Probiotic bacteria for hatchery production of Greenshell™ mussels, *Perna canaliculus*

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Thesis submitted for the Degree of Doctor of Philosophy

April 2009

Certificate of Authorship / Originality

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

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ACKNOWLEDGMENTS

During the course of my research, numerous people have helped me in various ways. Initial mention should be made to my supervisors, **Dr. Heinrich (Henry) Kaspar** and **Dr. Lewis Gibson**. Henry allowed me freedom to explore my ideas and manage my time, yet maintained a constant eye on my progress which ensured things were moving forward throughout my research. He also supported my growth as a speaker at various conferences and supported my position at the Glenhaven Aquaculture Centre (GACL) where space and resources were often in demand by associate researchers. Lewis played the long-distance role of a Sydney-based supervisor in a New Zealand-based PhD. Although this was less than ideal, it was unavoidable. Nevertheless, Lewis's input on aspects of microbiology was valuable during his visits to New Zealand. My ability as a writer of scientific articles has improved also as a result of his help.

The ongoing advice and help from **Dr. Maria João (Josie) Lategan** cannot be thanked enough. From our first meeting at an aquaculture conference in Sydney, Josie was a friendly and helpful figure throughout my research. She provided advice on many ideas and greatly helped my development as a researcher of probiotic bacteria. I truly value her contribution to my thesis.

During the initial stages, **Dr. Rodney Roberts** provided advice on the design of experiments which helped in the developmental stages. **Nick King** was a friendly associate who helped in many aspects of mussel rearing and setting up experiments in the hatchery. Additionally, **Ellie Watts**, **Jonathan Morrish**, **Dr. Norman Ragg** and **Nicky Roughton** helped during certain aspects of my hatchery experiments. **Dan McCall** and **Andy Elliot** are thanked for constantly supplying me with mussel larvae. For certain repairs to equipment, **Henk Beek**, **Phil Spencer** and **David Read** are all thanked. In addition to the aforementioned people at the hatchery, I would like to thank the other **GACL staff** for providing a great environment to work in, which was always a nice change to laboratory work.

Dr. Andrew Fidler is thanked for his help during the PCR work and GenBank familiarisation. **Dr. David Harte** was most helpful in sequencing of the 16S rRNA. **Dr. Steve Webb** is thanked for his help with the histopathology and **Rasma Vilkens** is thanked also for processing of the histological samples. A thank you goes to **Kirsty Smith** for teaching me how to construct phylogenetic trees using MrBayes.

Ron Fyfe is thanked for his help with many microbiology questions during the early stages. Additionally, staff in the **Microbiology Department** at Cawthron Institute are thanked for putting up with my using some of their equipment, sharing the autoclave and washing my dishes.

Throughout my research I have had too many flatmates to recall. All provided me with a world away from science which was valuable.

This thesis was supported by Foundation for Research Science & Technology (FRST, New Zealand) contract CAWX0303.

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LIST OF ABBREVIATIONS

AB	Antibiotic
AD	Antimicrobial drug
ARB	Antibiotic resistant bacteria
ATCC	American Type Culture Collection
BLIS	Bacteriocin-like inhibitory substance
CFU	Colony forming unit
DO	Dissolved oxygen
EDTA	Ethylenediaminetetraacetic acid
ETOH	Ethanol
EU	European Union
GACL	Glenhaven Aquaculture Centre Limited
GIT	Gastrointestinal tract
GSM	Greenshell™ mussel
H+E	Haematoxylin eosin
LAB	Lactic acid bacteria
MB	Marine broth
MIC	Minimum inhibitory concentration
TSA	Tryptone soy agar
TSB	Tryptone soy broth
MEPD	Minimum effective pathogenic dose
MH	Mueller-Hinton
NZ	New Zealand
ONPG	*o*-Nitrophenyl-β-D-galactopyranoside
R & D	Research and development
RDP	Ribosomal Database Project II
TCBS	Thiosulphate citrate bile sucrose
TCD	Tissue culture dish
US	United States of America
UV	Ultraviolet
vs	Versus

ABSTRACT

The Greenshell™ mussel (GSM), *Perna canaliculus*, industry in New Zealand (NZ) is the largest aquaculture sector in the country. In 2006, the export earnings were valued at US$145 million which represented 65% of NZ aquaculture earnings. Historically, and at present, GSM production involves the capture of wild mussels on ropes followed by on-growing of these animals to market size (approximately 14 months). However, hatchery production of GSM has been developed in recent years. Hatchery production will alleviate the seasonal uncertainties of current techniques and allow the benefits of selective breeding programs. To date, efforts to produce commercial quantities of GSM in hatcheries have been hampered by unreliable larval rearing. These problems were often alleviated by antibiotic use, which implied bacterial pathogens as the cause. Yet, the ongoing use of antibiotics is not sustainable because of increasing legislative restrictions on their use and the possible emergence of antibiotic resistant bacteria. Hence, the identification and use of novel probiotics was investigated as an alternative.

Because of a lack of previous work, it was necessary to investigate the bacterial pathogenesis of GSM larvae in the initial stages and, hence, to determine the cause of disease against which the probiotics would be active. Twenty-two bacterial strains, isolated from compromised larvae, were screened for larval toxicity using a larval bioassay. Two strains were identified as potential pathogens. Sequencing of the 16S rRNA gene identified *Vibrio splendidus* and *Vibrio* sp. DO1, a *Vibrio coralliilyticus/neptunius*-like isolate, as pathogens of GSM larvae. These strains had the

ability to cause 83 and 75% GSM larval mortality *in vitro* respectively, at a concentration 10^2 CFU ml^{-1}. Histopathology indicated the route of infection was via the digestive system. Using healthy larvae as target hosts, Koch's postulates were confirmed for the two isolates.

Although two bacterial pathogens were identified, the successful design and implementation of protective measures in the hatchery still required an understanding of the dynamics of the infection process. Developing an *in situ* experimental model for infection was therefore paramount. The minimum effective pathogenic dose (MEPD) of *V. splendidus* (10^5 CFU ml^{-1}) and *Vibrio* sp. DO1 (10^6 CFU ml^{-1}) was demonstrated for GSM larvae during hatchery production. In a flow-through water hatchery system, larvae given 1-2 hours of static water exposure with these pathogen doses, after which flow-through processes resumed, averaged 58% and 69% cumulative mortality, respectively, on the fourth day following pathogen exposure. Larvae exposed to a dosage one order of magnitude greater than the MEPD, had higher mortalities of 73% and 96% for *V. splendidus* and *Vibrio* sp. DO1 respectively. These four levels of mortality were significantly greater than those of the non-exposed control larvae, averaging 23% in the experiments involving *V. splendidus* and 35% with *Vibrio* sp. DO1. Experiments were repeated four times to establish reproducibility. The infection models were reproducible and provided a tool to assess measures for the protection of GSM larvae against infection in the hatchery environment.

A bioassay was developed to screen and select bacterial strains as potential probiotics for GSM larvae. Sixty-nine isolates originating from a GSM hatchery environment were tested for probiotic activity in larval pathogen-challenge bioassays conducted in tissue culture dishes (TCDs). *Vibrio* sp. DO1 and *V. splendidus* were the tested pathogens. Forty of the tested isolates afforded larval survival significantly greater than pathogen controls ($p < 0.05$). The bioassay technique achieved a 58% success rate in searching for putative probiotics and highlighted the benefit of including the host animal in the first stage of the screening procedure. The time of inoculation of putative probiotic strains prior to pathogen challenge influenced the outcome of the assay. A pre-exposure period of 20 hours revealed a greater number of potential probiotics than a two-hour pre-exposure period. Pilot challenge tests, under normal hatchery conditions, confirmed the usefulness of the TCD screening method in recognising effective probiotics.

Following hatchery pilot trials, two probiotic strains were chosen for further study, namely strains 0444 and 0536. Sequencing of the 16S rRNA gene and phylogenetic analysis identified the strains as *Alteromonas macleodii* 0444 and *Neptunomonas* sp. 0536. Both probiotics were evaluated separately in a GSM hatchery facility during routine larval rearing and when the larvae were challenged with a high and low pathogenic dose of *Vibrio* sp. DO1 and *V. splendidus*. In all experiments, probiotic application significantly improved larval survival, if administered prior to pathogen exposure. Across all experiments, larvae that were exposed to the high and low dosages of pathogens averaged 14% and 36% survival respectively on the fourth day following pathogen exposure. If the probiotics were administered prior to pathogen challenge, larval

survival averaged 50% and 66% respectively. Non-inoculated control larvae and larvae administered the probiotic alone demonstrated 67% and 79% survival respectively. In a repeat experiment, these benefits were reproduced, with the exception of *A. macleodii* 0444 trialled against *V. splendidus*. *Neptunomonas* sp. 0536 appeared to suppress naturally occurring vibrios in the culture environment of healthy GSM larvae. This was the first time *A. macleodii* and *Neptunomonas* sp. were demonstrated as probiotic bacteria.

Many studies document probiotic application in aquaculture under conditions of pathogen attack, yet few describe the use of probiotics during routine production. The effects of administering the probiotic, *A. macleodii* 0444, during routine GSM larvae production, were compared against larvae from the same cohort that were not treated with the probiotic. The probiotic was administered daily for the first 11 days of the larval period and was provided at two concentrations, 10^7 CFU ml^{-1} and 10^8 CFU ml^{-1}. Measures of larval swimming activity, gut colouration, lipid levels, larval survival, larval size and settlement success were recorded. There were minimal differences in all parameters between larvae provided the probiotic and control larvae. Probiotic treated larvae consumed more food and had higher lipid levels at the end of the larval period, but these were not statistically significant. All treatments completed the larval phase and settled successfully after metamorphosis. Survival at the end of the larval period was 37.2%, 38.8%, and 34.8% for control, 10^7 CFU ml^{-1} and 10^8 CFU ml^{-1} treatments respectively. The probiotic was still detected in larvae seven days after the final addition to the tanks.

Animals were further grown in the field at a commercial farm. The probiotic was not detected in mussels at four months after leaving the hatchery.

Combination use of the two probiotics, *A. macleodii* 0444 and *Neptunomonas* sp. 0536, was investigated to determine whether additive protection against pathogen attack with *Vibrio* sp. DO1 and *V. splendidus* was afforded to GSM larvae. The effects of combination administration were compared with larvae administered each probiotic as single strains and non-inoculated larvae. Additionally, two concentrations were tested for each probiotic, both singly and in combination, 10^7 and 10^8 CFU ml^{-1}. Larvae were administered probiotics daily for the first six days, challenged with pathogens on the third day and then reared until settlement (day 19). Although protection against pathogen attack was observed in combination treatments, when compared with single-strain administration, additive protection was not apparent. Administration of 10^8 CFU ml^{-1} levels of probiotics, both singly and in combination, afforded larval survival slightly better than 10^7 CFU ml^{-1} levels, although this was rarely statistically significant. On the other hand, the higher levels of probiotic led to smaller larvae and lower feed rates for the majority of the 19-day trial. At the end of the study, larval sizes were smaller in the treatment applied a combination of probiotics at 10^8 CFU ml^{-1} than those of the other treatments. Additionally, towards the end of the larval period, feed consumption in the combination 10^8 CFU ml^{-1} treatment was similar to that witnessed in the other probiotic treatments one day previously. This suggested that either the larvae were compromised or they were growing slower. Despite a lack of additive protection against a single strain pathogen attack being demonstrated, the potential benefit of multi-strain probiotics, as

prophylactic measures against every-day microbial encounters in larviculture, would remain. Although 10^8 CFU ml^{-1} levels appeared to protect against pathogen attack slightly better, they were also potentially detrimental to normal larval rearing when administered in combination. Following the successful completion of the larval period and pathogen protection afforded with a combination of probiotics at 10^7 CFU ml^{-1}, this level was recommended as the best concentration of each probiotic where combination administration would be applied.

