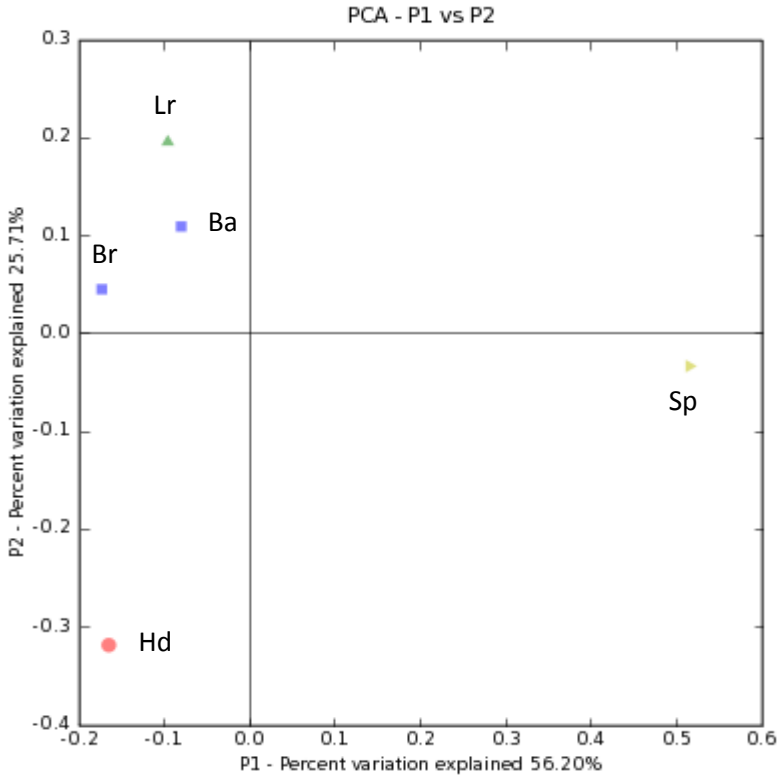

Figure 7.3.8. Phylogenetic similarity of cultivable *Vibrio* communities associated with different mussel hatchery water sources based on partial *atpA* gene sequences. The first two principal coordinates of a UniFrac PCO are shown. (Ba=bay seawater, Br= Broodstock culture seawater= Br, Lr= Larval culture seawater, Sp= Spat culture seawater= Sp, Hd=Header tank seawater).

The data collected and presented here represent a snapshot of cultivable *Vibrio* during normal operations in the hatchery. Our data mirror findings from other molluscan and crustacean hatchery studies of *Vibrio*. For example at least 10 different *Vibrio* genotypes were detected among 39 isolates associated with lobster hatchery culture environments (Bourne et al. 2004). When combined with other published studies of shellfish hatchery *Vibrio* populations, it is clear that all stages of juvenile shellfish production are associated with a substantial community of cultivable *Vibrio*, even when stock mortalities are low, or within normal commercial production limits. The development of pathogenicity and disease thus involves not only changes in the total community abundance and also the dominance and diversity of the *Vibrio* community.

Overall, the diversity analyses indicate that mussel hatchery cultivable *Vibrio* communities are predominantly composed of *V. splendidus* group bacteria that are also highly diverse and highly dynamic across the various culture environments. The communities appear to undergo substantial shifts in abundance and phylogenetic characteristics that are presumably dependent on the level and quality of organic enrichment of the culture systems, and also their interaction with the highly diverse and complex non-*Vibrio* community in the culture systems. The high levels of genetic diversity indicate that there may be similar levels of diversity and variation in larval virulence as well as interactive and synergistic effects between genotypes (Gay et al. 2004; see next section).

It is also important to note the disparity observed between culture-based and culture independent studies of microbial communities, including hatchery environments. While *Vibrio* spp. are frequently recovered from culture-based studies, they are rarely dominant members of the total or even cultivable marine heterotrophic bacterial community recovered using culture-independent approaches (e.g. PCR-cloning and DGGE studies). As a result it has been suggested that their importance in the bacterial community may be over-estimated (Giovannoni and Rappe 2000) and it is possible that their role in hatchery disease processes is also over-estimated. Recent culture-independent studies of the microbial communities associated with oyster larval rearing indicated that *Vibrio* were rarely dominant taxa in hatchery culture environments even during mortality events (e.g. Bourne et al. 2004, Chapman et al. 2012). However, a distinct “spike” in relative abundance of larvae-associated cultivable *Vibrio* (25-100% of total viable count), and culture water to a lesser extent, always coincided with onset of disease and/or mortality of commercial hatchery-reared larvae (Chapman et al. 2012). This indicates that the cultivable *Vibrio* community plays at least an indirect role in disease and mortality, perhaps in combination with other members of the bacterial community and additional behavioural factors such as increased larval aggregation (Chapman et al. 2012).

Pathogenicity of mussel hatchery Vibrio *isolates to mussel pediveligers*

Mussel pediveliger pathogenicity assays using *Vibrio* isolates 86, 125 and 169 indicated that all three isolates induced pediveliger mortality compared to negative (no bacteria) controls (Fig. 7.3.9). All controls exhibited less than 5% mortality in both 24 and 48 hr challenge experiments. Relative

to controls, mean pediveliger survival decreased with increasing *Vibrio* concentration in all assays (Fig. 7.3.10) with survival in 48h assays universally lower than observed in 24h assays.

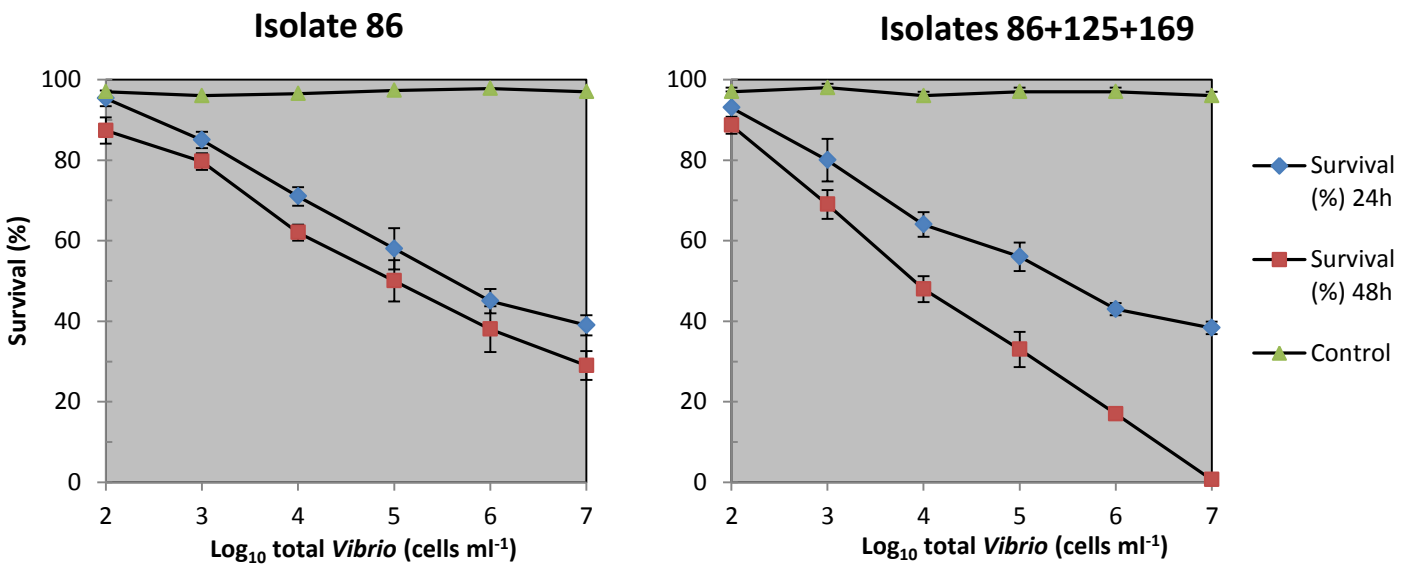

Figure 7.3.9: Survival of mussel pediveligers (± SE) in 24h and 48h exposure to varying total concentration of purified *Vibrio* isolates from SBS Hatchery. Data from assays with other strains and combinations are contained in Appendices.

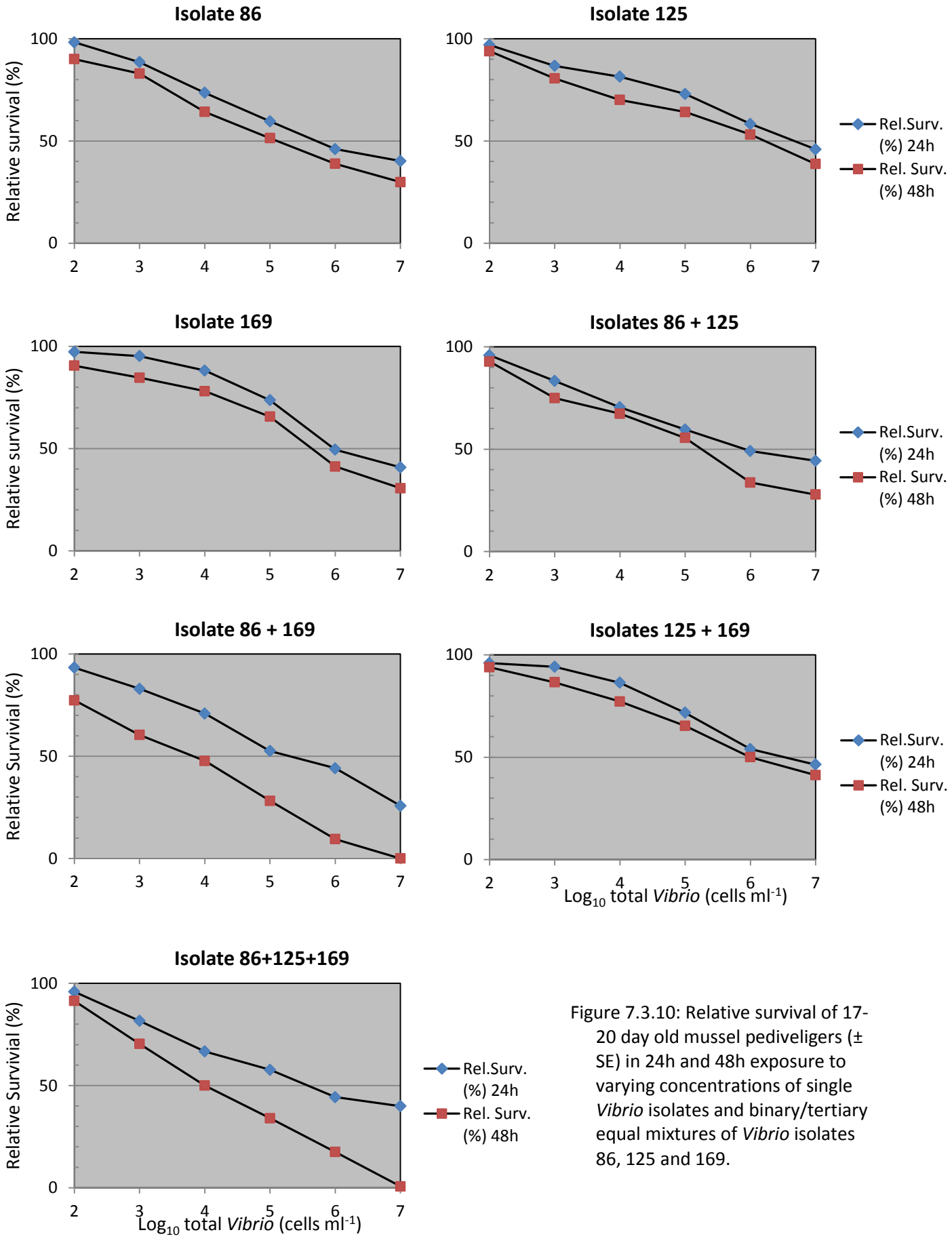

Figure 7.3.10: Relative survival of 17-20 day old mussel pediveligers (± SE) in 24h and 48h exposure to varying concentrations of single *Vibrio* isolates and binary/tertiary equal mixtures of *Vibrio* isolates 86, 125 and 169.

Virulence (as mean LC50) of isolates to pediveligers varied between isolates and mixtures of isolates in both 24h (F=40.721, df 6,14, *P*<0.001), and 48 h (F=126.228, df 6,16, *P*<0.001) pediveliger assays, ranging from approximately log 5.1 to 6.6 cells mL-1, and 4.0 to 6.2 respectively (Fig. 7.3.11).

Isolate 86 was more virulent than either isolate 125 or 169 in single isolate challenges. All binary and tertiary combinations which included isolate 86 were equally or more virulent than single isolates, whereas combinations of isolate 125 and 169 were no more virulent to pediveligers than the when applied as single challenge assays.

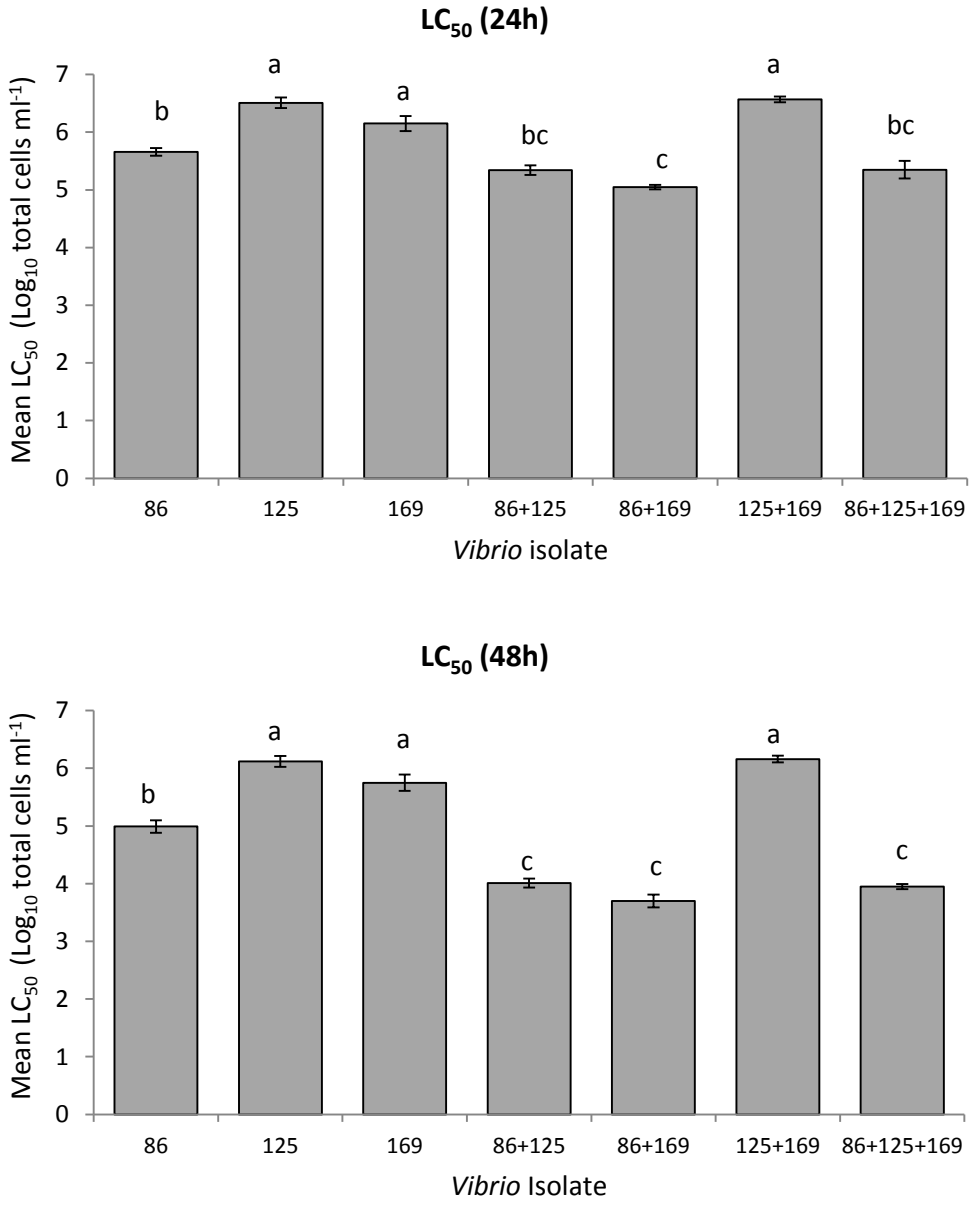

Figure 7.3.11: Virulence (LC_{50} ± SE, Log cells mL^{-1}) of three hatchery *Vibrio* isolates to 17-20 day old mussel pediveligers in 24 and 48h pediveliger challenge assays. Isolates were assayed singly and in combination at equal concentration. Letters indicate significantly different means.

Isolates 86 and 125 are both allied with the *V. splendidus*-group which has been widely reported as pathogenic to wide range of adult and juvenile shellfish including scallop larvae (*Pecten maximus*; Nicolas et al. 1996), carpet shell clams and cockles (*Ruditapes decussates*, *Fulvia mutica*; Fujiwara et al. 1993) turbot larvae (Farto et al. 1999; Gatesoupe et al. 1999). While both induced mortality, the virulence of the isolates differed by an order of magnitude. The most virulent isolate tested (86) showed similar virulence to that of *V. splendidus* (Biovar II) against pacific oyster larvae (*Crassostrea gigas*) where 10^4–10^5 cells mL^{-1} caused 100% mortality (24h assay) of 5-day old larvae (Sugumar et al. 1998). Strain 169 clustered with *V. orientalis*, *V. mediterranei* and *V. pectinicida*, with the latter species a known cause of bacillary necrosis in scallop larvae (*P. maximus*).

Studies of *Aeromonas* pathogenicity against salmonids established LC_{50} values of 10^4–10^5 cells mL^{-1} to be considered virulent and an LC_{50} >10^8 cells mL^1 to be considered avirulent (Mittal et al. 1980). Total cultivable *Vibrio* associated with "healthy" larval and spat tank waters range up to approx. 10^4 CFU mL^{-1} during normal bivalve hatchery rearing (this study, Sainz-Hernandez et al. 2005; Mechri et al. 2012), therefore from our LC_{50} estimates it would be reasonable to classify all three isolates as pathogenic to mussel pediveligers, particularly when assayed in combination with strain 86 where LC50 values of 10^4 CFU mL^{-1} or less were observed all cases. Similar interactive effects have been reported in in previous studies (Gay et al. 2004, Pasmore and Costerton 2003) and suggests that independent pathogenic mechanisms or toxic factors can act additively or synergistically to establish "collective virulence" of the *Vibrio* community.

Antagonistic effects between *Vibrio* genotypes are also known (Gay et al. 2004; Austin et al. 1995) as observed here where isolate 125 was less virulent when in combination with strain 169. For example, strains of *V. alginolyticus* can reduce the virulence of *V. anguillarum* and *V. ordalii* in fish (Austin et al. 1995) or *V. parahaemolyticus* in shrimp (Garriques and Arevalo 1995), and may act as probiotics in a similar manner to lactic acid bacteria (LAB) through production of bacteriocins (Gildberg et al. 1997, Gatesoupe 1994), competition for attachment sites, the ability to metabolize toxic factors produced by pathogenic strains (Verschuere et al. 2000). Another mechanism of interaction acts via blocking of gene regulation and cell signalling such as quorum-sensing pathways (Pasmore and Costerton 2003). A number of virulence genes regulate the expression of *Vibrio* pathogenicity, such as *toxR*, and *toxRS*, which activate transcription of virulence genes in *V. cholerae* (Pfau et al. 1998) or *tlh* (thermolabile haemolysin), *trh* (TDH-related haemolysin), and *tdh* (thermostable direct haemolysin) in *V. parahaemolyticus* (Iida et al. 1998; McCarthy et al. 1999). The transfer of virulence genes between pathogenic and non-pathogenic strains is also possible (Boyd et al. 2000; Faruque and Nair 2002).

In terms of managing hygiene and disease in the hatchery, it is clear from the studies conducted here and the published literature, that bacillary necrosis (vibriosis) is a complex disease not easily understood and managed through current approaches that concentrate on monitoring total *Vibrio* and non-*Vibrio* loadings. Due to the high *Vibrio* diversity encountered in this study, it is also neither feasible nor logical to concentrate on the detection and quantification of any single pathogenic

strain when it represents just one among hundreds of potentially pathogenic bacterial types present in the hatchery rearing systems.

Improved understanding of the disease process, including mechanisms of pathogenicity and dominant factors influencing virulence of *Vibrio*, particularly the *V. splendidus* group, may offer improved ways of detecting and managing hatchery disease. The production of extracellular metalloproteases have recently been demonstrated to be major virulence factors of *V. tubiashi* encoded by *Vtpa* gene (Hasegawa et al. 2008) and also strains of *V. splendidus* where it is encoded by a similar gene called *Vsm* (Binesse et al. 2008). Similar metalloproteases appear to be major virulence factors also in *V. anguillarum*, *V. aestuarianus*, and *V. vulnificus* (Labreuche et al. 2006a, 2006b; Valiente et al. 2008; Hasegawa et al. 2009), and a major factor in bleaching and necrosis of marine corals by *V. corallilyticus* (Sussman et al. 2008). Additional challenge studies also show a high correlation between virulence to adult oysters when administered via injection, and the production of metalloprotease-like activity (Saulnier et al. 2010). This suggests that excretion of extracellular metalloproteases is a common feature of marine necrotic diseases and that detection of specific metalloprotease activity/genes may be a more direct method to detect and manage bacillary necrosis, regardless of the causative organism/s and aquaculture species involved.

Group-specific PCR for Vibrio splendidus *group*

A diagnostic PCR was developed to specifically detect *V. splendidus* group bacteria and validated against a range of *V. splendidus*, other *Vibrio* species, and other marine bacteria. A total of eight primers were designed using seven sequence regions identified in phylogenetic analyses as conserved only in the *V. splendidus* group (Table 7.3.5).

Table 7.3.5: Sequences and characteristic of 8 PCR primers designed for specific amplification of *V. splendidus* group , based on *atpA* gene sequences . Melting temperature (T_m; Wallace et al. 2001) and degeneracy calculated using OligoCalc (Kibbe, 2007).

Primer	Sequences (5'-'3)	Tm (°C)	Degeneracy
1F649	GCACGGCGCACTRGCWAACACTG	61-62	4
2F715	CCTTGCACCGTATGCTGG	53	0
4F801	GCAAGCKGTAGCTTACCGTCAA	55-57	2
5R944	TGAAYTTTTCTACGTATGCTTCGC	52-54	2
6R1151	GCGCCGAACCACCTACACGWGAA	61	2
7R1242	CCGAWGAGAAYTGWGCAAATGC	53-55	8
9R823	TTGACGGTAAGCKACMGCTTGC	55-59	4
10F920	GCGAAGCATACGTAGAAAARTTCA	52-54	2

Examination of primers for potential cross-reactivity showed that the first ten returns with 100% sequence matches were all *V. splendidus* group sequences: *V. cyclitrophicus*, *V. pomeroyi*, *V. kanaloe*, *V. tasmaniensis*, *V. crassostreae* and *V. lentus*, *V. gallaecicus* and *V. gigantis*. When combined in priming sets with the *atpA* gene primers of Thompson et al. (2007), six *V. splendidus*

group-specific PCR primer pairs were developed for diagnostic PCR amplification (Table 7.3.6). These were targeted against sections in the distal half of the *atpA* gene from which sequence data was collected in this study, and predicted to amplify partial *atpA* gene products ranging from 130bp to 280bp (Fig. 7.3.12).

Table 7.3.6: Diagnostic PCR primer sets targeting *atpA* gene of *Vibrio splendidus* group. Primers atpA760F and atpA1204 are *Vibrio* generic primers (Thompson et al. 2007).

Primer pair	Pair T_m (°C)	Product size (bp)
(a) 2F715 & 5R944	52-54	180
(b) 10F920 & 7R1242	52-55	280
(c) atpA*760F* & 5R944	52-56	140
(d) 4F801 & 5R944	52-57	100
(e) 10F920 & atpA*1204*	52-60	240
(f) 1F649 & 9R823	55-62	130

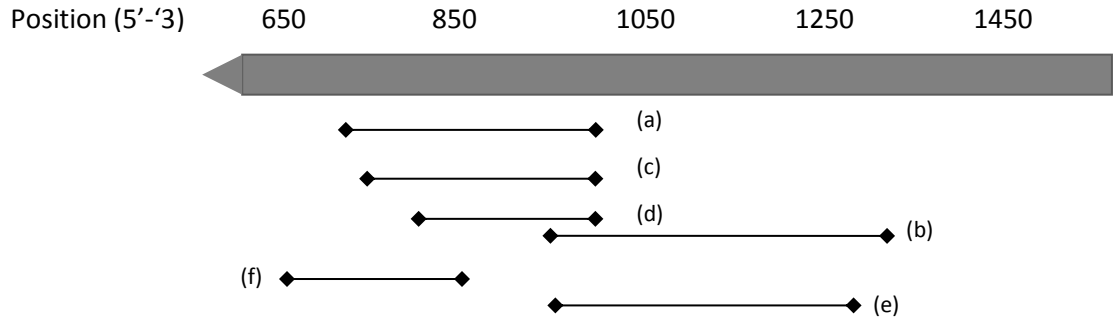

Figure 7.3.12: Schematic illustration of relative positions and predicted PCR products amplified by the 6 primer sets, labelled (a) to (f), targeting conserved regions of *atpA* gene of *V. splendidus* group strains.

The initial primer screening at 50°C was sufficient for primer set (a), (b) and (c) to amplify the expected *atpA* target region specifically and efficiently producing single strong band product of 180, 280 and 140 bp respectively (Fig. 7.3.13) of all five SBS *V. splendidus* group strains. There was no amplification product for *V. calviensis*, *V. proteolyticus*, *V. alginolyticus*-like and *V. harveryi*-like strain, or the five randomly picked bacterial colonies from the *Tetraselmis suecica* alga culture, subsequently identified by 16S rDNA gene sequencing as *Alteromonas genovensis*, *Flexibacter* sp., *Flexibacter tractuosus*, *Flavobacterium sp*. and *Planococcus* sp. Successful amplification of the 16S rRNA gene (approx 1400 bp) from most reactions indicated that the lack of the target product was not likely due to poor DNA quality.

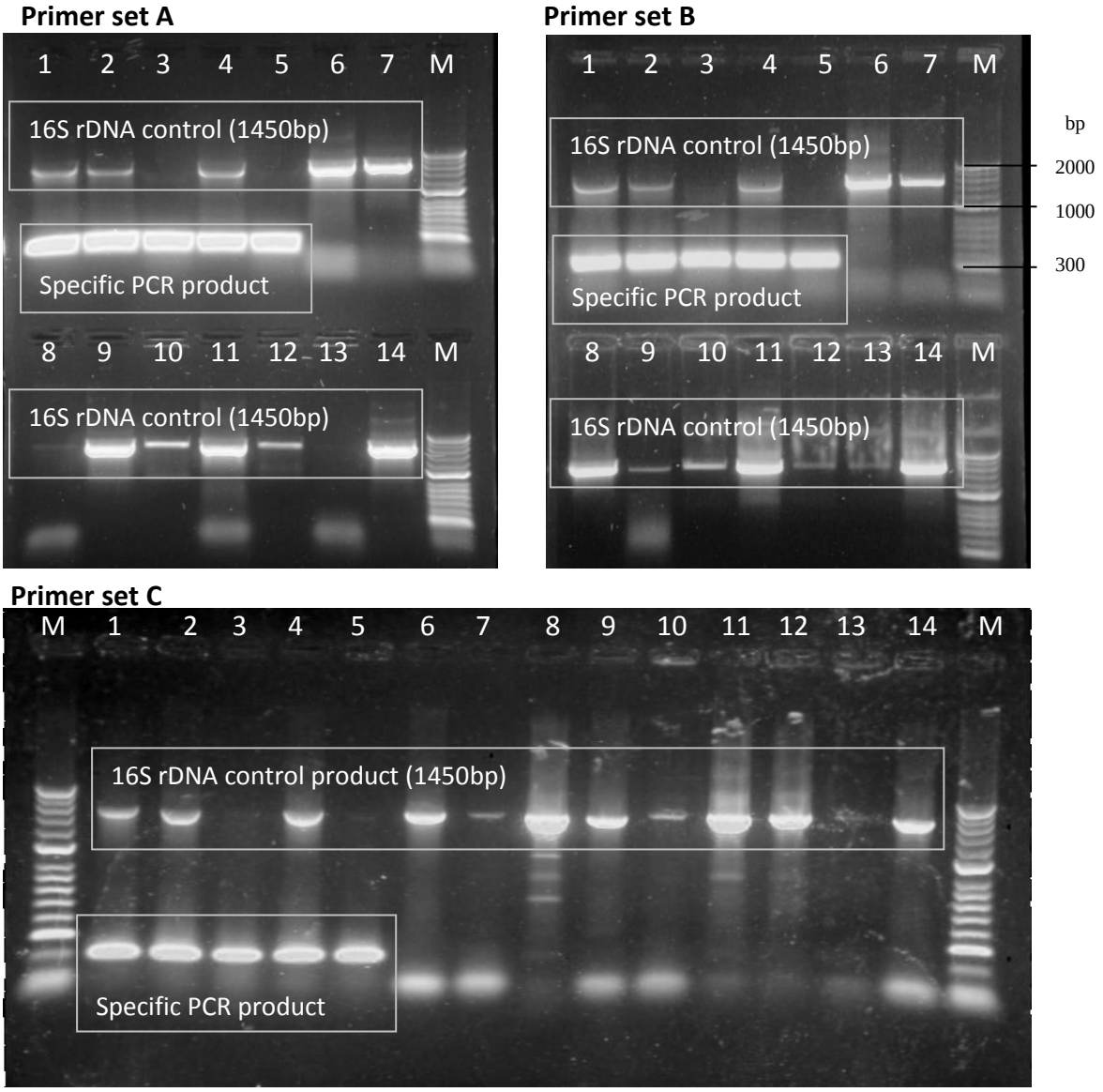

Figure 7.3.13: Specificity of *V. splendidus* group specific PCR primer set (a), (b) and (c) at annealing temperature of 50°C. Lanes 1 – 5: strains of the *V. splendidus* group; Lanes 6-10: random pick of bacterial colonies from non-axenic *Tetraselmis suecica* alga culture; Lanes 11-14: Non- splendidus *Vibrio* species (11=*V. calviensis*; 12=*V. proteolyticus*; 13= *V. alginolyticus*-like; 14=*V. harveyi*), M= marker DNA.