The work presented in this thesis supports the use of *A. macleodii* 0444 and *Neptunomonas* sp. 0536 in the routine rearing of GSM larvae. The ability to produce settled juvenile mussels, equal in numbers to those produced in normal healthy conditions, plus the benefits against pathogen attack led to the recommendation of their use on a routine prophylactic basis in GSM larval rearing. Their use for this purpose is intended in the near future. A provisional patent has been prepared and will be submitted shortly. It is anticipated that future work will continue with these probiotic strains to determine their potential benefit for other aquaculture species.

Chapter 1

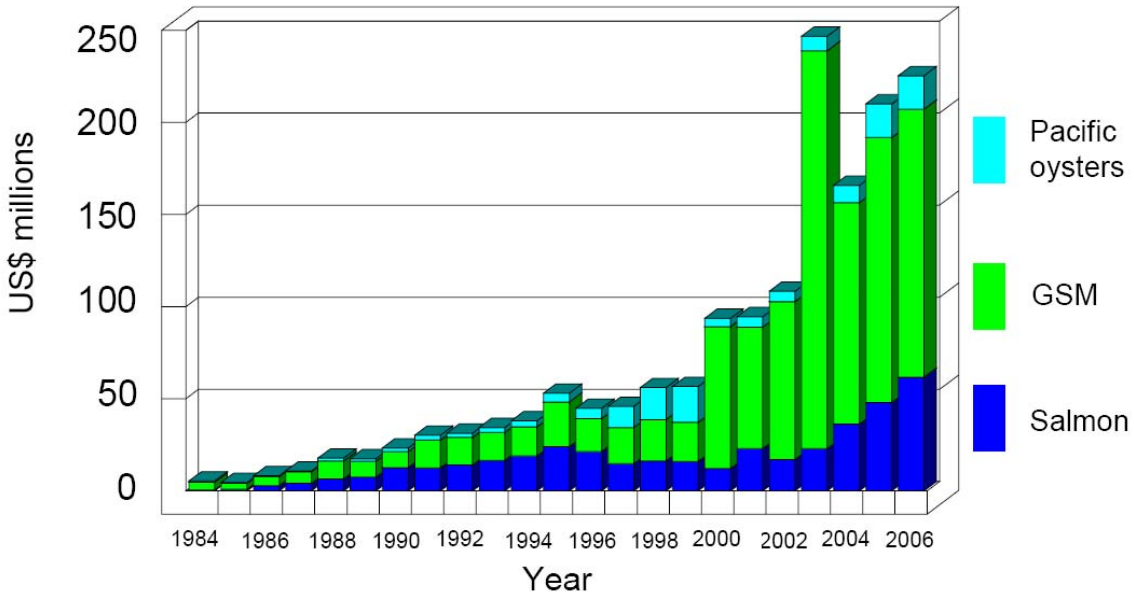
Literature Review

1.1 Introduction

Mussel farming in New Zealand (NZ) is a massive business. In particular, the main species, *Perna canaliculus*, has gained so much attention that its common name has been trademarked as Greenshell™ mussel (GSM). The GSM is native to NZ, has an aesthetic appeal to the eye (Fig. 1.1), is large and fleshy, grows in water deeper than that in which most other mussel species grow and reaches market size in 14 to 18 months (Fisher *et al*., 1993). Interest in cultivation of GSM has increased greatly in recent decades; production levels reached were 1,000 tonnes in 1970, 1,570 t in 1980, 10,860 t in 1985, 24,000 t in 1990, and 97,000 t in 2006 (FAO, 2007). GSM production figures for 2006 added US$145 million to the export earnings of NZ, representing 65% of NZ aquaculture earnings (Fig. 1.2).

Figure 1.1 Greenshell™ mussel, *Perna canaliculus*

Figure 1.2 Aquaculture production in New Zealand between 1984-2006.



The current practice of farming GSM would be viewed as extensive, since it involves gathering naturally spawned juveniles (spat) from the wild, hanging them in lines, and harvesting after approximately 14 months. However, collecting spat from the wild relies on environmental parameters and consequently the location and timing of mussel spat is often unpredictable and inconsistent (Jeffs *et al*., 1999). These limitations represent a risk to the farmer and industry and undermine assurance in expansion of GSM farming in NZ.

Previously, the life cycle of GSM was closed (Buchanan, 1998), i.e. mussels can be artificially induced to spawn in captivity and spat can be produced from these gametes. Although hatchery production of GSM is still in the R & D stage, the application of such techniques would alleviate the reliance on wild spat, ensure a constant supply of animals and increase production numbers. Furthermore, in combination with certain methods, such as selective breeding and cryopreservation, controlled spawning would improve

fitness, growth traits and other desirable properties of GSM in order to obtain a faster-growing, superior animal (Adams *et al*., 2004; King *et al*., 2004; King, 2005). Effectively, this technology could be worth hundreds of millions of dollars to NZ by facilitating another step-up in production figures, as was seen in the 80s and 90s (Fig. 1.2).

As with most larviculture, the practice of hatchery producing GSM larvae involves the intensive conditions of high stocking densities, high organic loads and a greater potential for opportunistic pathogens. A review by Olafsen (2001) discussed the intricate relationship that exists between bacteria and aquatic larvae in aquaculture. At present, small-scale hatchery practices for GSM rely on the occasional use of antibiotics at certain critical periods of the larval development. Without this treatment, larvae suffer extensive mortality, suggesting bacterial pathogenesis as a cause of the problems.

In aquaculture, diseases caused by *Vibrio* spp. and *Aeromonas* spp. are commonly implicated in episodes of mortality. When faced with disease problems, the common response has been to turn to antimicrobial drugs (hereafter referred to as ADs). The livestock and aquaculture industries have experienced widespread use of ADs in their practices. While the use of such products has an obvious benefit for the treatment of animals infected by bacterial disease, the use of ADs has been either prophylactic (preventative), or for growth enhancement (Van den Bogaard & Stobberingh, 2000). Certain ADs have a positive influence on the growth of livestock and, hence, have been used widely (Acar *et al*., 2000; Witte, 2000; Wierup, 2001; Phillips *et al*., 2004). Given

this, and the desire to prevent the establishment of pathogenic bacteria, it is argued that ADs have been widely overused (Aarestrup, 1999; Schwarz *et al*., 2001). Schwarz *et al*. (2001) provided a good overview of AD use in animals and the potential hazards associated with this.

The use of ADs in agriculture and aquaculture has led to the emergence of antibiotic resistant bacteria (hereafter referred to as ARB) (Schwarz *et al*., 2001; Akinbowale *et al*., 2006). In aquaculture this was felt most dramatically in the shrimp industry when massive increases in production, overcrowding of animals and unchecked antibiotic usages led to the emergence of numerous ARB and production crashes in many Asian countries (Karunasagar *et al*., 1994; Moriarty, 1999). For example, production figures for shrimp in the Philippines dropped by 55% in two years; from 90,000 t to 41,000 t between 1995 and 1997. In fact, it has never recovered and, in 2006, a mere 40,000 t was produced. An industry previously worth US$760 million is now worth only $285 million (FAO, 2007). Similarly, Thai shrimp production dropped by 40% between 1994 and 1997 as a consequence of disease problems (Moriarty, 1999), involving bacterial pathogens and shrimp viruses. Within aquaculture, there are numerous reports of ARB of farm origin (Karunasagar *et al*., 1994; Son *et al*., 1997; Molina-Aja *et al*., 2002; Chelossi *et al*., 2003; Sahul Hameed *et al*., 2003; Alcaide *et al*., 2005).

However, the risk is not just the potential loss to the farmer. The emergence of ARB on aquaculture farms could pose a risk to human health. There are many reports illustrating the transferral of resistant genes between bacteria (Son *et al*., 1997; Aarestrup, 1999; Van

den Bogaard & Stobberingh, 2000; Witte, 2000; Schwarz *et al*., 2001). This process means ARB originating from a shrimp farm could potentially transfer plasmids conferring resistance to bacteria involved in human health problems. This is an area of current debate. Studies point to a farm animal origin in certain ARB genes that have made their way into bacteria associated with humans (Van den Bogaard & Stobberingh, 2000; Witte, 2000; Schwarz *et al*., 2001). However, other reports argue against this (Acar *et al*., 2000; Phillips *et al*., 2004). The argument is based on the view that, although ARB have arisen in animal husbandry through use of antimicrobials, there is insufficient data to show a linkage to resistant gene transfer into bacteria of human concern. They argue in favour of the beneficial role antibiotics play in farming and caution against premature, unscientific decisions in the restriction of antibiotic usage.

Regardless of which argument represents the true situation, governments and organizations have introduced much tighter restrictions for antibiotic usage in animal production. The European Union (EU) initially put a ban on the use of avoparcin in 1997, and in 1999, included virginiamycin, spiramcin, tylosin and bacitracin as banned growth promoters in animal feed (Turnidge, 2004; Delsol *et al*., 2005). In 2005, the EU implemented a ban on the use of all non-therapeutic antimicrobials in animal production (Delsol *et al*., 2005). The US has been less stringent. There was a proposal in 2000 to introduce a ban on the use of fluoroquinolone and there was concern also about the use of virginiamycin (Nawaz *et al*., 2001). More recently a bill called “Preservation of Antibiotics for Medical Treatment Act (PAMTA)” was presented in the US congress. If passed this act would see a ban on the non-therapeutic use of any drug intended for

human use, in the production of feed animals. This act would be enforced two years from the date of being passed (UCS, 2009).

Other countries which currently have less antibiotic control, such as many of the Asian countries, are likely to be pressured through foreign trade restrictions, via the import markets being tightly controlled for antibiotic-contaminated products. Despite chloramphenicol being banned in Thailand since 1999, as a result of worldwide concern over its use in animal production, trace levels were still being detected in shrimp from Thailand, causing a temporary ban by the EU for Thai shrimp (Heckman, 2004). Chloramphenicol has also been detected in shrimp from Myanmar, India, Pakistan and Vietnam, highlighting the continuing misuse of antibiotics in Asian shrimp farming.

A leading example in the eradication of antibiotic use can be seen in the Norwegian salmon industry. After concern about the use of antibiotics in the late 1980s, there was a 95% drop in usage from 50 tonnes to one tonne annually. During the same period, salmon production increased 10-fold from about 5500 tonnes to 55000 tonnes. Reasons for the turnaround have been attributed to the use of vaccines, better husbandry and selective breeding programs (Maroni, 2000).

There is a developing social attitude against the unnecessary use of ADs and, where possible, it is the move away from non-essential AD use that the responsible farmer now seeks. Given the threat that both ADs and bacterial pathogens pose to farmers, as well as

in human health, alternatives are being sought. The use of probiotics is one field commanding considerable attention.