Primer set (a) was further tested across an additional two *V. splendidus* strains, *V. anguillarum* and *V. penaeicida*, together with 13 additional bacterial strains isolated from mussel spat seawater. Specific amplification was observed for all *V. splendidus* group strains and no product was obtained for all non-*V. splendidus* group strains, with the exception of a *V. penaeicida* isolate from *Jasus* phyllosoma (supplied by UTAS Tas. Aquaculture and Fisheries Institute) (Fig. 7.3.14). This isolate returned a clear positive with *V. splendidus*-group specific primer sets (d), (e) and (f) (Fig. 7.3.15), and also at annealing temperatures up to 55°C for primer sets (a) to (d). Subsequent sequencing of the 16S rDNA of this showed that it this isolate was phylogenetically allied with the *V. splendidus*-group (data not shown).

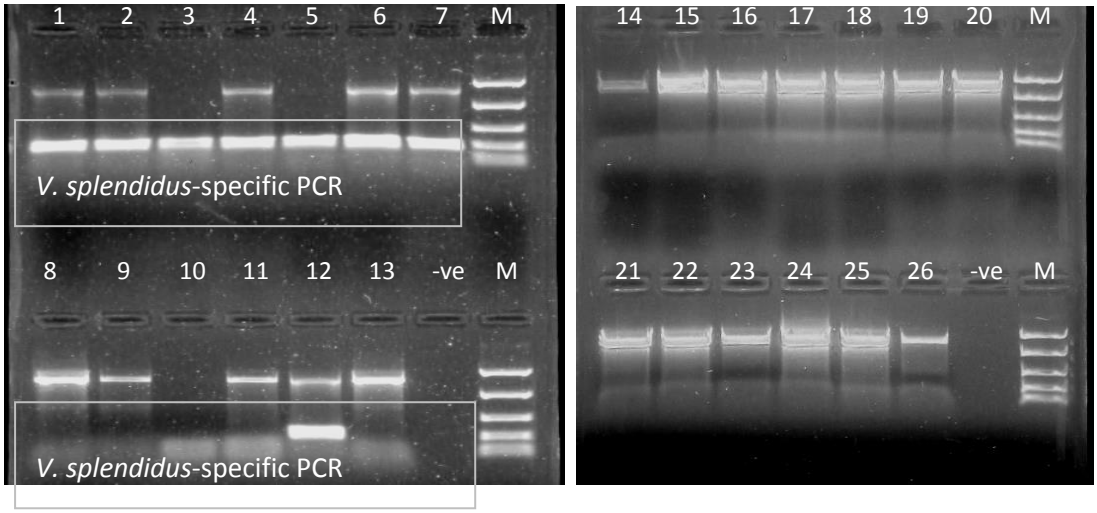

Figure 7.3.14: Specificity of *V. splendidus* group specific PCR primer set (a). Expected *V. splendidus*-group product indicated in boxes; larger PCR product (1450 bp) is the expected 16S DNA quality internal control. Lanes 1-7=*V. splendidus* group; Lane 8=*V. calviensis*; Lane 9=*V. proteolyticus*; Lane 10= *V. alginolyticus*; Lane 11= *V. harveyi*; Lane 12=*V. penaeicida* (TAFI); 13=*V. anguillarum*; Lanes 14-26=random bacterial colony picks mussel spat tank seawater; M:=DNA size marker.

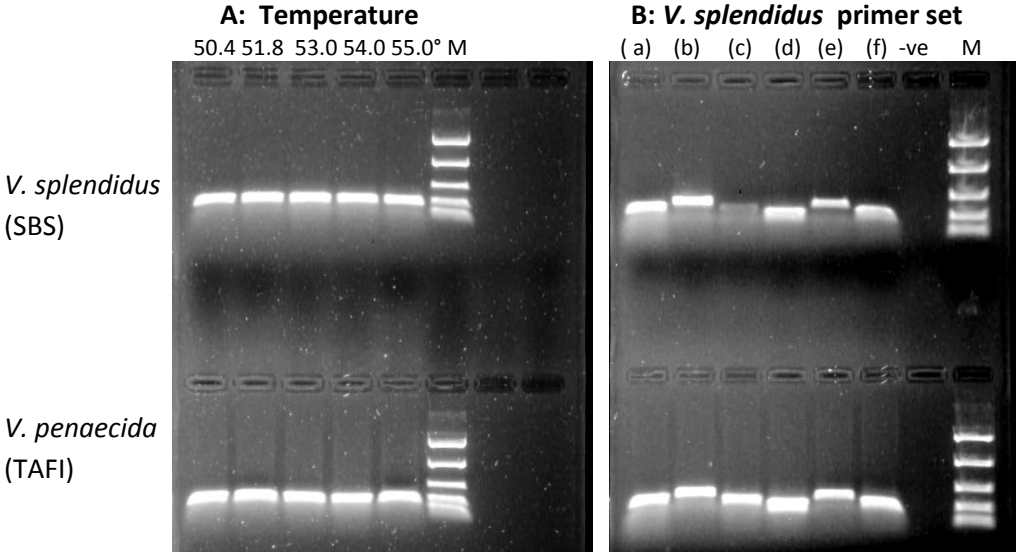

Figure 7.3.15: Performance of *V. splendidus*-specific PCRs applied to *V. splendidus* strain and *V. penaeicida* (TAFI) (lower half). A. Effect of increased annealing temperature on PCR performance. B. Performance of the six *V. splendidus* group primer sets (a) to (f) at annealing of 55°C. Both strains produce the expected products of 180, 280, 140, 100, 240 and 130 bp in size. The *V. penaeicida* was subsequently confirmed as allied to *V. splendidus* group by 16S rRNA gene sequencing.

Subsequent 16S rDNA sequencing of the 13 random isolates used in the specificity studies indicated that they were allied with the non-*Vibrio* Gamma-proteobacteria (*Pseudomonas putida*, *Acinetobacter radioresistens* and *Oceanobacter kriegii*) and the Beta-proteobacteria (*Comamonas*).

While the primer designs were based on the 40 *atpA* genotypes detected in this study, the *atpA* genotypes included represent each of six discrete clusters within the group, therefore we have

confidence that the primers effectively amplify all of the major clusters identified within the *V. splendidus* group. The specificity testing carried out also indicates that they reliably distinguish *V. splendidus* group genotypes from other *Vibrio* species/groups, and related non-*Vibrio* gamma-proteobacteria.

The capacity to rapidly detect and quantitatively monitor the abundance of this and other *Vibrio* groups would be valuable for both hatchery management and research. Our investigations indicate that *V. splendidus* group genotypes are an overwhelming majority (98%) of the cultivable *Vibrio* present during normal hatchery operations. In addition, *V. splendidus*-group biovars/genotypes have been associated with every larval/spat mortality event at SBS where the bacterial community has been characterised (I. Duthie, pers. comm.). Therefore, the priming regions and sequences identified could be adapted to quantitative PCR (qPCR) to provide rapid detection/quantification of the *Vibrio splendidus* group in the hatchery environment (Table 7.3.7, Fig. 7.3.16).

Table 7.3.7: Potential qPCR primers and TaqMan hydrolysis probes for *V. splendidus* group.

Primer pair	Pair T_m (°C)	Probe (position)	Probe sequence (5'-'3)[a]	Probe T_m (°C)	Product Size (bp)
1. 2F715 & 5R944	52-54	P4(801)	*FAM*-TCTATCTAAGCAAGC KGTAGCTTACCGTCA A-*BHQ1*	60-62	180
2. atpA760F & 5R944	52-56	P4(801)	*FAM*-TCTATCTAAGCAAGC KGTAGCTTACCGTCA A-*BHQ1*	60-62	140
3. 1F649 & 9R823	55-62	P2(715)	*FAM*-CGCTGCAATACCTTG CACCGTATGCTGGT -*BHQ1*	64	130

a: FAM= 6-fluorescein; BHQ1=Black Hole-1 quencher dye

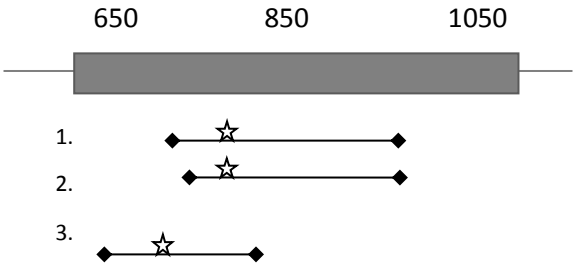

Figure 7.3.16: Relative position of three potential *V. splendidus* group qPCR primer/probe sets targeting conserved regions of the distal end of the *atpA* gene. Primer and predicted PCR product shown as diamond and line respectively; probe position shown by a star.

Equivalent published hatchery studies that have sampled cultivable *Vibrio* genetic diversity at the same scale and taxonomic detail are rare, however *V. splendidus* is the most regularly reported

Vibrio from molluscan hatcheries and appears to be the dominant cultivable *Vibrio* in all hatchery systems and environments. Adoption of the priming designs developed here for qPCR could thus be a suitable proxy for monitoring total *Vibrio* in a wide range of molluscan hatchery systems. While not all *Vibrio*-related bivalve mortality is caused by *V. splendidus* group genotypes, additional *Vibrio*-specific or group-specific qPCRs (e.g. *V. harveyi* group) could be used in combination with the primers here to reduce the risk of other pathogenic *Vibrio* remaining undetected. Their use in combination with a quantitative detection method for *Vibrio* metalloproteases would offer a powerful combination to understand the dynamics and development of bacillary necrosis, and with development of cheaper, more robust and user-friendly PCR platforms, also be used to assist management of the disease in marine hatcheries.

7.4 Replacement live foods for larval culture

Strain selection, lipid and essential fatty acid and profiles

After discussion with mussel hatchery industry representatives (Spring Bay Seafoods, Victoria DPI), fours strains were selected for assessment along with *C. calcitrans* CS-178 and *C. muelleri* CS-176 for comparison (Table 7.4.1). Both the latter strains are used at SBS and their performance in SBS algal culture systems is known.

Table 7.4.1: Lipid characteristics of *Chaetoceros calcitrans* CS-178, *Chaetoceros muelleri* CS-176, and the four diatom strains selected for growth assessment and treatment to remove bacteria.

Strain/ Species	Primary lipid classes and essential fatty acids							
	∑ SFA	∑ MUFA	∑ PUFA	ARA (20:4n6)	EPA (20:5n3)	DHA (22:6n3)	n3/n6 ratio	(DHA+EPA) /ARA ratio
Comparison strains*								
Chaet. calcitrans CS-178[1]	30.2	33.8	33.7	5.7	11.1	0.8	1.8	2.1
Chaet. calcitrans CS-178[3] (400L)	40.6	30.4	29.0	2.4	17.4	1.6	5.1	7.9
Chaet. muelleri CS-176 [1]	38.7	40.0	19.8	4.5	4.6	0.3	0.9	1.1
Chaet. muelleri CS-176 [3] (400L)	35.2	40.8	23.9	5.7	13.0	1.3	2.1	2.5
Candidate replacements								
Chaetoceros sp. CS-365/01	29.1	27.9	40.6	1.4	13.7	0.5	3.7	10.1
Chaetoceros sp. CS-365/02	31.0	24.6	41.5	0.5	12.6	0.4	4.4	26.0
Thalass. pseudonana CS-20 [2]	35.0	33.8	22.5	tr	10.2	1.3	51.3	>100
Thalass. pseudonana CS-173 [1]	27.2	19.5	52.6	0.3	19.3	3.9	32.1	77.6

*From published data: [1]Volkman et al. 1989, [2]Mansour et al.2005; [3] 400L bag culture (from Pettersen et al. 2010); tr=trace amount

The essential lipid/fatty acid profile of *T. pseudonana* CS-20 and CS-173 differed substantially from *C. calcitrans*, however, the balance of saturated (SFA) to monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) was relatively similar and both strains contained detectable amounts of the essential fatty acids (EFA) arachidonic acid (ARA) , eiscosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Table 7.4.1). Strain CS-20 contained only trace amounts ARA. Lipid profiles of *Chaetoceros* sp. 365/01 and 365/02 show that both strains have essentially the same lipid/EFA composition. This result is not unexpected as they originate from the same source material. While similar to *C. muelleri* and *C. calcitrans*, both *Chaetoceros* sp. CS-365 strains have a greater proportion of PUFA, less MUFA, and less ARA. The omega-3 to omega-6 ratio of all four candidate strains was substantially higher than *C. calcitrans* and *C. muelleri* (generally but not always in range of 1-2); both *Chaetoceros* sp. CS365 strains were most similar (Table 7.4.1).

All four species exhibited high (DHA+EPA/ARA) ratios compared to *C. calcitrans* and *C. muelleri* grown under similar culture conditions or in 400L bag culture (Table 7.4.1). While proportions of total SFA, MUFA and PUFA appeared to be relatively stable, considerable variation in profile and proportion of individual fatty acids is evident, particularly those at relatively low proportions. The concentration of PUFA and EFA is known to vary as much as 2-3 fold in even well characterized

species such as *C. calcitrans* and *C. muelleri*. Lipid and PUFA proportions vary considerably depending on culture conditions and nutrient status but, generally, both lower irradiance and cooler temperatures lead to increased proportion of PUFA and EFA in microalgae (Thompson et al. 1990; Thompson et al. 1999; Renaud et al. 2002). Increased proportion of DHA in the body of mussel larvae is correlated with reduced larval mortality, and higher EPA+DHA to ARA ratios are correlated with increased settlement success (Pettersen et al. 2010). While the cause-effect relationship is unclear, this suggests that EFA ratios are important factors in overall success of larval production and should be considered in selection of any species used as a dietary replacement for *C. calcitrans*. While Pettersen et al. (2010) reports relatively higher EPA and DHA and relatively lower ARA proportions for *C. calcitrans* and *C. muelleri* (see Table 7.4.1) their algal cells were grown in standard 400L bag batch cultures at lower temperature (16°C) and would have received considerably lower irradiance than the lab-scale flask cultures analysed here. Should similar proportional changes occur under 400L bag culture conditions all four candidate species might be expected to exhibit increased DHA/ARA and EPA/ARA ratios.

Both *Chaetoceros* sp. CS-365 strains appear to be suitable replacement for *C. calcitrans* in larval diets. Larval diets for *Mytilus edulis* containing *Chaetoceros muelleri* perform equally as well, in terms of growth rate and survival, as diets containing *C. calcitrans* whether used as a single species diet or in combination with other species (Galley et al. 2012). Therefore *Chaetoceros* sp. CS-365 strains would be expected to perform similarly or perhaps better due to the slightly smaller cells being more efficiently ingested by young mussel larvae. The EFA balance of the strains is also suitable, especially given increases in DHA+EPA/ARA ratio would be expected in larger volume bag cultures. Greater ratios may enhance pediveliger settlement; however the lower proportion of ARA may present problems, particularly for *T. pseudonana* CS-20, if used as a single diet species throughout larval rearing.

Antibiotic purification of candidate cultures

Low concentration antibiotic cocktails reduced the diversity of colony types detected on spread plate from all cultures, but were unsuccessful at removing all detectable bacteria (Table 7.4.2). Additional rounds of purification undertaken at medium (med-AB) and high concentration (high AB) resulted in no cultivable bacteria detected in either *T. pseudonana* strains using the three solid screening media. However, a small number of small white colonies were detected only on AOC medium for both *Chaetoceros* sp. strains (Table 7.4.2).

Solid media plate-purification methods removed all bacteria detectable on standard marine agar, however, in almost all cases, spread plating and incubation on AOC medium (7 days, 22°C) resulted in very small (microscopic) clear bacterial colonies for all but one of the *Chaetoceros* sp. CS-365/01 and CS-365/02 subclones (Table 6). Cultivable bacteria were not detected in four *T. pseudonana* CS-20 and five *T. pseudonana* CS-173 subclones, however, PCR-based detection assays detected bacterial DNA in all 24 plate-purified subclones (Table 7.4.3, Fig. 7.4.1). All *T. pseudonana* samples produced two distinct products, the target product and an additional product approximately 30bp

longer than expected, likely to be caused by cross-reaction of the primers with the 16SrDNA gene located on either the chloroplast or mitochondrial DNA of *T. pseudonana*. Comparative alignments of *T. pseudonana* cpDNA and mtDNA gene (Genbank EF067921, DQ186202) indicate that the targeted priming regions are sufficiently similar to allow PCR amplification (data not shown).

Table 7.4.2: Relative bacterial contamination of original and antibiotic-treated isolates of the four candidate cultures on three different solid media and by PCR of bacterial ribosomal RNA genes using polymerase chain reaction (PCR). (+++=>100 colonies, ++= <100 colonies, +=colonies detected; -=not detected).

		Relative bacterial contamination (ZM1, ZM10 & AOC medium)			
Strain	Original	1x AB low	2x AB	2x high AB	Comment
Chaetoceros sp. CS365/01	+++	+	+	+	small white colonies
Chaetoceros sp. CS365/02	+++	+	No recovery	+	small white colonies
T. pseudonana CS-20	++	-	-	-	No bacteria detected
T. pseudonana CS-173	+	-	-	-	No bacteria detected

Table 7.4.3: Bacterial screening of 24 plate-purified sub-clones of the four candidate cultures (6 sub-clones from each), on solid media (AOC), and by PCR of bacterial ribosomal RNA genes using polymerase chain reaction (PCR). (+=PCR product detected, -=PCR product not detected).

Strain	Bacteria (AOC)	Comments	PCR detection
C. sp. 365/01 C5x2 D6 LA	+	many small visible clear colonies	+
C. sp. 365/01 C5x2 D6 LB	+	microscopic clear colonies	+
C. sp. 365/01 C5x2 D6 LC	+	microscopic clear colonies	+
C. sp. 365/01 C5x2 D6 PPA	+	microscopic clear colonies	+
C. sp. 365/01 C5x2 D6 PPB	+	microscopic clear colonies	+
C. sp. 365/01 C5x2 D6 PPC	+	microscopic clear colonies	+
C. sp. 365/02 C4x2 D6 LA	+	microscopic clear colonies	+
C. sp. 365/02 C4x2 D6 LB	+	microscopic clear colonies	+
C. sp. 365/02 C4x2 D6 LC	+	microscopic clear colonies	+
C. sp. 365/02 C4x2 D6 PPA	-	no bacteria detected	+
C. sp. 365/02 C4x2 D6 PPB	+	microscopic clear colonies	+
C. sp. 365/02 C4x2 D6 PPC	+	microscopic clear colonies	+
T. ps. 20 C5x2 D6 LA	-	no bacteria detected	+
T. ps. 20 C5x2 D6 LB	+	microscopic clear colonies	+
T. ps. 20 C5x2 D6 LC	+	microscopic clear colonies	+
T. ps. 20 C5x2 D6 PPA	-	no bacteria detected	+
T. ps. 20 C5x2 D6 PPB	-	no bacteria detected	+
T. ps. 20 C5x2 D6 PPC	-	no bacteria detected	+
T. ps. 173 C5x2 D6 LA	-	no bacteria detected	+
T. ps. 173 C5x2 D6 LB	-	no bacteria detected	+
T. ps. 173 C5x2 D6 LC	-	no bacteria detected	+
T. ps. 173 C5x2 D6 PPA	-	no bacteria detected	+
T. ps. 173 C5x2 D6 PPB	+	microscopic clear colonies	+
T. ps. 173 C5x2 D6 PPC	-	microscopic clear colonies	+

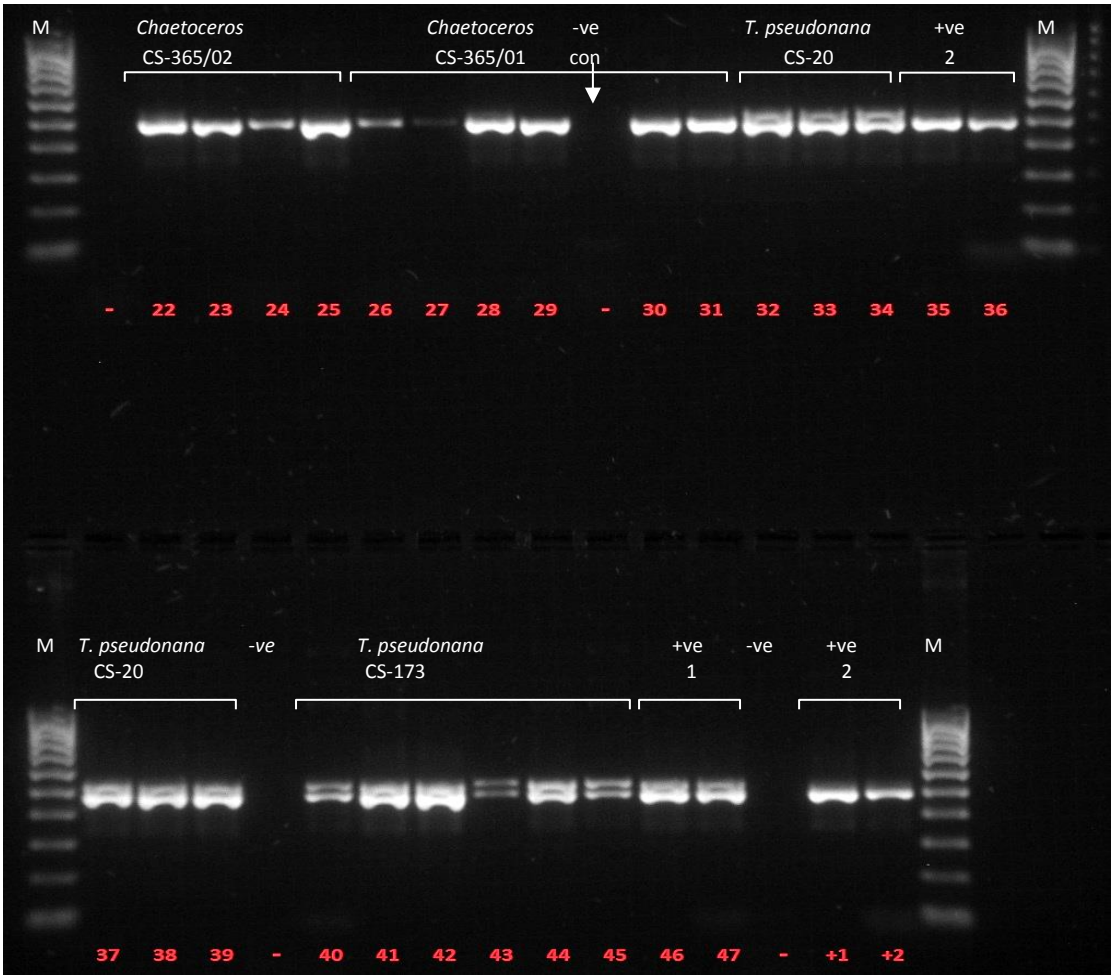

Figure 7.4.1: Detection of bacterial ribosomal RNA genes from 22 plate-purified subclones of the four candidate species. (–ve)=negative control; (+ve1)=DNA extracted from original bacteria contaminated cultures; (+ve2)=DNA extracted from a known non-axenic algal culture of a different species).

While attempts to completely remove all bacteria from the four strains were ultimately unsuccessful, the concentration and diversity of the cultivable bacterial community were substantially decreased and in most cases the remaining bacteria were not detectable on standard marine agar medium used to detect total viable bacteria. Slow–growing bacteria were present that appear adapted to using algal organic carbon (growth on AOC medium) rather than the peptone and yeast extracts contained in most general marine media. Spread-plating of subclones on TCBS indicate that presumptive *Vibrio* are also absent, therefore the purified subclones present a low risk as food for juvenile oysters. Due to their similarity to *C. calcitrans* in size and EFA content, they are potentially viable and safe replacements or supplementary food for hatcheries.

Performance of unpurified strains in hatchery culture systems

Attempts to grow *C. calcitrans* CS-178 in 500L bag cultures consistently failed, a common response reported anecdotally by a number of shellfish and other marine hatcheries. However, stable and reliable growth was achieved in both 20L carboys and 40L hanging polyethylene bags, suggesting

that failure in larger bags is not related to water quality, or polyethylene bag materials, but may instead be related to reduced light intensity or changes in gas exchange.

All four candidate replacement strains performed consistently in the three hatchery culture systems examined. Growth curves, exponential growth rates, and maximum cell densities in 20L carboys were less than *C. calcitrans* (F=5.434, df 5, 6 *P*=0.031) and similar to *C. muelleri* (Figs 7.4.2, 7.4.3; Tables 7.4.4, 7.4.5). All strains achieved concentrations of > 10^6 within 5 days of inoculation, indicating sufficient growth performance in hatchery systems to be viable food species in marine hatcheries.

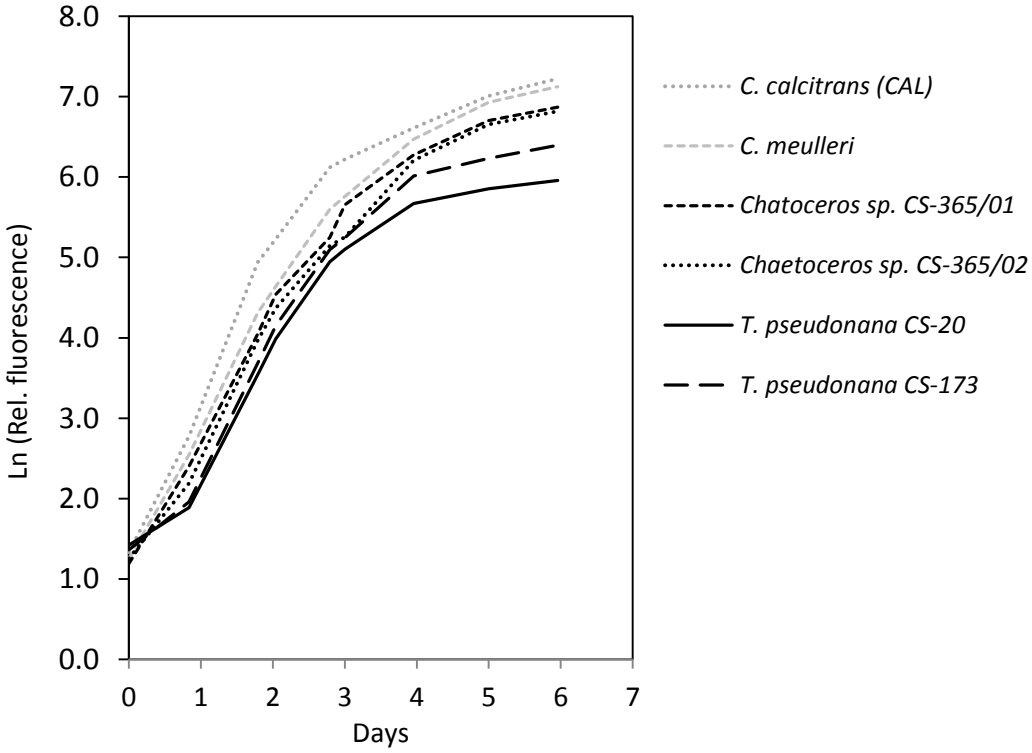

Figure 7.4.2: Growth dynamics of four candidate strains in 20L polycarbonate carboys (blue water cooler type) relative to *C. calcitrans* CS-178 and *C. muelleri* CS-176.

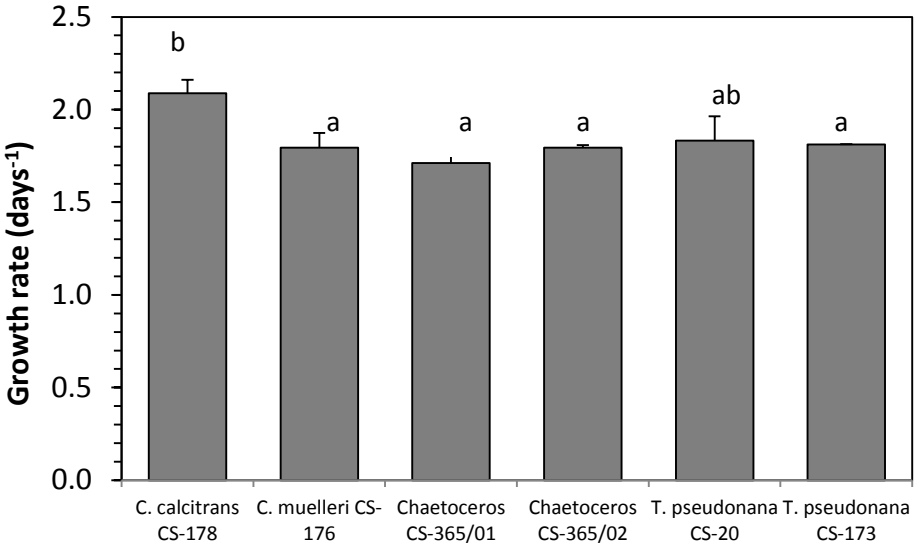

Figure 7.4.3: Mean (± SE) growth rate of candidate algal strains grown in 20L carboys (n=2, 20-22°C, 150 µmoles photons $PARm^{-2}$ s^{-1} at carboy surface). Means with different letters are significantly different from one another.

In 500L bags, the growth performance of *Chaetoceros* sp. CS-365/01 and CS-365/02 was almost identical to that of *C. muelleri* CS-176 (Fig. 7.4.4; Table 7.4.4). All four candidate strains achieved concentrations of $>10^6$ cells mL^{-1} within five days of inoculation. Cell concentrations at early stationary phase ranged from 2.0-6.5 $x10^6$ cells mL^{-1} for all four candidate strains (Table 7.4.4), however both *T. pseudonana* strains exhibited a short stationary phase (< 2 days) followed by rapid a population crash (see Fig. 7.4.4). Subsequent experiments indicate that crashes could be avoided by harvest and refilling with medium at early stationary phase (days 5-6, data not shown).