1.2 Probiotics: definition and principles

The term, probiotic, simply means "for life", originating from the Greek words "pro" and "bios" (Gismondo *et al*., 1999). The most widely quoted definition was made by Fuller (1989). He defined a probiotic as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance". This definition is still widely referred to, despite continual contention with regard to the correct definition of the term. Current probiotic applications and scientific data on mechanisms of action indicate that non-viable microbial components act in a beneficial manner and this benefit is not limited just to the intestinal region (Salminen *et al*., 1999). Fuller's definition was a revision of the original probiotic concept which referred to protozoans producing substances that stimulated other protozoans (Lilly & Stillwell, 1965). Yet, although probiotics have been an area of much interest and research in the past 30 years, the original idea was possibly formed by Metchnikoff in the early 1900s. Metchnikoff (1907) theorized that human health could be aided through the ingestion of fermented milk products. The concept of probiotic activity has its origins in the knowledge that active modulation of the microbiota of the gastrointestinal tract (GIT) could confer antagonism against pathogens, help development of the immune system, provide nutritional benefits and assist the intestinal mucosal barrier (Vaughan *et al*., 2002).

Today probiotics are quite commonplace in health-promoting "functional foods" for humans, as well as therapeutic, prophylactic and growth supplements in animal production and human health (Mombelli & Gismondo, 2000; Ouwehand *et al*., 2002; Sullivan & Nord, 2002; Senok *et al*., 2005). Typically, the lactic acid bacteria (LAB) have been widely used and researched for human and terrestrial animal purposes. Moreover, LAB are known to be present in the intestine of healthy fish (Ringø & Gatesoupe, 1998; Hagi *et al*., 2004). Interest in LAB stemmed from the fact that they are natural residents of the human GIT with the ability to tolerate the acidic and bile environment of the intestinal tract. LAB also function to convert lactose into lactic acid, thereby reducing the pH in the GIT and naturally preventing colonization by many bacteria (Mombelli & Gismondo, 2000; Klewicki & Klewicka, 2004). The most widely researched and used LAB are the lactobacilli and bifidobacteria (Corcoran *et al*., 2004; Ross *et al*., 2005; Senok *et al*., 2005).

Other commonly studied probiotics include the spore forming *Bacillus* spp. and yeasts. *Bacillus* spp. possess adhesion abilities, produce bacteriocins (antimicrobial peptides) and provide immunostimulation (Cherif *et al*., 2001; Cladera-Olivera *et al*., 2004; Duc *et al*., 2004; Barbosa *et al*., 2005). *Bacillus* strains appear to be effective probiotics and commercial products containing such strains have been demonstrated to improve shrimp production to a level similar to that when antimicrobials are used (Decamp & Moriarty, 2006). The application of *Bacillus* spp. as probiotics holds added interest because they can be kept in the spore form and therefore stored indefinitely on the shelf (Hong *et al*., 2005). The yeast, *Saccharomyces cerevisiae,* also has been commonly studied whereby

immunostimulatory activity was demonstrated and production of inhibitory substances shown (Castagliuolo *et al*., 1999; Dahan *et al*., 2003; Van der Aa Kühle *et al*., 2005).

A number of mechanisms exist in which probiotics could be beneficial and these could act either singly or in combination for a single probiotic bacterium. These include: inhibition of a pathogen via production of antagonistic compounds, competition for attachment sites, competition for nutrients, alteration of enzymatic activity of pathogens, immunostimulatory functions and nutritional benefits such as improving feed digestibility and feed utilization (Fuller, 1989; Fooks *et al*., 1999; Bomba *et al*., 2002). It has been reported that a probiotic must be adherent and colonize within the GIT, it must replicate to high numbers, it must produce antimicrobial substances and it must withstand the acidic environment of the GIT (Ziemer & Gibson, 1998; Dunne *et al*., 1999; Gismondo *et al*., 1999; Mombelli & Gismondo, 2000). However, these descriptions are misleading. These beliefs are based on the understanding that a probiotic must become a permanent member of the intestinal flora. Whilst bacteria with this capacity are common and much probiotic research focused on attachment capacity of bacteria, it was demonstrated that transient bacteria can also exert beneficial effects (Isolauri *et al*., 2004). Additionally, contrary to the requisite of being able to attach to mucus and produce antimicrobial substances, a probiotic needs to possess only one mode of action. Multi-strain and multi-species probiotics have proven that it is possible to provide synergistic bacteria with complementary modes of action to enhance protection (Timmerman *et al*., 2004).

1.3 Probiotics in aquaculture

1.3.1 Extended definition

When looking at probiotics intended for an aquatic usage it is important to consider certain influencing factors that are fundamentally different from terrestrial based probiotics. Aquatic animals have a much closer relationship with their external environment. Potential pathogens are able to maintain themselves in the external environment of the animal (water) and proliferate independently of the host animal (Hansen & Olafsen, 1999; Verschuere *et al*., 2000a). These potential pathogens are taken up constantly by the animal through the processes of osmoregulation and feeding. A study with Atlantic halibut, *Hippoglossus hippoglossus*, showed the transition from a prevailing *Flavobacterium* spp. intestinal flora to an *Aeromonas* spp./*Vibrio* spp. dominant flora which occurred when first feeding commenced (Bergh *et al*., 1994). This study highlighted the impact that the external environment and feeding had on the microbial status of the fish. However, the same study also found that the larvae did maintain a specific intestinal flora different to that of the external tank flora. This showed that, although there were ever-present external environmental factors influencing the microbial flora inside an aquatic animal, they could still maintain a host specific flora at any given time. It was suggested that this ability did not apply to bivalve larvae (Jorquera *et al*., 2001). Their work demonstrated that the transit time of bacteria in bivalve larvae was too short to allow the establishment of a bacterial population different from that of the surrounding water.

Based on the intricate relationship an aquatic organism has with the external environment when compared with that of terrestrial animals, the definition of a probiotic for aquatic environments needs to be modified. Verschuere *et al*. (2000a) suggested the definition "a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment". Apart from the requirement of the probiotic to be a live culture, this definition is a somewhat lengthy way of describing a probiotic as defined by Irianto & Austin (2002a) thus "a probiotic is an entire or component(s) of a micro-organism that is beneficial to the health of the host". The latter definition is in accordance with that given by Salminen *et al*. (1999). The non-requirement of being a live culture would allow for certain suggested immunostimulants (Itami *et al*., 1998; Smith *et al*., 2003), which are bacterial derivatives, such as peptidoglycan and lipopolysaccharides, to be included as probiotics. Although there is some dispute about what an aquatic probiotic actually is, all definitions differ from that of Fuller (1989) in that there is no longer the requisite for the probiotic to be acting in the GIT. Therefore, modes of action such as competition for nutrients and production of inhibitory substances could occur in the culture water. Additional effects of probiotic action should also be considered, given the modified definition, including change of the water quality and interaction with phytoplankton (Verschuere *et al*., 2000a).

Phytoplankton need to be considered when dealing with aquatic probiotics for two reasons. The first is that microalgae are capable of producing substances toxic to bacteria

and could potentially act in a beneficial manner. For example, *Skeletonema costatum*, a common microalga used in mollusc and crustacean larviculture, produces an organic extract capable of inhibiting the growth of *Vibrio anguillarum* and three other vibrios (Naviner *et al*., 1999).

Perhaps of more importance is the consideration of what effect adding a probiotic bacterium will have upon phytoplankton. Microalgae are required for most larviculture in aquaculture and, in fact, certain bacteria can stimulate microalgal growth (Haines & Guillard, 1974; Ukeles & Bishop, 1975; Barker & Herson, 1978; Fukami *et al*., 1992; Suminto & Hirayama, 1996; Fukami *et al*., 1997; Suminto & Hirayama, 1997). Thus, probiotics could be specifically targeted for microalgae production; however, the subsequent effects of such bacteria on the larvae must be established. The more realistic approach would be to use probiotics aimed at improving the health of the larvae and then determine whether these bacteria had an effect upon the microalgae. It would be very desirable to discover a probiotic that benefited the larvae and was either beneficial to or did not impair the microalgae. Consequently, the bacteria could be co-cultured with the microalgae before entry into the larviculture system. This was done by Gomez-Gil *et al*. (2002) who found the shrimp probiotic, C7b, could be co-cultured with shrimp larvae food, *Chaetoceros muelleri*, without affecting the microalga. Similarly, Avendaño & Riquelme (1999) investigated the growth of seven bacterial strains with *Isochrysis galbana*. Four of these strains did not affect growth of the microalgae, while co-culture significantly improved ingestion of bacterium C33 by larval scallop, *Argopecten purpuratus*.

1.3.2 Modes of action

Several studies have demonstrated certain modes of probiotic action in effect in the aquatic environment. Bairagi *et al*. (2002) assessed aerobic bacteria associated with the GIT of nine freshwater fish. They determined that selected strains produced digestive enzymes, thus facilitating feed utilization and digestion. Ramirez & Dixon (2003) reported on the enzymatic properties of anaerobic intestinal bacteria isolated from three fish species, showing the potential role a probiotic could play. In another paper by Bairagi *et al*. (2004), the benefit of adding *B. subtilis* and *B. circulans* to the diet of rohu, *Labeo rohita,* was shown. In the search to replace fish meal with leaf meal in fish feed, they found that addition of the two fish intestinal *Bacillus* spp. increased performance as judged by a number of factors including growth, feed conversion ratio, and protein efficiency ratio. They attributed this to the extracellular cellulolytic and amylolytic enzyme production by the bacteria.

Although competition for adhesion sites has been widely suggested as a mode of action, there is little evidence in the literature to demonstrate this. There have been studies reporting adhesion of certain bacteria to intestinal mucus *in vitro*, but transferral of these to *in vivo* models did not produce supporting results (Hansen & Olafsen, 1999). Attachment ability of potential probiotics seen *in vitro* cannot be assumed to demonstrate the real effect *in vivo*. Additionally, whilst studies to date have demonstrated the ability of certain bacteria to adhere to intestinal mucus *in vitro* (Krovacek *et al*., 1987; Olsson *et al*., 1992; Garcia *et al*., 1997, Jöborn *et al*., 1997), they failed to assess a competitive exclusion effect. More recently, Vine *et al*. (2004a) demonstrated a competitive exclusion

effect when testing five probiotics versus two pathogens on fish intestinal mucus. They found that the presence of one of the probiotics on the mucus inhibited the attachment of one of the pathogens tested. Interestingly, pre-colonization with the other probiotics encouraged attachment of the two pathogens. However, in other tests, when mucus was pre-colonized with the pathogens, attachment of the probiotics was also enhanced. This suggests that conditions of the assay might have favoured attachment of any bacterium added second, regardless of attachment competition capabilities.

Although not directly concerning attachment competition, Yan *et al*. (2002) demonstrated that the production of antibiotic substances by two seaweed-associated *Bacillus* spp. was dependent on biofilm formation by the bacteria. This study highlighted a factor which might be important for some bacteria to be effective probiotics, i.e. surface attachment. This observation concurred with Fuller's (1989) definition of a probiotic, i.e. the requirement for GIT colonisation. It has been proposed that the mechanism of competitive exclusion for attachment sites could be aided via addition of probiotic bacteria during the initial egg fertilization step of larviculture, thereby "getting in there first" (Irianto & Austin, 2002a). This concept was not supported by Makridis *et al*. (2000) who found no difference between the concentrations of two bacteria in the gut of turbot larvae when these bacteria were administered at hatching and two days post-hatching.

Several studies have attributed a probiotic effect to competition for energy sources (Rico-Mora *et al*., 1998; Verschuere *et al*., 1999; Verschuere *et al*., 2000b). Beneficial growth and survival was found in *Artemia* sp. pre-exposed to nine strains of bacteria before

challenge with *V. proteolyticus* (Verschuere *et al*., 1999). It was concluded that the effect was not caused by extracellular products, but required the live bacterial cell. Although it was not specifically tested, they hypothesized that the protective effect probably resulted from competition for energy sources and for adhesion sites. Competition for iron has been reported as an important factor in marine bacteria (Verschuere *et al*., 2000a). Iron is needed by most bacteria for growth, but is generally limited in the tissues and body fluids of animals and is present in the insoluble ferric, Fe^{3+}, form (Verschuere *et al*., 2000a). Iron-binding agents, siderophores, allow acquisition of iron suitable for microbial growth. Siderophore production is a noted mechanism of virulence in some pathogens (Gram *et al*., 1999). Equally, a siderophore-producing probiotic could deprive potential pathogens of iron under iron limiting conditions. This was shown by Gram *et al*. (1999), who found that a culture supernatant of *Pseudomonas fluorescens*, grown in iron-limited conditions, inhibited growth of *V. anguillarum*, whereas the supernatant from iron-available cultures did not.

Itami *et al*. (1998) found that addition of *Bifidobacterium thermophilum*-derived peptidoglycan to kuruma shrimp increased significantly their survival when they were challenged with *V. penaeicida*. They attributed this to an immunostimulatory effect, as the phagocytic activity of shrimp granulocytes was significantly higher in the treated shrimp compared with those of the control animals. A study by Gullian *et al*. (2004) differed slightly in its approach of an immunostimulating probiotic. Rather than testing bacterial derivatives such as glycans or lipopolysaccharides, they tested immunostimulation by a live *Vibrio* sp. (P62) and *Bacillus* sp. (P64), using *V.*

alginolyticus as a positive control. They concluded that P64 and *V. alginolyticus* were immunostimulants. However, this conclusion was based on only two of the nine immunological parameters they presented showing significant differences between treatments, and stemmed from standardizing all parameters into one index for one statistical conclusion, i.e. immunostimulatory or not. A review by Smith *et al*. (2003) provided important information on the potential problems associated with immunostimulants in crustacean aquaculture. They argued that the prolonged use of immunostimulants was, in fact, detrimental to the host and that much more research was needed before their use during critical periods could be considered safe.

Possibly the most studied mode of probiotic action in aquatic animals is the production of inhibitory substances; this will be discussed in the next section.

1.3.3 Previous research and methodology

A summary of past research into aquaculture probiotics is given in Table 1.1, pages 22-28. It should be reiterated that, by definition, a probiotic need only be beneficial to the host and that this benefit could also be nutritional or a change in the host's immediate environment, namely tank water in the case of aquaculture. Yet, screening to date has concentrated on the search for probiotic bacteria active against a pathogen; perhaps because of the problems bacterial pathogens can cause in the aquaculture environment. In screening for potential probiotics, most of this research employed identification of inhibitory activity *in vitro* (Dopazo *et al*., 1988; Westerdahl *et al*., 1991; Sugita *et al*., 1996a; 1996b; Bly *et al*., 1997; Sugita *et al*., 1997a; 1997b; Sugita *et al*., 1998; Burgess

et al., 1999; Jorquera *et al*., 1999; Spanggaard *et al*., 2001; Chythanya *et al*., 2002; Sugita *et al*., 2002; Hjelm *et al*., 2004a; 2004b). Currently, there are four agar-based methods commonly employed to screen for inhibitory substances *in vitro*; the double layer method, the well diffusion method, the cross-streak method and the disc diffusion method. All methods are based on the principle that a bacterium (the producer) produces an extracellular substance which is inhibitory to itself or another bacterial strain (the indicator). The inhibitory activity is displayed by growth inhibition of the indicator in agar medium.

In some cases, initial *in vitro* screening was followed by small scale testing of shortlisted candidates *in vivo* for either pathogenicity to the host (Makridis *et al*., 2000; Chythanya *et al*., 2002; Hjelm *et al*., 2004a) or host protection when challenged with a pathogen (Rengpipat *et al*., 1998; Robertson *et al*., 2000; Gram *et al*., 2001; Irianto & Austin, 2002b; Lategan & Gibson, 2003; Vaseeharan *et al*., 2004; Lategan *et al*., 2004a; 2004b). Apart from the study by Gram *et al*. (2001), a positive protective effect was seen in all *in vivo* studies following positive antagonism assays *in vitro*. In other studies, *in vitro* shortlisted probiotics were tested further for properties such as bile resistance (Chabrillón *et al*., 2006), attachment capacity (Olsson *et al*., 1992; Hjelm *et al*., 2004a), immunostimulation (Gullian *et al*., 2004; Rengpipat *et al*., 2000; Irianto & Austin, 2003), competition for adhesion sites (Vine *et al*., 2004a; Chabrillón *et al*., 2006) and competition for nutrients (siderophore production) (Gram *et al*., 1999). In practice, these latter studies test whether or not a probiotic that produces diffusible inhibitory substances also possesses other modes of probiotic action.

Screening for production of an inhibitory substance *in vitro* and then taking likely candidates into the further testing stage limits the shortlist to those isolates which exhibit only one of the various modes of probiotic action, namely production of diffusible inhibitory substances. Although production of an inhibitory substance has been shown to work very well in probiotics and this screening method has identified very good probiotics in aquaculture (Irianto & Austin, 2002b; Lategan & Gibson, 2003; Vaseeharan *et al*., 2004; Lategan *et al*., 2004a; 2004b), there are two major limitations to this approach. The first is that other modes of probiotic activity (e.g. immunostimulation, digestive enzymes production, competition for attachment site, or nutrients) will not be expressed in the laboratory on an agar plate and, hence, a major source of potential beneficial action will be overlooked. The second drawback is that positive results *in vitro* fail to determine the real *in vivo* effect. This means that a bacterium which is antagonistic in the laboratory might not be inhibitory when associated with the animal in question. For example, using *P. fluorescens* strain AH2, a probiotic proven to be successful in protecting rainbow trout from *V. anguillarum*, Gram *et al*. (2001) found that this bacterium was also inhibitory to the salmon pathogen *A. salmonicida in vitro*. However, no protective effect was found when transferring the probiotic to an *in vivo* challenge experiment with salmon and this pathogen. The same effect was seen by Ruiz-Ponte *et al*. (1999), when *in vitro* antagonism was not able to protect scallop larvae challenged by a pathogen in an *in vivo* situation. Similarly, a bacterium which is not inhibitory in the laboratory might actually be antagonistic *in vivo*. This occurrence was shown in a study with rainbow trout where a commercial probiotic product, BioPlus2B, was assessed in rainbow trout challenged with *Yersinia ruckeri* (Raida *et al*., 2003). Although the product

was shown to enhance survival of the challenged fish, no inhibitory effect was found via *in vitro* antagonism assays.

The most likely reasons for the research approaches taken in the past are cost, ease of experimentation and availability of test animals and space. Setting up initial screening experiments involves very large numbers of tests in order to screen as many isolates as possible in the hope of obtaining good probiotics. In reality, setting up this phase of experimentation with a suitable number of animals per replicate, a sufficient number of replicates per treatment and screening even the modest number of 100 isolates, presents a huge demand for number of animals needed and also the space and resources to carry out these experiments *in vivo*. In view of this, it is not surprising that a laboratory component is added to the screening before challenging animals with a substantially reduced shortlist. Contrary to the *in vitro* approach of identifying probiotics, Makridis *et al.* (2005) adopted a direct *in vivo* approach. They isolated six bacteria from healthy cultures of gilthead sea bream larvae food, *Artemia* sp. and rotifers. They then tested these food-sourced bacteria with the sea bream larvae. They found that addition of the bacteria significantly improved larval survival. Similarly, search for *Artemia* sp. probiotics by Verschuere *et al*. (1999) implemented *in vivo* experiments using bacteria sourced from healthy *Artemia* sp. cultures. Based on growth and survival figures of these monoxenic cultures, nine out of eighteen strains tested were chosen for *in vivo* challenge experiments against *V. proteolyticus* CW8T2 (Verschuere *et al*., 2000b). All nine strains demonstrated a significant protective effect. The encouraging results from these two studies highlighted the benefit of including test animals at the initial stages of the screening process.

Another popular approach used for identifying aquaculture probiotics included testing of proven human and agricultural probiotics such as LAB and yeasts. The research approach consisted of either selecting and testing of LAB from the GIT of aquatic animals (Stoffels *et al*., 1992; Gildberg *et al*., 1995; 1997; Gildberg & Mikkelsen, 1998; Shiri Harzevili *et al*., 1998; Tovar *et al*., 2002; Bairagi *et al*., 2004), or using probiotics developed for terrestrial animals (Nikoskelainen *et al*., 2001a; 2001b; 2003; Lara-Flores *et al*., 2003; Patra & Mohamed, 2003; Panigrahi *et al*., 2004; 2005; Planas *et al*., 2004; Venkat *et al*., 2004; Aubin *et al*., 2005). Such research, therefore, limits the identification of novel probiotic bacteria. However, research of this type is definitely warranted as the evidence to date has shown LAB to be just as useful in aquatic animals as in terrestrial animals.

Table 1.1 Summary of research towards probiotics for aquaculture.