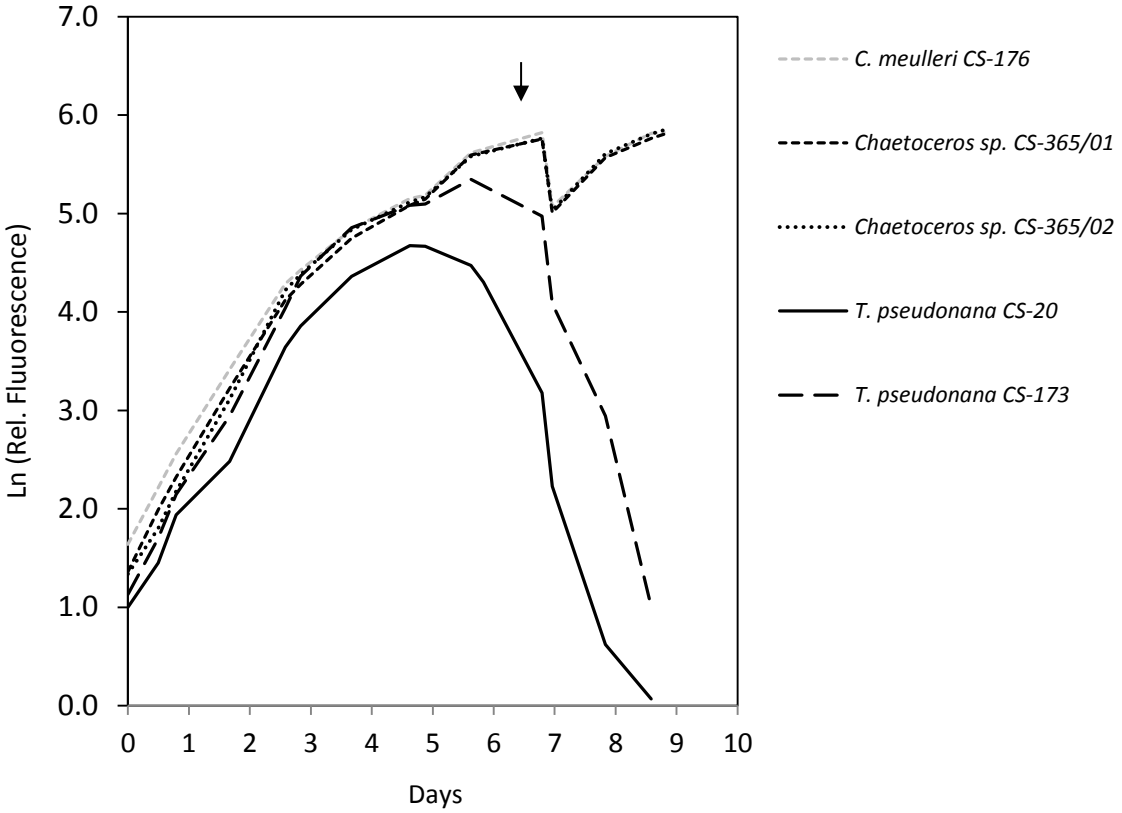

Figure 7.4.4: Growth of four candidate strains in 500L polyethylene bags relative to *C. muelleri* CS-173 (20-22°C, 120 µmoles photons m^{-2} s^{-1} at bag surface). Arrow indicates 70% harvest and refill with fresh growth medium.

Table 7.4.4: Maximum batch culture growth rates (k_{max}) and stationary phase harvest concentration of cultures grown in 20L carboys and 500L bags.

Algal Strain	20L carboy		500L bag	
	k_{max} (div. day^{-1})	Harvest conc. (cells mL^{-1})	k_{max} (div. day^{-1})	Harvest conc. (cells mL^{-1})
C. calcitrans CS-178	3.01	1.97 x10^7	no growth	no growth
C. muelleri CS-176	2.59	9.54 x10^6	1.49	1.85 x10^6
Chaetoceros sp. CS-365/01	2.47	7.71 x10^6	1.54	1.95 x10^6
Chaetoceros sp. CS-365/02	2.59	8.51 x10^6	1.64	2.44 x10^6
T. pseudonana CS-20	2.64	2.13 x10^6	1.72	4.50 x10^6
T. pseudonana CS-173	2.61	6.50 x10^6	1.63	6.05 x10^6

Successful growth of all strains (including *C. calcitrans* CS-178) was achieved in 40L hanging bags of SBS size/design (Figs 7.4.4, 7.4.5). Maximum exponential growth rates ranged from 1.53 to 2.08 div. day^{-1}, faster than 500L bags, but slower than 20L carboys, (compare Table 7.4.5 with Table 7.4.4).

Table 7.4.5: Maximum growth rate and harvest concentration at early stationary phase of cultures grown in 40L hanging bags (20-22°C, 120 µmoles photons m^{-2} s^{-1} at bag surface).

Algal Strain	k_{max} (div. day^{-1})	Harvest (cells mL^{-1})
C. calcitrans CS-178	2.54	3.23×10^7
C. muelleri CS-176	2.29	2.75×10^7
Chaetoceros sp. CS-365/01	2.32	2.11×10^7
Chaetoceros sp. CS-365/02	2.31	1.91×10^7
T. pseudonana CS-20	2.18	4.17×10^6
T. pseudonana CS-173	2.21	1.45×10^7

Mean growth rates across three semi-continuous harvest/refill cycles varied considerably (particularly *T. pseudonana* CS-20) such that growth rates of the six strains were not significantly different (F_{strain}=1.684; df 5, 10; *P*=0.226) (Fig. 7.4.4). Growth rates of the four candidate strains were decreased on-average by 20% after the first harvest/refill cycle (F_{phase}=5.844; df 2, 10; *P*=0.021) and 38% (23-60%) by the third growth phase (Fig. 7.4.5).

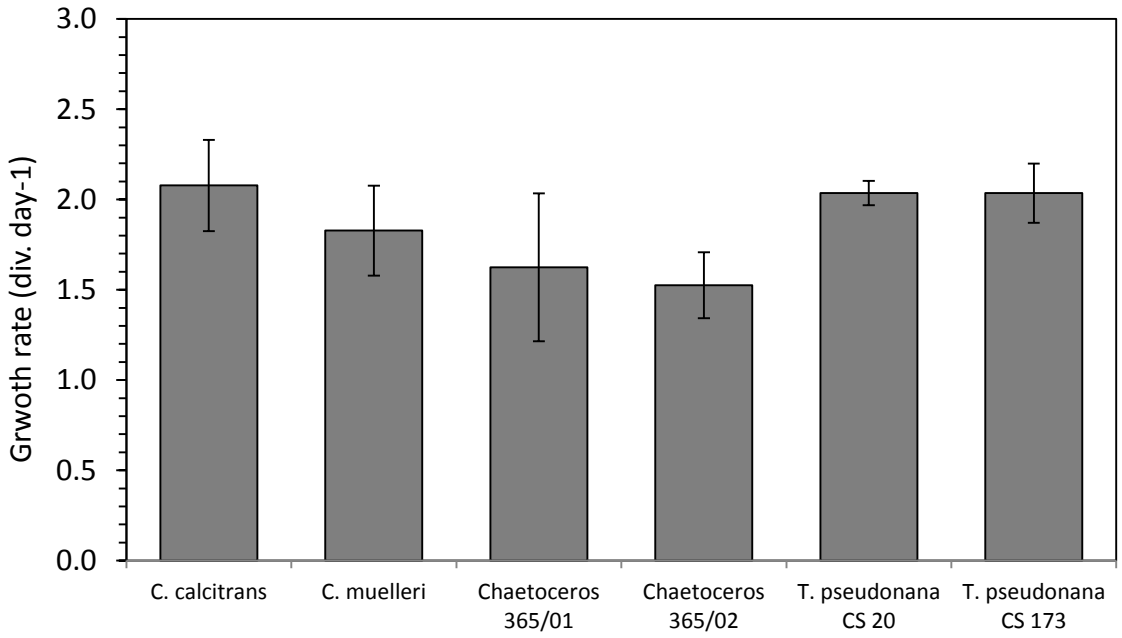

Figure 7.4.4: Mean growth rate (±SE) of four candidate strains in 40L polyethylene hanging bags relative to *C. calcitrans* CS-178 and *C. muelleri* CS-173 (20-22°C, 120 µmoles photons m^{-2} s^{-1} at bag surface).

Cell harvest concentration (early stationary phase) exceeded 10^7 cells mL^{-1} in all cases except for *T. pseudonana* CS-20 (4.2×10^6 cells mL^{-1}) (Table 7.4.5). The stationary phase of *C. calcitrans* and *T. pseudonana* CS-20 strains in 40L bags was generally short (<3 days), whereas *T. pseudonana* CS-173, *C. muelleri*, both *Chaetoceros* sp. strains exhibited a stable stationary phase exceeding 9 days (Table 7.4.6).

Table 7.4.6: Stationary phase duration (days) of cultures grown in different hatchery scale culture systems. Plus signs indicate minimum estimates due to termination of the experiment. Stable stationary phase may have continued for much longer in some cases.

	Stationary phase length (days)		
Algal Strain	10L carboy	400L bag	40L bag
C. calcitrans CS-178	7+	3+	3
C. muelleri CS-176	8+	3+	11
Chaetoceros CS-365/01	8+	3+	14
Chaetoceros CS-365/02	5	3+	10
T. pseudonana CS-20	4.5	3	1
T. pseudonana CS-173	4.5	3	9

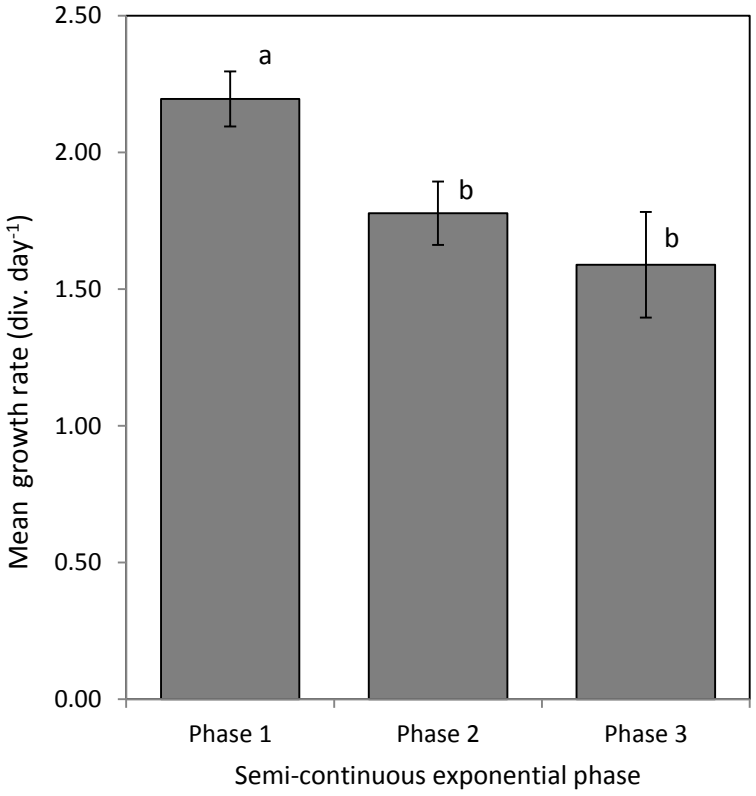

Figure 7.4.5: Mean exponential growth rate (±SE) of candidate strains over three semi-continuous growth phases in 40L polyethylene hanging bags (20-22°C, 120 µmoles photons m^{-2} s^{-1} at bag surface). Means with different letters are significantly different from one another.

The faster maximum growth rate achieved in 40L bags compared to 500L bags was undoubtedly due to the much shorter light path of hanging bags (only 18-20cm) resulting in greater light penetration and higher net irradiance per cell. Shorter light paths also reduce effects of self-shading at high cell concentrations in the latter half of exponential growth phase. For comparison,

maximum growth (in early exponential phase) prior to the onset of self-shading was typically 0.5 div. day^{-1} faster than the mean growth rate across multiple harvest cycles.

Reduction in growth performance of bag cultures over subsequent harvest/refill cycles has been noted anecdotally in most hatcheries, but to our knowledge this is the first time the scale of the reduction has been quantified. While many commercial mollusc hatcheries operate a version of the Bayes bag system on a continuous culture basis, 70-90% bag harvest/refill remains a common harvest method. Individual bags are replaced regularly (typically "bag life" of 3-5 weeks) primarily to limit bacterial infections and bag fouling. The declining performance in continuous systems is likely caused by the same mechanisms operating in semi-continuous culture systems. Reduced growth performance has often been attributed to increased bacterial load, exacerbated by bag fouling with moribund and dead algal/bacterial biomass, which in-turn also reduces light penetration. Experience in these trials (and from routine bag culture in the UTAS Aquaculture Centre) has demonstrated that polyethylene bag cultures are impossible to maintain in an axenic state. Bacterial infection is typically detectable within a few days of inoculation and may explain the 20% reduction in growth performance of axenic *C. calcitrans* over harvest/refill cycles.

Other contributing factors may also be repeated or extended cycles of nutrient limitation that degrades the physiological state of the cell biomass acting as an inoculum for subsequent growth phases. However, it is important to note that bacterial communities associated with phytoplankton cultures are phylogenetically diverse (e.g. Green et al. 2010) and dynamic (e.g. Grossart 1999), and that their effect on phytoplankton growth dynamics is complex (Doucette et al. 1998), not always inhibitory, and they can even be essential for growth (Bolch et al. 2011). For example, the addition of a *Chaetoceros*-associated *Flavobacterium* (strain DN10) improved the exponential growth rate of *Chaetoceros gracilis* by 10% and extended the length and stability of stationary phase (Suminto and Hirayama 1997). The same effect was not evident when added to cultures of the haptophyte *I. galbana*, illustrating the species-specificity of many phytoplankton-bacteria interactions.

Performance of antibiotic purified sub-clones in hatchery culture systems

Antibiotic purified sub-clones of all four cultures were grown successfully in semi-continuous 40L hanging bags for multiple growth phases (Figs 7.4.6, 7.4.7). General growth dynamics of treated sub-clones was similar to unpurified parent cultures, however exponential growth rate of purified sub-clones was reduced by 37% relative to the unpurified parent cultures ($F_{ABtreat}$=18.042; df=1,14; *P*=0.001) (Fig. 7.4.8); interaction between strain and AB treatment was not significant ($F_{strain*ABtreat}$=0.47, df=3,14, *P*=0.708). As in earlier experiments, exponential growth rate consistently declined with each harvest/refill phase for all strains resulting in a lower mean growth rate than the observed maximum growth rates in 40L bags (Fig. 7.4.9). The mean growth rates of the *Chaetoceros* sp. CS-365 strains were slower than *T. pseudonana* strains (F_{strain}=4.23; df 3, 14; *P*=0.025) (Fig. 7.4.9).