Animals tested	Potential probiotic	Pathogen tested or type of study conducted	Test method	Reference
	A. media	*Ed. tarda, V. anguillarum, Y. ruckeri, A. salmonicida, Lactococcus garvieae, Saprolegnia parasitica*	*in vitro*	Lategan *et al*., 2006
	Alt. haloplanktis	*V. anguillarum, V. alginolyticus, V. ordalii, A. hydrophila*	*in vitro*	Riquelme *et al*., 1996a
	Aerobic bacteria from GIT of freshwater fish	Enzyme production study	*in vitro*	Bairagi *et al*., 2002
	Antibiotic producing *Alteromonas* sp.	Non-antibiotic producing *Alteromonas* sp.	*in vitro*	Lemos *et al*.,1991
	Bacillus spp.	*V. anguillarum, V. vulnificus, Pa. piscicida, Ent. seriolicida*	*in vitro*	Sugita *et al*., 1996a
	Carp intestinal bacteria	*A. hydrophila, A. salmonicida, E. coli, S. aureus*	*in vitro*	Sugita *et al*., 1997b
	Carnobacterium piscicola	*Carnobacteria, lactobacilli, pediococci, lactococci*	*in vitro*	Stoffels *et al*., 1992
	Freshwater bacteria	*Aeromonas* spp.	*in vitro*	Sugita *et al*., 1996b
	6 terrestrial LAB (*L. rhamnosus* (ATCC 53103), *L. rhamnosus* (LC705), *L casei*, *L. bulgaricus, L. johnsonii, Bif. lactis, Ent. faecium*	*A. salmonicida, V. anguillarum, Fl. psychrophilum*	*in vitro*	Nikoskelainen *et al*., 2001a
	Marine bacteria	*V. anguillarum*	*in vitro*	Westerdahl *et al*., 1991
	Marine bacteria	*V. anguillarum*	*in vitro*	Olsson *et al*., 1992
	Marine bacteria	*Lactococcus garvieae, Pa. piscicida, V. anguillarum, V. vulnificus*	*in vitro*	Sugita *et al*., 2002
	Marine bacteria	*A. hydrophila, V. alginolyticus*	*in vitro*	Vine *et al*., 2004a
	Marine bacteria	*A. hydrophila, V. alginolyticus*	*in vitro*	Vine *et al*., 2004b
	Psalt. undina	IHNV, *V. anguillarum*	*in vitro*	Maeda *et al*., 1997
	Psalteromonas spp., *Bacillus* spp.	*A. hydrophila, V. anguillarum, S. epidermidis, Proteus* spp., *Ca. albicans, Ent. faecalis*	*in vitro*	Ivanova *et al*., 1998

	Ps. flourescens	Saprolegnia	*in vitro*	Bly *et al*., 1997
	Pseudomonas spp., *Alteromonas* spp.	*Vibrio* spp., *Aeromonas* spp., *Pasteurella* spp., *Edwardsiella* spp., *Y. ruckeri*, *Ps. aeruginosa*	*in vitro*	Dopazo *et al*., 1988
	Pseudomonas spp., *Aeromonas* spp., *Vibrio* spp.	IHNV	*in vitro*	Kamei *et al*., 1988
	Pseudomonas spp.	*A. hydrophila*	*in vitro*	Das *et al*., 2006
	Roseobacter sp.	*Proteobacteria* spp., *Flavobacterium* spp., *Actinobacteria* spp.	*in vitro*	Brinkhoff *et al*., 2004
	Roseobacter spp., Vibrionaceae	*V. anguillarum, V. splendidus*	*in vitro*	Hjelm *et al*., 2004b
	V. anguillarum	Growth in salmon mucus study	*in vitro*	Garcia *et al*., 1997
	Vibrio sp. (strain NM10)	*Pa. piscicida*	*in vitro*	Sugita *et al*., 1997b
	Vibrio spp.	IHNV, OMV	*in vitro*	Direkbusarakom *et al*., 1998
	Vibrio spp., *Bacillus* sp., coryneform	*V. vulnificus*	*in vitro*	Sugita *et al*., 1998
	Vibrio sp.	*V. anguillarum*	*in vitro*	Jorquera *et al*., 1999
	123 *Vibrio* spp.	*V. tapetis*	*in vitro*	Castro *et al*., 2002
	V. mediterranei 1	*V. parahaemolyticus*	*in vitro*	Carraturo *et al*., 2006
FINFISH				
Atlantic cod	*Carnobacterium divergens*	*V. anguillarum*	*in vitro & in vivo*	Gildberg *et al*., 1997
Atlantic cod	*Carnobacterium divergens*	*V. anguillarum*	*in vitro & in vivo*	Gildberg & Mikkelsen, 1998
Atlantic salmon	*L. plantarum*	*A. salmonicida*	*in vitro & in vivo*	Gildberg *et al*., 1995
Atlantic salmon	*Carnobacterium* sp. (K1)	*V. anguillarum, A. salmonicida*	*in vitro & in vivo*	Jöborn *et al*., 1997
Atlantic salmon	*Ps. fluorescens*	*A. salmonicida*	*in vitro & in vivo*	Gram *et al*., 2001

Atlantic salmon, rainbow trout	*Carnobacterium* sp.	*V. anguillarum, V. ordalii, Y. ruckeri, A. salmonicida*	*in vitro* & *in vivo*	Robertson *et al*., 2000
Eel	Commercial product: Cernivet® LBC (*Ent. Faecium* SF68), Toyocerin® (*B. toyoi*)	*Ed. tarda*	*in vivo*	Chang & Liu, 2002
Eel	*A. media*	saprolegniosis	*in vitro* & *in vivo*	Lategan & Gibson, 2003
Eel	*A. media*	saprolegniosis	*in vivo*	Lategan *et al*., 2004b
Gilthead sea bream	*Cytophaga* sp., *Roseobacter* sp., *Ruergeria* sp., *Paracoccus* sp., *Aeromonas* sp., *Shewanella* sp.	Natural larval survival study	*in vivo*	Makridis *et al*., 2005
Gilthead sea bream	*Vibrio* spp., *Micrococcus* sp.	*V. anguillarum*	*in vitro* & *in vivo*	Chabrillón *et al*., 2006
Goldfish	Dead cells of *A. hydrophila,*	*A. salmonicida*	*in vivo*	Irianto *et al*., 2003
Indian major carp	*B. subtilis*	*A. hydrophila*	*in vivo*	Kumar *et al*., 2006
Nile tilapia	*Str. faecium, L. acidophilus, Sacc. cerevisiae*	Growth study	*in vivo*	Lara-Flores *et al*., 2003
Pollack	Commercial product: Bactocell (*Pediococcus acidilactici*), Levucell (*Sacc. cerevisiae*)	Pollack growth study using enriched Artemia	*in vivo*	Gatesoupe, 2002
Rainbow trout	*Ps. fluorescens*	*V. anguillarum*	*in vitro* & *in vivo*	Gram *et al*., 1999
Rainbow trout	*L. rhamnosus*	*A. salmonicida* ssp. *salmonicida* (furunculosis)		Nikoskelainen *et al*., 2001b
Rainbow trout	*Ps*. spp.	*V. anguillarum*	*in vitro* & *in vivo*	Spanggaard *et al*., 2001
Rainbow trout	*A. hydrophila, V. fluvialis, Carnobacterium* sp.	*A. salmonicida*	*in vitro* & *in vivo*	Irianto & Austin, 2002a
Rainbow trout	Dead cells of *A. hydrophila, V. fluvialis, Carnobacterium* sp.	*A. salmonicida*	*in vivo*	Irianto & Austin, 2003
Rainbow trout	*L. rhamnosus*	Immune enhancement paper	*in vivo*	Nikoskelainen *et al*., 2003

Rainbow trout	Commercial product: BioPlus2B (*B. subtilis, B. licheniformis*)	*Y. ruckeri*	*in vivo*	Raida *et al*., 2003
Rainbow trout	*L. rhamnosus*	Natural immunostimulation measured	*in vivo*	Panigrahi *et al*., 2004
Rainbow trout	*Pediococcus acidilactici, Sacc. boulardii*	Prevention of vertebral column compression syndrome	*in vivo*	Aubin *et al*., 2005
Rainbow trout	*A. sobria*	*Lactococcus garvieae, Str. iniae*	*in vivo*	Brunt & Austin, 2005
Rainbow trout	*L. rhamnosus*	Natural immunostimulation measured	*in vivo*	Panigrahi *et al*., 2005
Rohu	*B. circulans, B. subtilis*	Digestive enzyme study	*in vivo*	Bairagi *et al*., 2004
Sea bass	*Debaryomyces hansenii, Sacc. cerevisiae*	Digestive enzyme study	*in vivo*	Tovar *et al*., 2002
Senegalese sole	Vibrionaceae, Pseudomonodaceae, *Micrococcus sp.*	*V. harveyi*	*in vitro & in vivo*	Chabrillón *et al*., 2005
Silver perch	*A. media*	saprolegniosis	*in vivo*	Lategan *et al*., 2004b
Tilapia	Commercial product: Alchem Poseidon, Korea	*Ed. tarda*	*in vivo*	Taoka *et al*., 2006
Turbot	2 unidentified marine bacteria	GIT colonization study	*in vivo*	Makridis *et al*., 2000
Turbot	Marine bacteria	Natural survival study	*in vivo*	Huys *et al*., 2001
Turbot	*Roseobacter* spp., *Vibrio* spp.	*V. anguillarum, V. splendidus, Psalteromonas* sp.	*in vitro & in vivo*	Hjelm *et al*., 2004a
CRUSTACEANS				
F.W. prawns	*Lactobacillus* spp.	Gram negative bacteria	*in vivo*	Venkat *et al*., 2004
Shrimp embryos	*Alteromonas* sp.	*Lagenidum callinectes* (fungus)	*in vivo*	Gil-Turnes *et al*., 1989
Shrimp	*Bif. thermophilum* derived peptidoglycan	*V. penaeicida*	*in vivo*	Itami *et al*., 1998

Shrimp	Commercial product: DMS 1000, 1100, 2000	Pond culture survival study	*in vivo*	Moriarty, 1998
Shrimp	*Bacillus* sp.	*V. harveyi*	*in vivo*	Rengpipat *et al*., 1998
Shrimp	Commercial product: BioStart™ HB-1 (*B. subtilis, B. megaterium, B. polymyxa*) BioStart™ HB-2 (*B. licheniformis*)	Natural growth and survival study	*in vivo*	McIntosh *et al*., 2000
Shrimp	*Bacillus* sp. (S11)	*V. harveyi*	*in vivo*	Rengpipat *et al*., 2000
Shrimp	*Ps. aeruginosa*	*V. harveyi, V. fluvialis, V. parahaemolyticus, V. damsela, V. vulnificus*	*in vitro & in vivo*	Chythanya *et al*., 2002
Shrimp	*Bacillus* spp.	*V. harveyi*	*in vivo*	Meunpol *et al*., 2003
Shrimp	*B. subtilis*	*V. harveyi*	*in vitro & in vivo*	Vaseeharan & Ramasamy, 2003
Shrimp	*Vibrio* spp., *Bacillus* sp.	*V. harveyi*	*in vitro & in vivo*	Gullian *et al*., 2004
Shrimp	*Pseudomonas* sp (PM 11), *V. fluvalis* (PM 17)	Natural immunostimulation study	*in vitro & in vivo*	Alavandi *et al*., 2004
Shrimp	Commercial product: unidentified	Pond occurrence of *V. anguillarum* study	*in vivo*	Vaseeharan *et al*., 2004
Shrimp	*Pseudomonas* sp. PS-102	112 bacterial pathogens	*in vitro*	Vijayan *et al*., 2006
Shrimp larvae	*Arthrobacter* XE-7	*V. parahaemolyticus, V. anguillarum, V. nereis*	*in vitro & in vivo*	Li *et al*., 2006
Swimming crab larvae	*Thalassobacter utilis*	*V. anguillarum, Haliphthoros* sp. (fungus)	*in vivo*	Nogami *et al*., 1997
MOLLUSC				
Abalone	Unidentified: 1 yeast and 1 bacterium	Growth study and challenge with *V. anguillarum*	*in vivo*	Macey & Coyne, 2005
Pacific oyster larvae	*Alteromonas* sp. (CA2)	Growth and natural survival experiment	*in vivo*	Douillet & Langdon, 1993