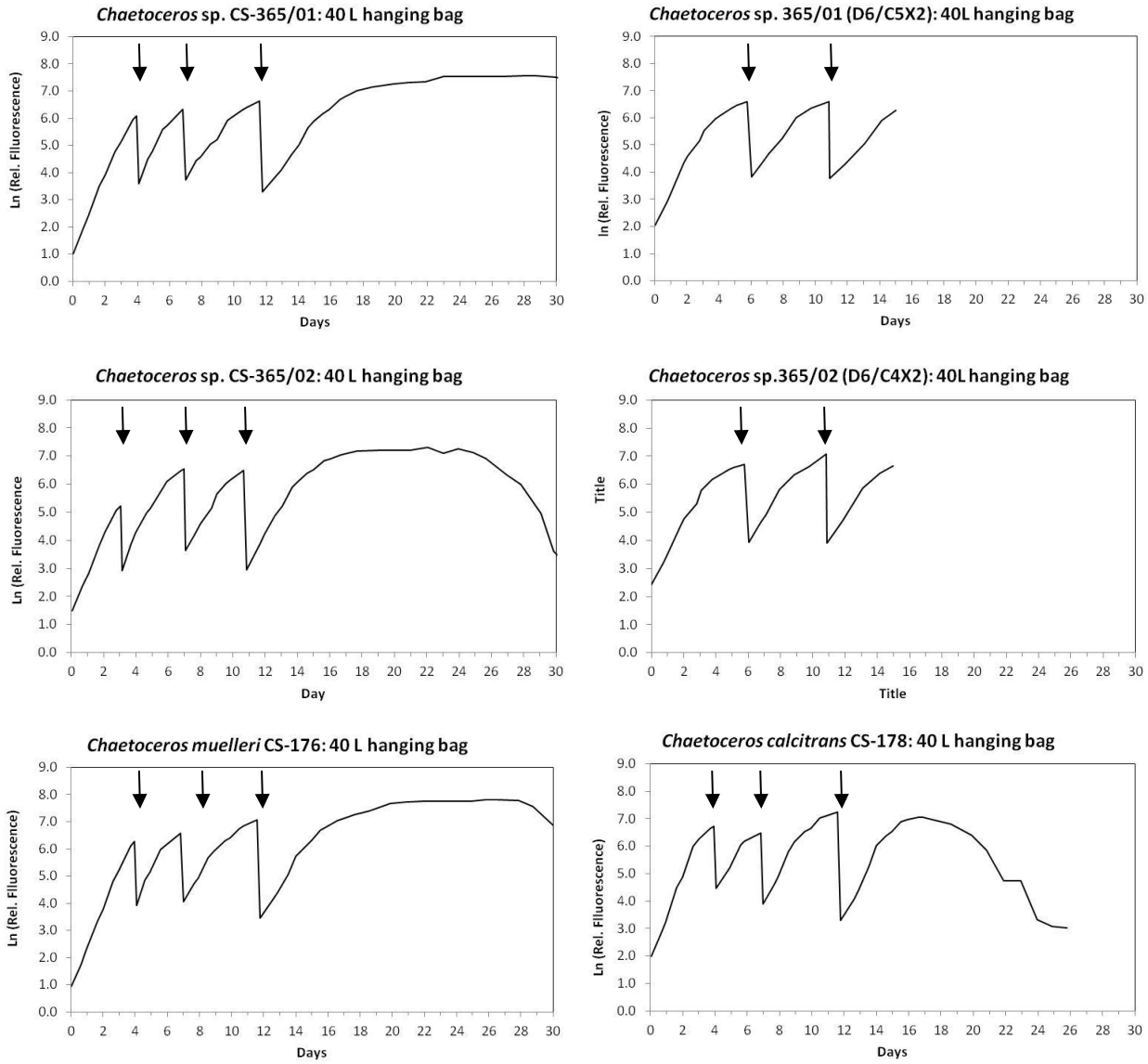

Figure 7.4.6: Pre-purification (left) and post- antibiotic purification (right) growth dynamics of *Chaetoceros* strains CS-365/01 and CS-365/02 compared to *C. calcitrans* CS-178 and *C. muelleri* CS-176 in 40 L hanging bags through 3-4 cycles of semi-continuous culture (20-22°C, 120 µmoles photons m^{-2} s^{-1} at bag surface). Arrows indicate 90% harvest and media replacement. Post-purification growth experiments were terminated at 15 days after three growth/harvest cycles.

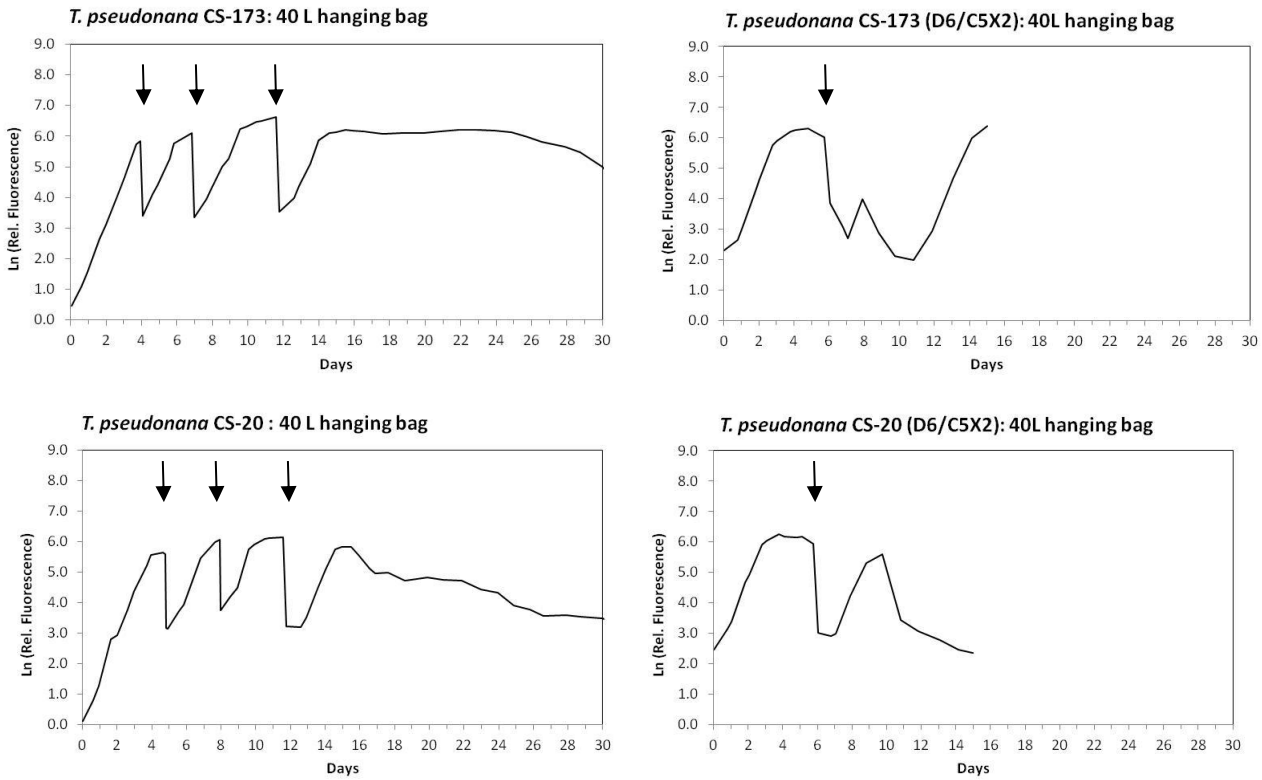

Figure 7.4.7: Growth dynamics of *Thalassiosira pseudonana* strains in 40 L hanging bags under semi-continuous culture conditions (20-22°C, 120 µmoles photons m^{-2} s^{-1} at bag surface) prior to (left) and after (right) antibiotic purification to reduce/remove bacterial community. Arrows indicate to 90% harvest and media replacement. Note that post-purification growth experiments were terminated at 15 days after two growth/harvest cycles.

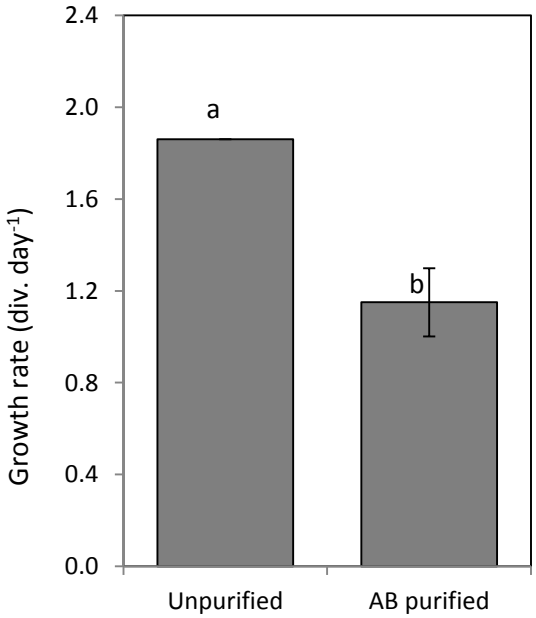

Figure 7.4.8: Mean growth rate in 40L hanging bags unpurified cultures compared to purified sub-clones of the four candidate species (averaged over three exponential growth phases). Bags were grown under semi-continuous culture conditions (20-22°C, 120 µmoles photons m-2 s-1 at bag surface). Means with different letters are significantly different from one another.

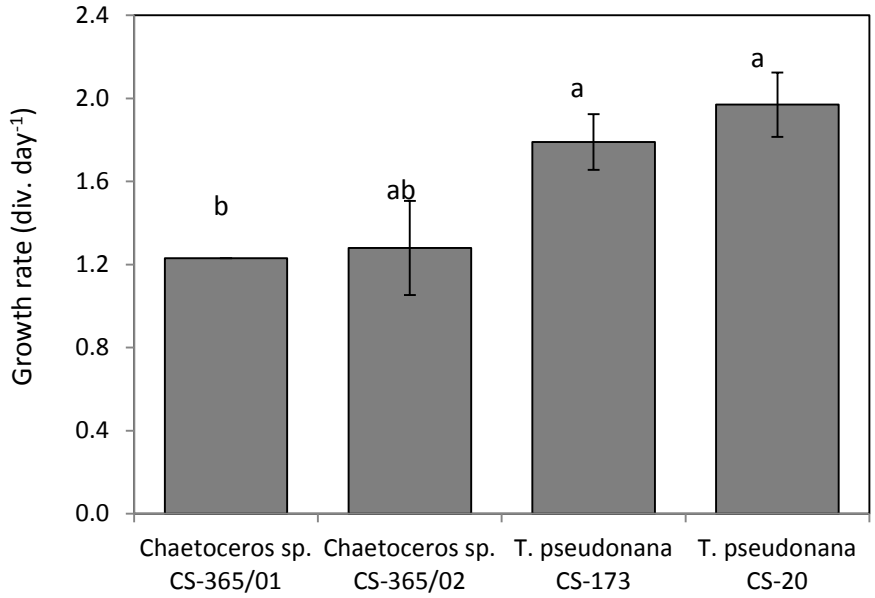

Figure 7.4.9: Mean growth rate of candidate species over three growth phases in 40L hanging bags. Bags were grown under semi-continuous culture conditions (20-22°C, 120 µmoles photons m-2 s-1 at bag surface). Means with different letters are significantly different from one another.

Stationary phase harvest concentrations of *T. pseudonana* CS-20 were lower than other strains (F_{strain}=10.84; df 3, 14; *P*=0.001) (Fig. 7.4.10). Harvest concentrations of purified sub-clones consistently increased by an average of 48% relative to unpurified parent cultures ($F_{ABtreat}$=4.97; df 1,14; *P*=0.043) (Fig. 7.4.11); interaction between strain and AB treatment was not significant ($F_{strain*ABtreat}$=0.59, df 3,14, *P*=0.634).

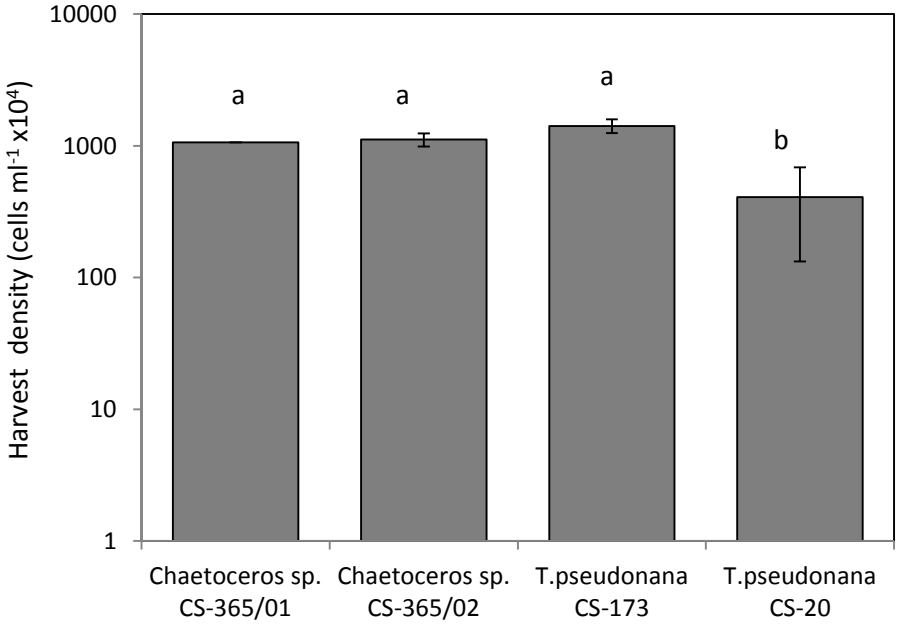

Figure 7.4.10: Mean harvest cell concentrations of 40L hanging bags at early stationary phase of the four candidate species. Bags were grown under semi-continuous culture conditions (20-22°C, 120 µmoles photons m^{-2} s^{-1} at bag surface). Means with different letters are significantly different from one another.

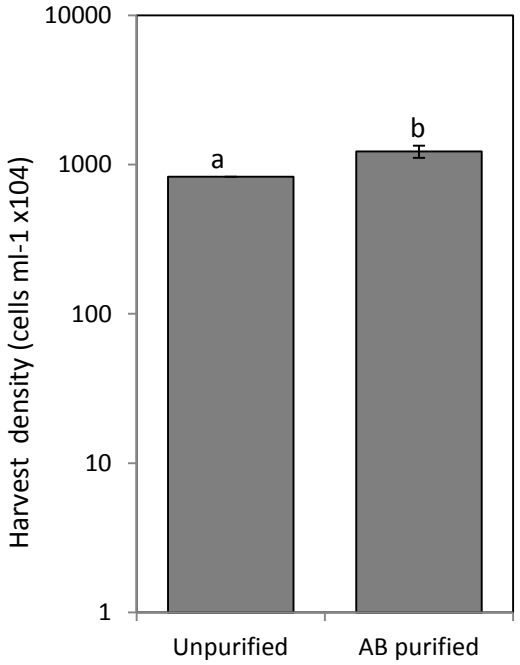

Figure 7.4.11: Mean early stationary harvest cell concentrations unpurified cultures and antibiotic purified subclones of the four candidate species grown in 40L hanging bags. Bags were grown under semi-continuous culture conditions (20-22°C, 120 µmoles photons m^{-2} s^{-1} at bag surface). Means with different letters are significantly different from one another.

The relative reduction in growth rate of purified cultures suggests that removal of the microbial community was detrimental to the performance of the strains in hatchery systems. Established phytoplankton physiological principles consider that growth rate of phytoplankton is primarily a function of temperature, which governs the rate of biochemical activity of the cells (Thompson 1999), and also light, which is required for photosynthesis. In the presence of sufficient light and nutrients (C, N, P, Si, Fe, trace metals and essential vitamins) growth should proceed at the maximum possible rate for a given temperature. However, more recent studies clearly show that bacterial community composition and abundance also have substantial independent effects on physiology and growth of phytoplankton (e.g. Fukami et al. 1991, Doucette et al. 1998, Grossart 1999, Bolch et al. 2011). It appears likely that many (or even most) species of phytoplankton are partially or completely reliant on specific members of the microbial community that facilitate nutrient uptake (e.g. Ferric ions, Amin et al. 2009) and other physiologically important functions.

Similar reductions in growth performance after purification attempts have been anecdotally reported for strains of *C. muelleri*. It may be impossible to completely remove all bacteria from algal cultures of many species without loss of culture viability, or degradation of growth performance to the point that they cannot grow in hatchery culture systems. Similar pre- and post-purification comparisons of other phytoplankton species show considerable variation in relative response (e.g. compare Dantzer and Levin 1997, Doucette and Powell, 1998, Hold et al. 2001, Uribe

and Espejo 2003, Maas et al. 2007), therefore it is difficult to predict the effect or outcome of further purification attempts. Should further degradation occur one possible solution could be to isolate growth-stimulating bacteria that can be added to enhance growth rates and stabilise the culture dynamics (Suminto and Hirayama 1997).