Pacific oyster larvae	*Alteromonas* sp. (CA2)	Growth and natural survival experiment	*in vivo*	Douillet & Langdon, 1994
Pacific oyster larvae	*A. media*	*Aeromonas* spp., *Vibrio* spp., *P. damsella*, *Y. ruckeri*, *V. tubiashii*	*in vitro & in vivo*	Gibson *et al*., 1998
Scallop larvae	Marine bacteria	*V. anguillarum*	*in vitro & in vivo*	Riquelme *et al*., 1997
Scallop larvae	Marine bacteria	*V. anguillarum*		Avendaño & Riquelme, 1999
Scallop larvae	*Roseobacter* sp.	Variety- including *Vibrio* spp., *Aeromonas* spp.	*in vitro & in vivo*	Ruiz-Ponte *et al*., 1999
Scallop larvae	*Vibrio* sp. (C33), *Pseudomonas* sp. (strain 11), *Arthrobacter* sp. (strain 77)	Natural survival and ingestion study	*in vivo*	Riquelme *et al*., 2000
Scallop larvae	*Vibrio* sp. (C33), *Pseudomonas* sp. (strain 11), *Bacillus* sp. (B2)	Natural survival experiment in mass culture	*in vivo*	Riquelme *et al*., 2001
LIVE FOOD				
Artemia	9 marine bacteria	Natural survival and growth study	*in vivo*	Verschuere *et al*., 1999
Artemia	Commercial product: 9 commercial products and 8 laboratory cultures (including mainly *Bacillus* and *Pseudomonas*)	Natural growth study	*in vivo*	Douillet, 2000a
Artemia	*Aeromonas* spp., *Vibrio* spp.	*V. proteolyticus*	*in vivo*	Verschuere *et al*., 2000a
Artemia	*Microbacterium* spp., *Exiguobacterium* sp.	Natural survival study	*in vivo*	Orozco-Medina *et al*., 2002
Artemia	*Sacc. boulardii* (yeast)	*V. harveyi*	*in vivo*	Patra & Mohamed, 2003
Artemia	LAB	*V. alginolyticus*	*in vitro & in vivo*	Villamil *et al*., 2003
Chaetoceros ceratosporum	Marine bacteria	Microalgae growth study	*in vivo*	Fukami *et al*., 1992
C. gracilis	*Flavobacterium* sp.	Co-culture study	*in vivo*	Suminto & Hirayama, 1996

C. gracilis, Isochrysis galbana, Pavlova lutheri	*Flavobacterium* sp.	Co-culture study	*in vivo*	Suminto & Hirayama, 1997
C. muelleri	*V. alginolyticus*	Co-culture study	*in vivo*	Gomez-Gil *et al*., 2002
I. galbana	7 inhibitory substance-producing marine bacteria	Co-culture study	*in vivo*	Avendaño & Riquelme, 1999
Rotifers	LAB	Turbot growth study	*in vivo*	Gatesoupe, 1991
Rotifers	Mixed culture	Growth experiment	*in vivo*	Hirata *et al*., 1998
Rotifers	*Lactococcus lactis*	*V. anguillarum*	*in vivo*	Shiri Harzevili *et al*., 1998
Rotifers	*Alteromonas* sp., 3 unidentified spp.	Growth study	*in vivo*	Douillet, 2000b
Rotifers	7 terrestrial LAB	Growth study	*in vivo*	Planas *et al*., 2004
Skeletonema costatum	Putative *Aeromonas* sp. (SK-05)	*V. alginolyticus*	*in vivo*	Rico-Mora *et al*., 1998

Note: *A.= Aeromonas*, *Alt.= Alteromonas*, *B.= Bacillus, Bif.= Bifidobacterium*, *Ca.= Candida*, *Ed.= Edwardsiella*, *Ent.= Enterococcus*, *Fl.= Flavobacterium*, IHNV=infectious hematopoietic necrosis virus, *L.= Lactobacillus,* OMV= *Onchorhynchus masou* virus, *P.=Photobacterium*, *Pa.= Pasteurella*, *Pr.= Proteus*, *Ps.= Pseudomonas*, *Psalt.= Pseudoalteromonas*, *S.=Staphylococcus*, *Sacc.= Saccharomyces*, *Str.= Streptococcus*, *V.= Vibrio*, *Y.= Yersinia*

1.3.4 Probiotic research in mollusc aquaculture

There has been moderate past research effort into probiotics for bivalve molluscs. This has included work on the Pacific oyster, *Crassostrea gigas*; the scallop, *Pecten maximus*; the Chilean scallop, *Argopecten pupuratus*; and the Manila clam, *Ruditapes philippinarum* (Table 1.1). It is noteworthy that, apart from Douillet & Langdon (1993; 1994), all work published to date on bivalve probiotics originated as a consequence of screening for diffusible inhibitory substances *in vitro*.

Work on larvae of the Chilean scallop has been the most sustained published information on probiotics in bivalve mollusc culture (Riquelme *et al*., 1996a; Riquelme *et al*., 1997; Avendaño & Riquelme, 1999; Jorquera *et al*., 1999; Riquelme *et al*., 2000; Jorquera *et al*., 2001; Riquelme *et al*., 2001). The initial published work (Riquelme *et al*., 1996a) identified a bacterium, *Alteromonas haloplanktis*, capable of reducing mortality when larvae were exposed to 10^3 colony forming units ml^{-1} (CFU ml^{-1}) of *V. anguillarum* (VAR). It was found in the same study that only stationary phase supernatants of the probiotic were inhibitory to VAR *in vitro* when compared with log phase supernatants. Despite this moderate success, *Alteromonas haloplanktis* was not pursued in further published research. Jorquera *et al*., (1999) then set out to isolate the antimicrobial fractions of C33 (*Vibrio* sp.) using thin layer chromatography. This bacterium had previously shown good antimicrobial activities *in vitro*. Avendãno & Riquelme (1999) tested the co-culture and administration of seven potential probiotics to larvae through the microalga *Isochrysis galbana*, including one bacterium (strain 11) capable of providing larval protection against VAR for 24 hours (Riquelme *et al*., 1997). They found that the

previously non-ingested bacterium, C33, was ingested by the larvae after co-culture thereby providing a vector for introduction. The next two published reports by this group provided useful information. Of the three bacteria tested (strains 11, 77 and C33), Riquelme *et al*. (2000) demonstrated that only two of these were ingested by the larvae (strains 11 and 77). They also determined that when the probiotics were given at 10^6 CFU ml^{-1}, a period of six hours was needed for significant ingestion to occur. Additionally, with the one strain tested further (strain 77), 24 hours was needed for it to become the dominant member of the larval microbiota when administered at 10^6 CFU ml^{-1} and 48 hours was required if given at 10^4 CFU ml^{-1}. The next report (Riquelme *et al*., 2001) incorporated probiotics into a commercial scale hatchery production using the bacterial strains C33 (*Vibrio* sp.), strain 11 (*Pseudomonas* sp.), and *Bacillus* sp. (strain B2). This study determined that the probiotics allowed completion of the larval cycle without the need to use antibiotics.

The first work on probiotics in bivalves was conducted by Douillet & Langdon (1993). Unlike most research of this nature, their approach did not include *in vitro* agar-based tests. Instead, they applied 21 strains of bacteria directly to axenic Pacific oyster larvae, *Crassostrea gigas*, to determine the growth and survival characteristics of the bacterium added. Larvae were never challenged by a pathogen under the experimental protocol; the study looked specifically at the monoxenic effect of each bacterium. Of the tested strains, CA2, a putative *Alteromonas* sp., was identified as consistently enhancing both larval growth and survival. This work was followed up by determining the effect of adding different concentrations of CA2 to non-axenic cultures of the larvae and the effect of

CA2 with different species of microalgae (Douillet & Langdon, 1994). They showed that although seasonal variation in seawater microbiota did not affect the growth advantages from CA2, seasonal variation in growth and survival caused by different broodstock cohorts was apparent. Based on the lower numbers of slow growing larvae in treatments receiving CA2, they also proposed that it might provide some nutritional benefit. Another study on *C. gigas* was conducted by Gibson *et al*. (1998). They found a bacterium, *Aeromonas media* (strain A199), capable of inhibiting 89 of the 90 strains tested *in vitro* using the cross-streak method. Tested strains mainly comprised vibrios and aeromonads, with also two strains of *Yersinia ruckeri*, two of *Photobacterium damsella*, three of *V. anguillarum* and one of *Enterococcus seriolicida*, the non-affected bacterium. This widespread antagonism was then tested in bioassays with oyster larvae. It was found that A199 was able to prevent larval death when challenged with up to 10^5 CFU ml^{-1} of *V. tubiashii,* if A199 was inoculated one hour earlier at 10^4 CFU ml^{-1}. More recently, Elston *et al*. (2004) identified two potential probiotics for *C. gigas* larval production, P02-45 and P02-1. They determined that both killed bacteria and cell-free extracts were inhibitory *in vitro*. They further tested the co-culture of these strains with microalgae, and found that a protective effect against *V. tubiashii* could be established via co-culture with *Isochrysis galbana* (T-Iso) and *Rhodomonas* sp. Probiotic addition in larval challenge experiments was at a concentration of 10^5 CFU ml^{-1} and was not detrimental to larval survival until exceeding a concentration of 10^7 CFU ml^{-1}.

Other mollusc research has been conducted on the scallop, *Pecten maximus* (Ruiz-Ponte *et al*., 1999). This research group found a strain of *Roseobacter* sp. (BS107) that

produced *in vitro* antagonism only when the probiotic was cultured in the presence of either another bacterium producing a proteinaceous molecule, or the molecule itself. This molecule was thought to effect the antibacterial activity of BS107. In larval bioassays, BS107 did not enhance survival either in monoculture or when larvae were challenged with *V. pectenicida*. However, BS107 cell extracts did enhance survival of larvae in normal culture, but not when challenged with a pathogen. This suggested that substances produced by live BS107 could have been toxic to the larvae and that BS107 was not effective when high concentrations of the pathogen were used.

Work on the Manila clam, *Ruditapes philippinarum*, investigated *Vibrio* spp. microbiota associated with the mollusc over a one-year period (Castro *et al*., 2002). The most common species were found to be *V. tubiashii*, *V. splendidus* and *V. harveyi*. In screening isolates against *V. tapetis*, a bacterium implicated in brown ring disease, they found that five strains of *V. tubiashii* or *V. tubiashii*-like bacteria were able to inhibit the growth of the pathogen. However, the significance of this finding remains to be seen as *V. tubiashii* alone has been shown to be pathogenic to certain bivalves (Paillard *et al*., 2004). An interesting observation to come out of this study was that the *in vitro* antagonism by *V. tubiashii* was demonstrated only when the producer was grown on Mueller-Hinton agar highlighting another consideration with *in vitro* based screening, i.e. a possible effect of the growth medium upon inhibitory substance production.

1.3.5 Developing probiotics for aquaculture

It has been widely published that a probiotic must possess certain properties (Verschuere *et al*., 2000a). These properties were proposed in order to aid in correct establishment of new, effective and safe products. The properties include:

1. the probiotic should not be harmful to the host it is desired for,
2. it should be accepted by the host, e.g. through ingestion and potential colonization and replication within the host,
3. it should reach the location where the effect is required to take place,
4. it should actually work *in vivo* as opposed to *in vitro* findings,
5. it should preferably not contain virulence genes or antibiotic resistance genes.

The list of these requisites is given to allow step-wise examination of potential probiotics. However, the sum of many of these properties could be tested quickly via *in vivo* experimentation with the target animal. In essence, these properties are describing one simple question, "does the potential probiotic provide an overall health benefit when given to the animal?"