The increased stationary phase harvest concentrations of purified cultures are difficult to explain, but may result from the interaction of physical and microbial interactive factors. Firstly, the availability of nutrients for algal biomass may be increased due by reduction of nutrient competition with the microbial community. Secondly, alteration of the microbial community is known to change the rate of trace metal uptake including iron (Amin et al. 2009), leading to reduced chlorophyll-a synthesis. The effect of this would be reduced pigment per cell, higher light penetration at high cell density, and higher cell densities before the onset of severe light limitation.

Larval feeding and survival experiments

Feeding experiments with plate-purified strains indicated that all four candidate strains were filtered and ingested at similar or higher efficiency over 48 h than *C. calcitrans* (Fig. 7.4.12) ($F=21.064$, df 5, $P<0.0001$). Despite no water changes during the experiment, larval mortality at 72h was less than 5% in all cases except for the treatment fed *C. calcitrans* at 3.5×10^4 cells mL^{-1} ($\chi^2 = 55.78$, df 5, $P<0.001$; Fig. 7.4.13). More ciliates were evident in this treatment and may have caused the elevated mortality. At the conclusion of the 72h feeding experiment, all four candidate strain treatments were maintained for a further 7 days at a daily ration of approximately 10^5 cells mL^{-1}. Water changes were carried out every two days and no substantial mortality was noted (data not shown).

The short-term feeding experiment carried out here indicate that all four purified strains are able to be filtered effectively by larval mussels and the low mortalities indicates that they are not directly harmful and do not contain bacteria that are highly virulent to young mussel larvae. Mussel larvae are thought to efficiency filter and ingest particles from 3-10 µm, however, our data indicate that the two *Chaetoceros* sp. CS-365 were less efficiently cleared than the smaller *C. calcitrans* and *T. pseudonana*. Both *Chaetoceros* sp. strains also possess moderate spines (approx. 3µm length) increasing effective cell size and perhaps decreasing the filtration efficiency by early mussel larvae.

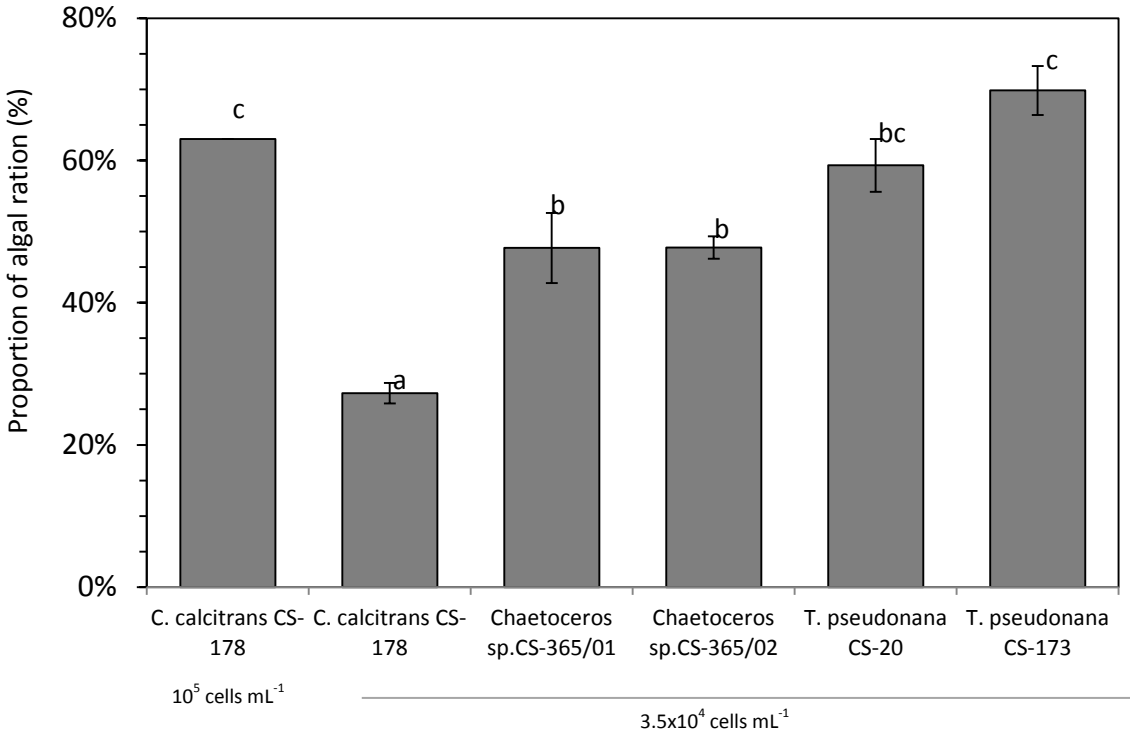

Figure 7.4.12: Proportion of total algal ration cleared in 48h by 7-9 day old mussel larvae fed single diets of the four candidate species and *C. calcitrans* CS-178. No water exchange carried out. Subclones used were *Chaetoceros* sp. CS365/01 C5X2 D6 LPA, *Chaetoceros* sp. CS365/02 C4X2 D6 PPB, *T. pseudonana* CS20 C5X2 D6 LPA, *T. pseudonana* CS20 C5X2 D6 PPA. Means with different letters are significantly different from one another.

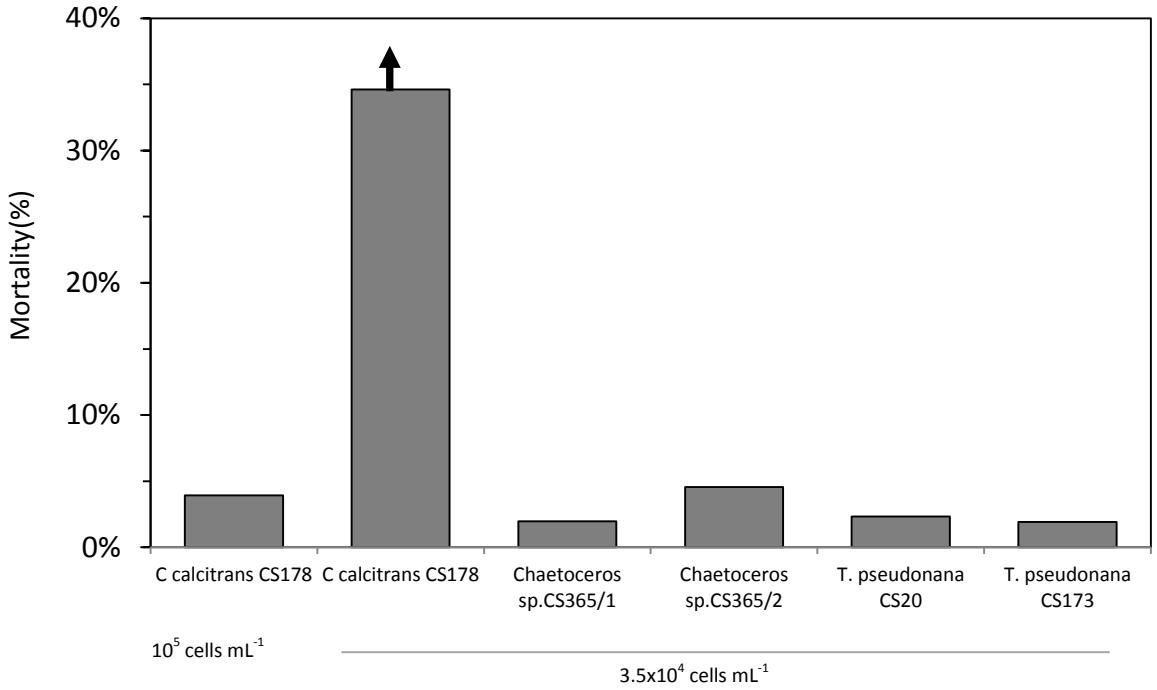

Figure 7.4.13: Mortality of 7-10 day old mussel larvae after 72h feeding on single algal diets of the four candidate species. The arrow indicates significantly greater mortality in that species. Purified sub-clones used for feeding were *Chaetoceros* sp. CS365/01 C5X2 D6 LPA, *Chaetoceros* sp. CS365/02 C4X2 D6 PPB, *T.* pseudonana CS20 C5X2 D6 LPA, *T. pseudonana* CS20 C5X2 D6 PPA.

8. BENEFITS AND ADOPTION

This project addressed several critical points and problem across the mussel production cycle. The beneficiaries of the research are the Australian mussel industry through an improved knowledge of factors influencing successful broodstock conditioning and hatchery-rearing of juvenile mussels. The outcomes also benefit marine molluscan hatcheries supporting production in other shellfish and crustacean sectors. The specific benefits and adoption of the research are outlined below:

1. *Spat settlement patterns and retention.* The project has provided an improved knowledge of settlement success and spat distribution in rearing systems at SBS hatchery. The data provides a basis from which to improve spat retention through the nursery phase and through transfer to sea.

2. *A reliable method for estimating spat density and settlement success*. An accurate method has been developed to estimate spat density on settlement tank rope substrate. The sampling principles and estimation method accommodates for the high spatial variation observed, has been tested on-site in the hatchery, and is ready for adoption by mussel hatcheries using similar settlement and spat husbandry approaches.

3. *Improved supplemental feeding for broodstock conditioning of mussels.* A series of dietary options have been tested and shown to be effective as partial replacements or supplemental food for bringing mussel broodstock into condition. This should allow the industry to widen the larval production window beyond the spring spawning season, particularly during the less reliable summer spawning season.

4. *Established operational Vibrio benchmarks in a mussel hatchery operational environment.* The project outcomes established major sources of cultivable *Vibrio* communities in the hatchery and provides quantitative operational benchmarks for cultivable *Vibrio* under commercial mussel hatchery conditions. The data benefits industry by providing a means to assess changes and disease risk associated with future changes in husbandry practice and to assess the value and success improvements in hatchery operational hygiene.

5. *Improved understanding of diversity of* Vibrio *communities associated with hatchery rearing of mussels.* The community of *Vibrio splendidus* associated with all stage of mussel rearing without causing significant mortality or disease highlights: 1) the limitations of current widespread focus on total *Vibrio* (n TCBS medium) and total bacterial loads as a tool for monitoring and management of Vibriosis; 2) The need for improved detection and quantification of *Vibrio* and other hatchery disease agents; 3) the need for additional research to understand factors and mechanisms that determine virulence of *Vibrio splendidus* to shellfish larvae.

6. *Proof-of-concept of rapid molecular detection for hatchery* Vibrio. The project established a suitable molecular target (the *atpA* gene), characterized target community diversity and completed initial laboratory validation of a rapid molecular detection for hatchery *Vibrio*. This research underpins future development of rapid detection and quantification of *Vibrio* for shellfish hatcheries.

7. *Four additional algal food strains suitable for mussel larvae and the broader hatchery sector.* The work carried out four purified strains developed during the project were extensively tested in the most common forms of hatchery-scale algal culture systems and shown to have suitable growth dynamics and performance for use in commercial hatcheries. Feeding trials indicated that the four strains can be fed to mussel larvae as young as 7 days without mortality. The safety of these four strains as food for mussel larvae is clearly demonstrated and removes a major barrier to industry uptake in the routine production cycle of mussels and other invertebrate hatcheries. Spring Bay Seafoods was supplied in August 2012 with the four purified and tested isolates for incorporation into their larval rearing and feeding protocols. All four purified strains were also lodged with Australian National Algal culture Collection (ANACC, CSIRO Fisheries and Atmospheric Research, Hobart, http://www.csiro.au/Organisation-Structure/National-Facilities/Australian-National-Algae-Culture-Collection.aspx), and are available for adoption by the broader Australian marine hatchery industry.

8. *Alternative live food to reduce/replace* Chaetoceros calcitrans *in early larval rearing phase of production*. The nutritional (lipid) characteristics and growth performance and safety of the two *Chaetoceros* sp. CS-365 have been shown to be suitable alternative live foods to partially or completely replace *Chaetoceros muelleri* CS-176 and *Chaetoceros calcitrans* CS-178 as food for mussel larvae. The strains and ready for adoption and integration in mussel (and other marine species) hatchery larval feeding protocols. Currently Spring Bay Seafoods hatchery is not experiencing substantial algal production problems with *C. calcitrans* CS-178, and is yet to incorporate *Chaetoceros* sp. CS-365 strains into their larval rearing and feeding protocols.

9. FURTHER DEVELOPMENT

9.1 Spat survival and retention

The logistics of determining the density and distribution patterns on the 500m continuous dropper rope are challenging for the commercial operation and in determining the processes determining the patch distribution and how this can be managed for better use of the space. It would be worth exploring the use of "sacrificial ropes" would allow easy sampling of settled spat for estimates of survivorship and density. These "sacrificial ropes" would be short 6cm sections of rope identical to the continuous dropper rope that are cable tied to the dropper rope at random locations along the continuous dropper rope. These "sacrificial ropes" could then be removed from the dropper rope when assessments of mortality and density were required, without the need to physically cut up the continuous dropper rope.

9.2 Mussel broodstock conditioning

In this study, when using exploring the use of different combinations of live and non-live diets successful spawning was achieved. When using live diets the binary diet *Isochrysis sp*. + *Chaetoceros muelleri* (70:30) resulted in the greatest mean fecundity and greatest larval survival rate. There was evidence that Instant Algae Shellfish Diet 1800® (SD) (Reed Mariculture) was very successful in facilitating broodstock conditioning. Cost is a major issue when using replacement diets, and there was evidence that partial replacement is possible allowing the use of non-live diets to supplement live microalgae. These experiments also highlight how important diet quality is in facilitating broodstock conditioning and being able to control the rate that animals come reproductively mature; therefore that are advantages of land-based broodstock systems with quality controlled diets. The experiments in this study used ambient water temperatures and as a result although the replacement diets allowed glycogen accumulation to occur, the conditions were not suitable to allow gametogenesis and spawning to occur. Given that temperature, particularly cooler temperatures that those used here, influence broodstock conditioning extending this work with a temperature component may allow cost effective diets to be identified that will produce spawning broodstock. A larger experiment manipulating temperature with supplemented feed will allow the rates of gametogenesis to be modelled as a function of temperature; this will effectively allow timing of broodstock conditioning to be controlled.

9.3 Vibriosis and hatchery disease

The studies here and elsewhere indicate that the nature of the rearing systems combined with animal husbandry are likely the over-riding drivers of changes in the relative and total abundance of the bacterial and the total *Vibrio* community. Given the substantial effort cost and logistic difficulties of sampling major mortality events in a commercial hatchery environment, future hatchery Vibriosis research may be more efficiently carried by deliberate experimental manipulation of rearing systems. This would provide a way to establish microbial community responses to particular changes in husbandry practice (e.g. microbial quality of algal food, feeding rates, water exchange rates, stocking density etc) and other factors that contribute to the

emergence of pathogenic microbial communities and high larval/spat mortality at the critical stages of juvenile production.

While small lab-scale experimental systems have a place in identifying and testing hypotheses, recent studies (e.g. Chapman 2012) suggest that the behaviour and responses of both the stock (mussels, oysters etc) and microbial communities are governed to some degree by changes in scale/volume and the rearing systems. Future research would be enhanced considerably by combination of lab and commercial scale experiments. Commercial-scale experiments based in a research hatchery would allow experiments without production-related constraints and run-to-run variation when experiments are reliant on commercial hatchery production for research material.