It was stated previously that there are inherent limitations with the past and current *in vitro* screening procedures and problems with changing the initial screening phase to *in vivo* experiments. Despite this, the possibility of being able to answer the question "does the potential probiotic provide a health benefit when given to the animal?" in the screening phase offers great simplicity, directness and an all-encompassing allowance for probiotics acting by any mode of probiotic action to be identified. For these reasons, the

prospect of including test animals in initial screening by means of challenge tests is very appealing. Such a screening model was recently described while using nematodes to screen for antimicrobials (Bhavsar & Brown, 2006). Twenty-five compounds were found to be effective in promoting nematode survival. In addition, they were shown to act by different mechanisms that may have been overlooked in a more classical screening procedure. Future research into novel probiotics for aquaculture would benefit from adoption of these principles as opposed to a total focus on screening for the production of inhibitory substances.

1.4 Objective of the study

The field of probiotics is definitely one of great interest and provides an avenue for alternatives to antibiotic use. In aquaculture, there is a great desire to develop and use probiotic products; there is already widespread use in many Asian countries. However, more species specific research is required to develop products for the farmer; discrepant results in the effectiveness of proposed probiotics suggest a "user designed" component as a factor in a product that works for a given animal. There is currently limited information on probiotic products for bivalve hatchery production and no literature exists concerning the use of probiotics for GSM larviculture. In consideration of the economic value of the GSM industry to NZ and the fact that, although the addition of antibiotics has been effective, it presents future risk, there is an urgent need to move away from antimicrobial drug management and to develop probiotic bacteria specific to GSM larval production. Given the wide range of proposed modes of action a probiotic may exhibit,

there is also a need to allow discovery of probiotics exhibiting all forms of activity, either singly or in combination.

1.5 Aims of the study

The purpose of this research program was to screen the environmental system of GSM larvae for potential probiotic bacterial strains that might be of benefit during the early growth period of the larvae. It specifically aimed to:

1. allow identification of probiotics exhibiting any mode of beneficial action,
2. isolate probiotics that allowed for at least 20% improved GSM larval survival when faced with a pathogen attack,
3. identify probiotics that allowed successful production of GSM larvae in the absence of antibiotics,
4. assess the benefit of the probiotics to larvae at a functional hatchery.

The focus was to find a probiotic that worked with GSM larvae, and the research was, therefore, industry driven for practical application.

Chapter 2

Development of a simple larval bioassay to enable screening for probiotics of Greenshell™ mussel larvae, *Perna canaliculus*

2.1 Introduction

In the search for new antimicrobials, common practice adopts initial *in vitro* screening methods. Such methods screen the effectiveness of test isolates in a controlled environment in the absence of the target host. This has also been witnessed in past research concerning probiotic bacteria for aquaculture (Spanggaard *et al*., 2001; Chythanya *et al*., 2002; Hjelm *et al*., 2004a). However, as previously mentioned, not all potential modes of probiotic action are elucidated when using standard *in vitro* screening methods initially and it is desirable to include target animals in the first screening stage for probiotic bacteria. Moreover, a positive or negative result *in vitro* does not always transfer into the *in vivo* environment. For example, *in vitro* protection was not witnessed *in vivo* (Ruiz-Ponte *et al*., 1999; Gram *et al*., 2001), and no *in vitro* antagonism occurred while there actually was *in vivo* protection (Raida, 2003). Ideally, before any test isolate is regarded ineffective, it is desirable to determine its actual effect on the intended target host. This potentially allows all probiotic modes of action to be witnessed.

In New Zealand bivalve research, this type of approach is aided by the microscopic size of the animals, the ready availability of larvae and absence of ethical/legislative issues using bivalve larvae in tests. To increase the likelihood of identifying probiotic bacteria effective in GSM larviculture, direct screening of test bacterial isolates using GSM larvae was preferred in the initial screening stages. The development of a small scale, replicable bioassay to enable future screening for probiotics of GSM larvae was investigated. In past studies involving larval aquatic animals, tissue culture dishes (TCDs, Fig. 2.1) have been

employed as experimental containers (Lovko *et al*., 2003; Hjelm *et al*., 2004b; Unuma *et al*., 2004). The feasibility of using TCDs with GSM larvae was tested herein.

Figure 2.1 12-well tissue culture dishes (TCDs).

2.2 Materials and methods

2.2.1 Experimental animals

Larvae used in experiments were obtained from the Glenhaven Aquaculture Centre Limited (GACL) hatchery facility, Glenduan, Nelson, New Zealand. GSM larvae were obtained as per hatchery protocol. Prior to spawning, broodstock were cleaned of external fouling and placed in a water bath 3°C above ambient (approximately 18°C) for three hours. A cold shock of 3°C below ambient (approximately 12°C) was then applied to the mussels. Some mussels required repeated shock and induction cues to induce spawning.

Once a mussel began to spawn it was placed into a separate container to hold its gametes separately prior to fertilization. Fertilization followed a sperm-to-egg ratio of approximately 10:1, in a volume of 20 l ambient seawater. Fertilization proceeded in this environment for 20 min, before fertilized eggs were transferred into a 170 l static water tank with aeration and ethylenediaminetetraacetic acid (EDTA, Sigma), final concentration 1 mg l^{-1} (Buchanan, 1998), for incubation. Incubation occurred in static water until D-veliger larvae were observed (approximately 36 hours), with food (0.5 l of 15,000 cells ml^{-1} *Chaetoceros calcitrans*) added to the tank after 24 hours. At this stage, larvae were screened on 45 μm nylon mesh to separate D-veliger larvae from trochophore larvae. D-veliger larvae were then placed into a 170 l flow-through banjo-filter system (Fig. 2.2). Water flow was continuous at 80 ml min^{-1} (providing two full water exchanges per hour in each tank), aeration was provided, water temperature was 19°C and larvae were fed a 2:1 mix of *Chaetoceros calcitrans*:*Isochrysis galbana* to provide a final algal concentration in tanks of 40 cells μl^{-1}.

Larvae were screened on nylon mesh size 55 μm for use in the experiments (approximately 72 hours culture in flow-through system). Screened larvae were transported to the Cawthron Institute immediately (approximately 10 min drive). Larvae were then washed five times with sterile seawater and re-suspended in sterile seawater for use in experiments.

Figure 2.2 Banjo-filter flow-through water GSM larval culture system.

2.2.2 TCD bioassay

The main purpose of future bioassay tests was to determine the one-on-one effect of bacteria on larvae without outside influences. Moreover, it was expedient to exclude food from the system to negate the possibility of microalgae decomposition and microalgae/bacteria interactions. It was preferable, also, to have no water exchange as it would not allow reliable recovery of all test animals following screening and replacing water. It was also not practical to provide aeration to each TCD well.

Establishing the feasibility of using TCDs without food, water exchange or air required two questions to be addressed: 1) what would be the effect on the larvae under conditions

of no water exchange, food or aeration (with and without addition of bacterial inoculum); 2), what would be the effect on water quality by having no aeration or water exchange (with and without addition of bacterial inoculum).

2.2.2.1 Larval survival in TCDs without food, aeration or water exchange (with and without additional bacterial inoculum)

Larvae were placed into the wells of a Falcon 12-well TCD (Becton Dickinson, USA). Each well contained 4 ml autoclaved seawater and larvae at a concentration of approximately 10 larvae ml^{-1}. Seven bacterial isolates (04146, 04160, CCT, 0444, 04145, 04139, 04150) previously isolated from the GACL were randomly chosen and separately inoculated into TCDs containing the larvae. Each isolate was added at three concentrations (10^2, 10^4, and 10^6 CFU ml^{-1}) to determine whether bacterial inocula had a deleterious effect in TCD bioassays. To avoid aerosol contamination between treatments, each bacterial concentration to be tested was dedicated a separate 12-well TCD. Each treatment contained six replicate wells, i.e. only six wells from each 12-well TCD were used. Control treatments included a TCD with larvae, sterile seawater and no addition of bacteria. No food or aeration was provided.

Tissue culture dishes were maintained at 19°C (Fig. 2.3) and larval survival measured over 13 days by microscopic observation. Cumulative percentage survival was calculated from the numbers of live and dead larvae in each well. Dead larvae were easily distinguished by lack of soft tissue and motility when compared with live larvae (Fig. 2.4). Moribund larvae (i.e., those that exhibited decomposition of soft tissue, were not

moving, or doing so in irregular circular motions) were considered alive for survival counts. These larvae always progressed to dead animals in the following 24-48 hours.

Prior to inoculation, bacteria were grown for 48 hours at 22°C on tryptone soy agar (TSA, Merck) made to 2% salinity using seawater (TSA-2%Sea), following the method of Buller (2004) and, hence, providing a medium similar to that of the isolates' environment. Bacteria were suspended in 10 ml of 2% sterile seawater to a concentration of 10^9 CFU ml^{-1} using MacFarland turbidity standards (Murano & Hudnall, 2001). These estimates were verified by aerobic, agar plate, colony counts of 10-fold dilutions. The appropriate bacterial suspension (400 µl) was added to TCD wells to yield concentrations 10^2, 10^4 and 10^6 CFU ml^{-1}.

Figure 2.3 Tissue culture dishes stacked in incubator.

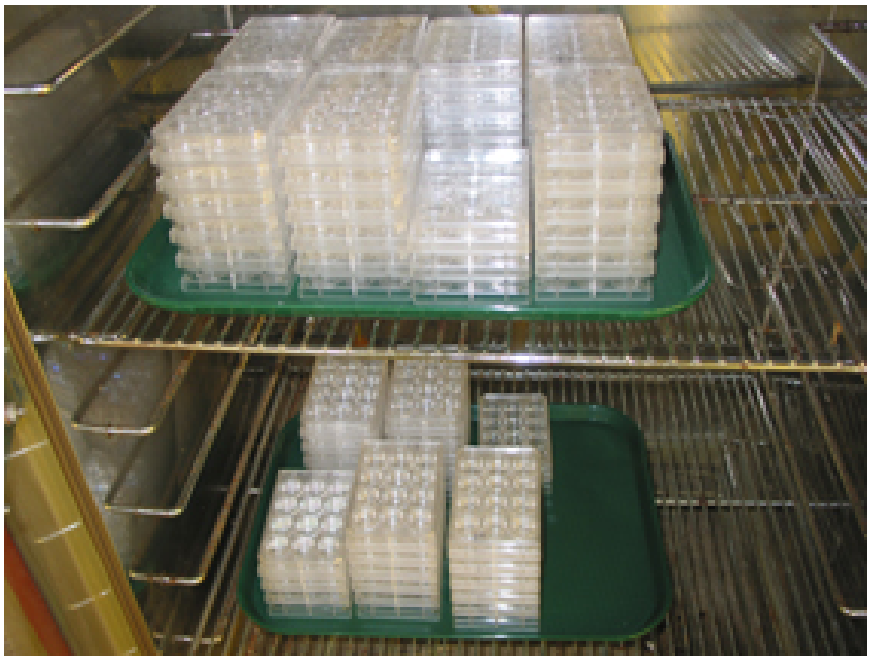
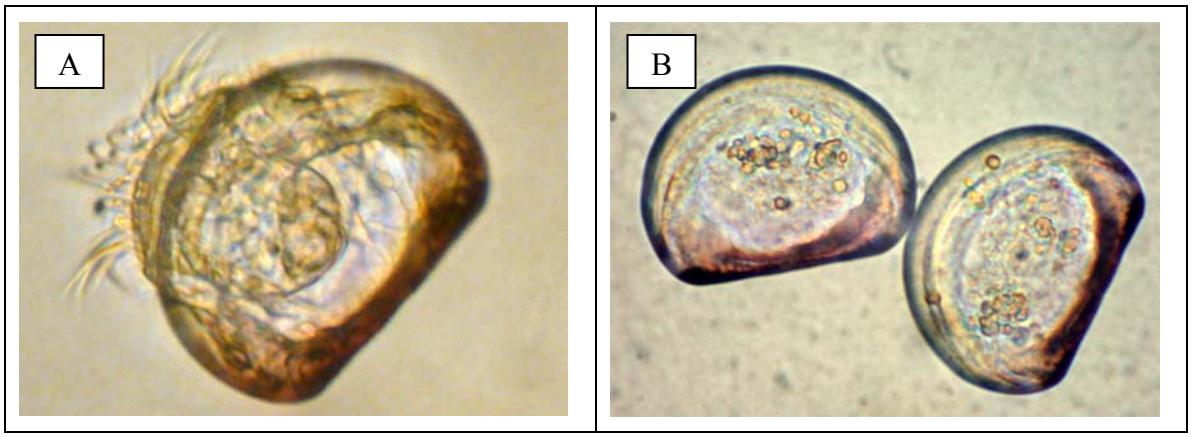


Figure 2.4 Live (A) and dead larvae (B). Live larvae are freely swimming with normal velum and stomach visible inside shell. Dead animals are more translucent due to absence of internal organs; a few microalgae cells are present.