Further development of molecular approaches to group-specific detection and quantification of *Vibrio* in marine hatchery culture systems would also benefit from combining laboratory-based and research hatchery approaches. Given recent advances identifying the primary role of metalloproteases as virulence factors for *Vibrio* to shellfish, the combination of group-specific detection (particularly of *V. splendidus* group) in combination with quantification of metalloprotease activity (and/or gene expression), would provide a powerful tool for improving knowledge of the relationship between *Vibrio* community diversity/abundance and total community virulence to mussel larvae. Such knowledge would also be directly relevant and transferable to hatchery culture of other invertebrate and vertebrate aquaculture species.

Of particular, importance for mussel production is to further examine changes in the structure and dominance of *Vibrio* and total microbial communities during key biological and operational stages in the production cycle. In particularly the settlement substrate harbours a significant microbial community, and the rope conditioning and settlement process induces a major shift in the *Vibrio* dominance at a time when the animals may be physiologically compromised.

9.4 Live algal foods

This project addressed the major barriers to industry uptake of the four new food species (reliability and safety), Hatcheries are entirely reliant on the nutritional and microbial quality and the algal food and given the financial and operational disruption of larval rearing failures, hatcheries are understandably conservative when it comes to modifying successful procedures and protocols (“if it isn’t broke, don’t fix it”). For these reasons, hatchery uptake of new food species for production has traditionally been limited over the past 30 years. Additional larval feeding trials may encourage the industry to integrate the new species into their feeding protocols. In particular, experiments to determine the optimal point and level of dietary replacement with *Chaetoceros* sp. CS-365 may assist industry confidence of hatcheries to integrate these strains into their larval feeding protocols. However, it is instructive to note that similar studies have been carried out for *T. pseudonana* CS173 (=strain 3H) (Pettersen et al. 2010), but this research has not led to uptake of this strain for juvenile mussel production, or by hatcheries serving other sectors.

Further purification of the four algal strains could be undertaken and this may improve industry acceptance of the purified strains. However, outcomes of such attempts are unpredictable and likely to result in no net benefit in the quality of the strains as a food source. The data in this report suggest that further reduction/removal of the microbial community may instead reduce growth performance, nutritional quality and/or culture stability to the point that they are no longer useful or productive in hatchery systems.

10. PLANNED OUTCOMES

At the time the funding was granted for this project, the majority of mussel production was reliant on either natural spat fall or single hatcheries based in Victoria and SBS hatchery in Tasmania. Larval production was plagued by unpredictable and high mortality and disease at critical points in the juvenile production cycle. Addressing these issues aimed to provide seasonally independent production of quality-assured, hatchery-produced spat and ultimately, through better control of the reproductive cycle, allow the mussel industry to pursue benefits of selective breeding and triploid production.

Firstly, the project has led to improved knowledge of key factors controlling broodstock conditioning, and distribution and estimation of mussel larval/spat retention. The findings have led to a set of recommendations for incorporation into mussel hatchery standard operating procedures:

Recommendations for estimation of mussel spat density and survival on 500m dropper spools. Existing spat estimation protocols suffer from being unable to accurately estimate spat density on dropper ropes due to high spatial variance in the tank environment, and the potential mobility of mussel spat after settlement. The study successfully determined optimal dropper line sampling for reliable estimates of spat density. The sampling method results in precise mean estimates (precision of approx. 0.6) with commercially acceptable 95% confidence intervals. The key recommendations for spool sampling procedure are:

1. Rope sub-samples of 6 cm length should be used to estimate spat number on settlement spools.
2. A total of 8 random replicates from non-adjacent sections of the dropper rope.
    - 4 replicates from the top, outer wind of rope spool.
    - 4 four replicates at random positions on the rest of the spool. Exact position may be determined by the logistics of accessing the rope on the spool.

Recommendations for broodstock conditioning of female mussels. This project examined the dietary aspects of the broodstock conditioning, with a focus on replacing living microalgal diets with non-living diets. Partial or complement replacement of the live diets is possible, however, at ambient temperature (10-15^{o}C) none of the trialled diets allowed sufficient females to achieve gametogenesis and spawning condition within an eight week period and were insufficient for spawning at a commercial scale. However, replacement diets are logistically and economically feasible if conditioning is carried out at cooler temperatures that allow egg sufficient egg development to occur. The broodstock conditioning recommendations are explicitly for females brought into the hatchery in the spring and spawned. A second spawning can be achieved in 9-12 weeks by implementing the following:

1. Females should be held at 7-10^{o}C in a temperature-controlled recirculating seawater system.
    - Increasing the water temperature slows the rate of gonadal growth and egg development, and can be used to slow the rate of conditioning

- Conditioning temperature should remain below 16^{o}C.

2. A non-living commercial diet can be used to supplement or replace living microalgae.
    - Instant Algae Shellfish Diet 1800® (Reed Mariculture) at any level of replacement of live algal food is useful to maximise glycogen accumulation and gonadal growth at 11.5-15^{o}C.

A second output is improved understanding of *Vibrio* diversity and dynamics associated with mussel hatchery operational environments. Cultivable *Vibrio* are a normal member of the bacterial community associated with adults, larvae, spat and hatchery rearing systems throughout juvenile mussel production, and do not cause significant mortality or disease when present at the benchmark concentrations detailed in this study. The quantitative operational benchmarks established can be used to assess risk associated with changes in husbandry practice and guide improvements in operational hygiene in hatcheries. The project also established a suitable molecular target (the atpA gene) development of a rapid detection method for hatchery *Vibrio* in shellfish hatcheries.

A third output is the availability of four new algal strains with known growth performance and nutritional characteristics that are now available for uptake by mussel hatcheries and other marine hatcheries. At least two of the strains (*Chaetoceros* sp. CS-365 strains) appear to be a close nutritional and size match to act as partial or complete replacements for the problematic *Chaetoceros calcitrans* CS-178, which performs poorly or unreliably in standard hatchery culture systems.

The outputs of the research and recommendations for hatchery operations have been communicated to Spring Bay Seafoods as well as other research and industry mussel sector stakeholders through interim milestone and progress reports. Key aspects of the research findings are also being prepared for publication in scientific reviewed journals to disseminate key findings amongst the scientific community.

11. CONCLUSIONS

Suitable live algal and non-living powdered broodstock diets were explored to condition stock over the summer months, when reproductive condition is less synchronised and reliable. There was little evidence that diet influence the accumulation of glycogen stores, supporting the model for blue mussel that the second spawning even over the summer months is not fuelled by glycogen reserves, but directly from ingested food. The binary diet of *Isochrysis* sp. + *Pavlova lutheri* allowed females to become into spawning condition and produce larger eggs than other living diet combinations. Part or complete replacement of the living algal diet with non-living algal diet formulations improved accumulation of glycogen reserves and gonad growth. Instant Algae Shellfish Diet 1800® (Reed Mariculture) performed well as a non-living algae option to partially replace or supplement the live algal diet.

Settlement of larvae onto 500m continuous dropper lines, as practiced at SBS hatchery, has suffered from extreme patchiness leading to problems and stock loss after transfer the sea. While up to 50% of pediveligers successfully settled and 44% survived for 16 days on dropper line, as much as 40% of spat were unable to be accounted either due to not being fully competent or delayed settlement, or due to detaching and being lost to production. The distribution and size of spat varied throughout the tank with spat in the top half a meter being three times greater than in the lower part of the tank; spat were also larger near the top and outer part of the spools. A sampling approach was developed that would allow reliable estimates of blue mussel spat density settled on lines prior to deployment at the sea-based nursery. Eight replicate short lengths (6 cm) of the dropper line were recommended, with increased replication on the outside and top of the spool- where density and variation was highest.

The disease known as bacillary necrosis caused primarily by the marine bacteria of the genus *Vibrio*, are a source of unexplained and often catastrophic stock loss in larval and spat nursery phases. Molecular and selective culture methods were used to characterise the number and diversity of cultivable *Vibrio* associated with larval and spat phases of juvenile production. The number of *Vibrio* associated with mussel hatchery culture were similar to that encountered in other marine hatchery species and rearing environments, indicating that husbandry practices and rearing system design, water treatment, and stock and food densities are a significant controlling factor for bacterial and *Vibrio* abundance via the concentration, distribution and quality of organic carbon used for bacterial growth. Substantial populations (up to 10^5-10^6 cell ml^{-1}) were present at all stages of production, even when stock mortality was within normal commercial production limits, indicating that development of bacillary necrosis involves changes in relative dominance and diversity rather than simply increased *Vibrio* abundance.

DNA sequence studies showed that cultivable hatchery *Vibrio* were overwhelmingly (98%) dominated by *Vibrio splendidus* group bacteria. The communities were highly diverse (31 genotypes; 6 distinct clusters), with each rearing stage displaying distinctly mixtures of *V. splendidus* types. Different *V. splendidius* group genotypes showed significant differences (1.5 orders of magnitude) in virulence (LD_{50}; 24h, 48h) to larvae with the most virulent strain tested

associated with unfiltered hatchery intake seawater. Strains associated with healthy larvae rearing systems were less virulent suggesting that unfiltered and/or poorly treated intake water presents a higher disease risk to larvae. Mixtures of isolates showed synergistic effects indicating that community interactions also influence virulence to larvae. A diagnostic PCR approach was also developed to specifically detect *V. splendidus* group that could be form the basis of a rapid detection and quantification method for potentially pathogenic *V. splendidus* bacteria in marine hatcheries. The findings of the study indicate that water management and husbandry methods directly influence diversity and dominance of the *Vibrio* communities probably via the level/type of organic material enrichment associated with each stage of mussel rearing process. The study also demonstrates that development of bacillary necrosis is a complex interactive phenomenon that is not easily described or predicted using approaches that concentrate on total bacteria or total *Vibrio* load in rearing systems. Future studies should aim to determine common husbandry-related factors and mechanisms that promote pathogenesis/virulence and may benefit from better understanding of and detection of genes/proteins associated with virulence/pathogenesis of *V. splendidus* group bacteria. Future research would also be enhanced considerably by combination of lab and commercial scale experiments in a research hatchery, allowing experiments without production-related constraints and inevitable run-to-run variation inherent in commercial hatchery production.

Live food (microalgae) production for early stage (2-7 days) is heavily reliant on continuous and reliable production of microalgal strains of suitable size, nutritional profile and digestibility for successful growth and survival. For mussels, the early larval phase production relies heavily on a single diatom strain *Chaetoceros calcitrans* CS-178 whose growth in hatchery culture systems is unreliable. Four additional purified strains were developed and extensively tested in the most hatchery-scale algal culture systems. Lipid profiles of two diatom strains, *Chaetoceros* sp. CS-365/01 and CS-365/02 were found to be very similar to *C. calcitrans* CS-178 and growth performance was also similar to that of other algal strains commonly used as live foods in mussel hatcheries. Feeding studies showed that the two strains are ingested by readily by larvae as young as 7 days without significant mortality indicating that the four strains could be safely fed to mussel larvae. All four purified strains were also lodged with Australian National Algal culture Collection (ANACC, CSIRO Fisheries and Atmospheric Research) and are available for adoption by the hatchery industry.

The project has provided techniques, methods and knowledge to address critical hatchery production bottlenecks associated with broodstock management, high and unpredictable larval and spat mortality, and suitable and reliable live feeds for both the larval and spat phases of mussel production. The outcome of the research assists the future commercial viability of hatchery-based mussel production, allowing the industry to move from environmentally unsustainable reliance on collecting wild spat and realize the market potential of Australian mussels.

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13. APPENDICES

13.1 Appendix 1: Intellectual Property

The research contained is this report is for the public domain. The report, data and any resulting manuscripts are intended for wide dissemination. Several cultures suitable for use as live bivalve larval food were generated during the project. These cultures have been made available to the hatchery industry and research community through deposition with the Australian National Algal Culture Collection, CSIRO Marine and Atmospheric Research, Hobart, Tasmania.

13.2 Appendix 2: Staff and agencies that contributed to the project.

Name	Organisation	Funding
Christopher Bolch	University of Tasmania	FRDC and in-kind
Natalie Moltschaniwskyj	University of Tasmania	FRDC and in-kind
Jeremy Carson	DPIPWE, Tasmania	in-kind
Angela Williams	University of Tasmania	FRDC
Gaelle Salini	University of Tasmania	FRDC
Jo-Ann Fearman	University of Tasmania	FRDC , in-kind and Spring Bay Seafoods
Kwan Tzu Nin	University of Tasmania	FRDC and in-kind
Aqil Azizi	University of Tasmania	FRDC and in-kind
Vincent Ong	University of Tasmania	FRDC and in-kind
Phillip Lamb	Spring Bay Seafoods	In-kind
Ian Duthie	Spring Bay Seafoods	In-kind
Sereena Ashlin	Spring Bay Seafoods	In-kind

13.3 Appendix 3: Lipid profile of *Chaetoceros* sp. CS-365/01 and *Chaetoceros* sp. CS-365/02

	Proportion (%)	
Fatty Acid	***Chaetoceros* 365/01**	***Chaetoceros* 365/02**
12:0	0.1	0.2
14:0	12.1	14.9
15:0	1.1	1.1
16:0	14.9	14.1
17:0	0.1	0.1
18:0	0.7	1.0
22:0	0.1	0.0
Sum of saturated FA	**29.1**	**31.0**
14:1	0.2	0.4
15:1	0.2	0.1
16:1	22.0	18.7
16:1	0.7	0.7
18:1n9	1.4	1.8
18:1n7	3.3	2.9
Sum of monounsaturated FA	**27.9**	**24.6**
16:2	5.8	6.9
16:3	11.7	13.7
18:2n6	0.7	0.5
18:3n6	2.3	1.8
18:4n6	0.5	1.0
18:4n3	2.7	3.4
18:5n3	1.3	0.8
20:4n6	1.4	0.5
20:5n3	13.7	12.6
22:6n3	0.5	0.4
Sum Polyunsaturated	**40.6**	**41.5**
Other	**2.5**	**2.9**
Total	**100.0**	**100.0**

Note: Some double bond isomer positions remain unassigned. Assignment of polyunsaturated n3/n6 double bond designations are based on compound retention time.

13.4 Appendix 4: Details of purified algal cultures lodged with the Australian National Algal Culture Collection.

Species	ANACC Acc. No.	Strain code in report	Details
Thalassiosira pseudonana	CS-20/02	T.ps.20 C5x2 D6 LA	Re-isolated from CS-20 by C. Bolch & G. Salini; lodged 18/9/12
Thalassiosira pseudonana	CS-20/03	T.ps.20 C5x2 D6 PPC	Re-isolated from CS-20 by C. Bolch & G. Salini ; lodged 18/9/12
Thalassiosira pseudonana	CS-173/02	T.ps.173 C5x2 D6 PPA	Re-isolated from CS-173 by C. Bolch & G. Salini; lodged 18/9/12
Chaetoceros sp.	CS-365/03	365/01 C5x2 D6 LA	Re-isolated from CS-365/01 by C. Bolch & G. Salini; lodged 18/9/12
Chaetoceros sp.	CS-365/04	365/02 C4x2 D6 PPA	Re-isolated from CS-365/02 by C. Bolch & G. Salini; lodged 18/9/12
Chaetoceros sp.	CS-365/05	365/02 C4x2 D6 PPB	Re-isolated from CS-365/02 by C. Bolch & G. Salini; lodged 18/9/12