2.2.2.2 Water quality effects in a system with GSM larvae and no aeration or water exchange (with and without additional bacterial inoculum)

Due to the small volume of water in TCDs, containing just 4 ml, it was not possible to test water quality parameters. Therefore, tests were adapted to larger volume vessels wherein the other TCD bioassay parameters were maintained. Flat-bottom Florence flasks (1000 ml), containing 700 ml of sterile seawater and larvae at a concentration of 10 larvae ml^{-1}, were used. Flasks were maintained at 19°C. A previously isolated bacterium, 04287, originating from the GACL hatchery, was added to the flasks at a concentration of 10^3 CFU ml^{-1}. Control treatments included flasks set up without addition of 04287. No food was added to flasks. Both treatments (04287 and control) were set up in flasks with and without aeration to provide comparison between a normal situation and that without air. All treatments were run in triplicate. On the seventh day, bacterial concentrations were determined for each flask by means of the plate dilution technique, as was larval

survival, pH (SevenEasy™ pH meter S20, Mettler Toledo) and dissolved oxygen (DO, % saturation) (YSI Model 85, USA).

2.2.3 Pre-treatment of larvae with antibiotics prior to experimentation

Pilot trials suggested larvae-associated bacteria entering TCDs at the start of an experiment were responsible for large variation within treatments, i.e. high and low survival displayed within the six replicates of the same treatment. To remove the influence of extraneous bacteria and facilitate identifying a probiotic effect of any added inoculum, pre-treating the larvae with antibiotics (AB) prior to experimentation was tested. Four AB treatment regimes were tested to determine an optimum AB regime for allowing a reduction of bacterial numbers in non-sterile GSM larvae.

Streptomycin sulphate and penicillin G sodium were used. Each AB was provided at a concentration of 150 μg ml^{-1} suspended in 100 ml of sterile seawater. Four AB treatment regimes were tested: (i) larvae immersed in 100 ml of AB for 21 hours, (ii) larvae given two 3 ½ hour treatments of AB with 20 hours between treatments, (iii) larvae held for 24 hours, without feeding, to achieve partial removal of gut contents before two 3 ½ hour treatments of AB with 20 hours between treatments, (iv) larvae held for 48 hours, without feeding, again to achieve partial removal of gut contents before two 3 ½ hour treatments of AB with 20 hours between treatments. Control treatments included TCDs holding sterile seawater without larvae and TCDs with non-treated larvae.

All larvae were washed prior to and after AB treatment with sterile seawater in order to remove residual AB and dead bacteria. Larvae were re-suspended in sterile seawater for holding periods between successive AB treatments, and for post-treatment counts of larval concentration before subsequent addition to experimental TCDs at the required concentration.

Antibiotic-treated and non-treated larvae were placed into all wells of a 12-well TCD with 4 ml sterile seawater at a concentration of 10 larvae ml^{-1}. Each treatment was conducted in triplicate TCDs. Larvae were maintained at 19°C throughout the experiment. Each day for seven days, 0.1 ml from a randomly chosen TCD well from each TCD was surface-spread onto TSA 2%-Sea to detect bacterial presence/absence. The detection limit for this method was 10^1 CFU ml^{-1}. Often no growth occurred on the plates, i.e. samples were below the detection limit. Previously sampled TCD wells were excluded from future sampling to eliminate any potential contamination caused by earlier sampling. In essence, the sampling technique provided 21 individual observations, for each treatment, of bacterial presence over a seven-day period (three treatment replicates sampled daily for seven days). The percentage of observations which were below the detection limit for the seven-day duration was determined from each AB regime. Larval survival was measured on day 7.

2.2.4 Data analysis

Percentage survival figures were arc sin square root transformed to approximate normality and compared using ANOVA ($p = 0.05$). Post hoc comparisons followed

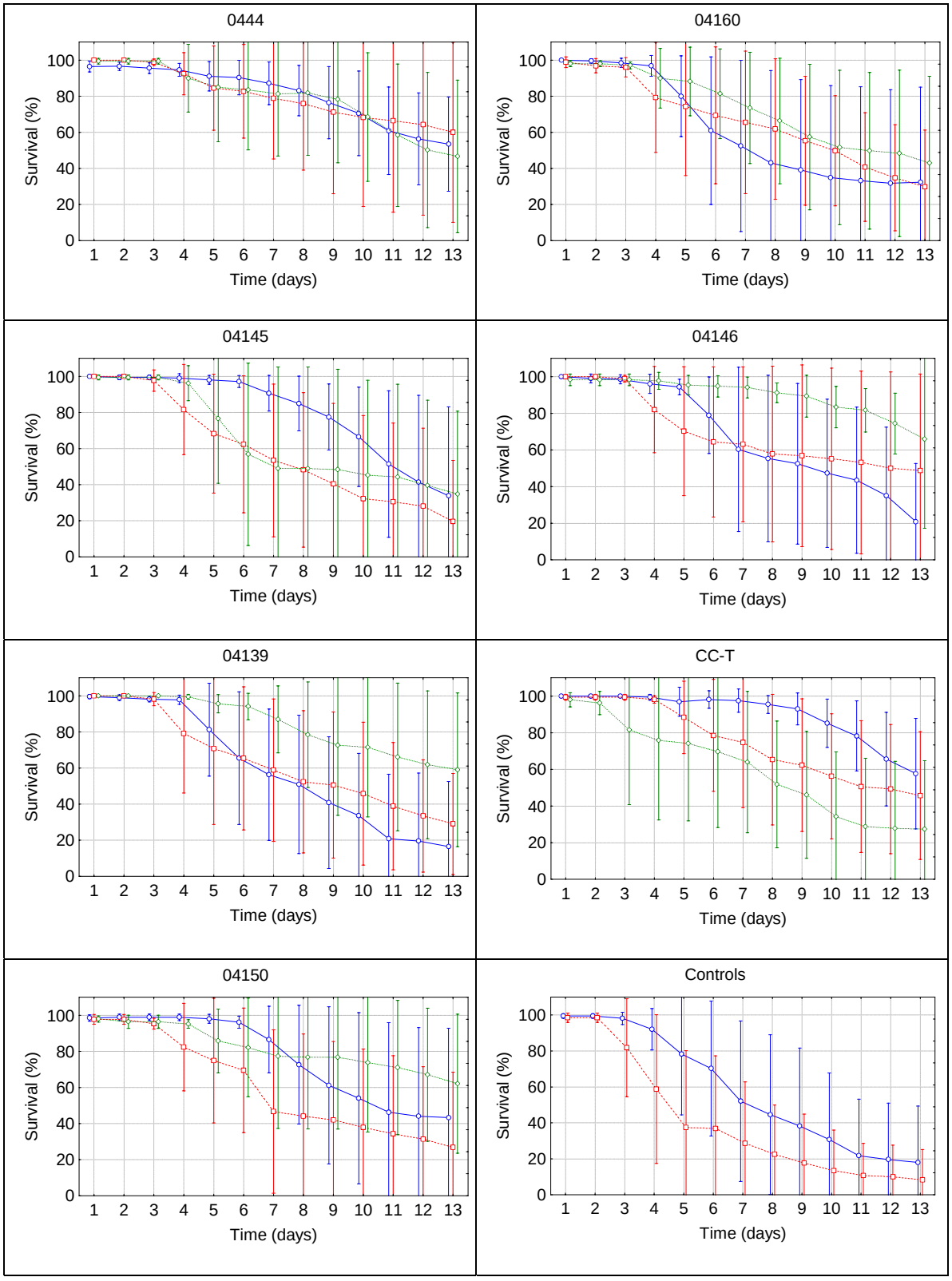
Tukey's test. In cases with unequal sample sizes, Tukey's HSD for unequal N was used for post hoc analysis. Where data did not meet ANOVA assumptions, data were compared using the non-parametric Kruskal-Wallis test ($p = 0.05$). STATISTICA (StatSoft, Inc.), version 7.1 was used for all data analyses contained within this thesis.

2.3 Results

2.3.1 Larval survival in TCDs without food, aeration or water exchange (with and without additional bacterial inoculum)

Tests incorporating bacterial isolates showed a large degree of variation of larval survival within treatments (Fig. 2.5 error bars). This was also observed within control treatments where no bacterial inoculum was added. Despite the variation, mean larval survival in TCDs was greater than 40% in all but one treatment and greater than 60% in 16 of the 24 tested treatments after seven days. Some larvae remained viable in each treatment upon day 13 completion of the experiment. Pilot studies had revealed that under conditions of no food, air or water exchange larvae were able to survive for over 24 days (Appendix 1).

Figure 2.5 Survival (%) of larvae over a 13-day period following inoculation with bacterial isolates. Each isolate is represented in a separate graph; blue line = 10^6 CFU ml^{-1}, red = 10^4 CFU ml^{-1}, and green = 10^2 CFU ml^{-1}. Error bars denote 95% confidence intervals.



2.3.2 Water quality effects in a system with GSM larvae and no aeration or water exchange (with and without additional bacterial inoculum)

Day 7 bacterial levels were at a magnitude of 10^5 CFU ml^{-1}, regardless of whether aeration or an initial bacterial inoculum was provided (Fig. 2.6). There was no statistical difference in bacterial levels. In terms of pH and DO, there were no statistical effects shown whether or not an initial bacterial inoculum had been added. In treatments without air, the pH was statistically higher and the DO statistically lower on day 7 when compared with those provided air (Table 2.1). Despite this, there was no effect on larval survival ($p > 0.05$) in treatments without air or the associated water parameter differences. In fact, the treatment without air plus an initial bacterial inoculum had statistically higher survival than all other treatments (Table 2.1).

Figure 2.6 Bacterial load (CFU ml^{-1}), pH, dissolved oxygen and larval survival at day 7 of flask cultures containing a bacterial inoculum (04287) or no inoculum, and with or without aeration.

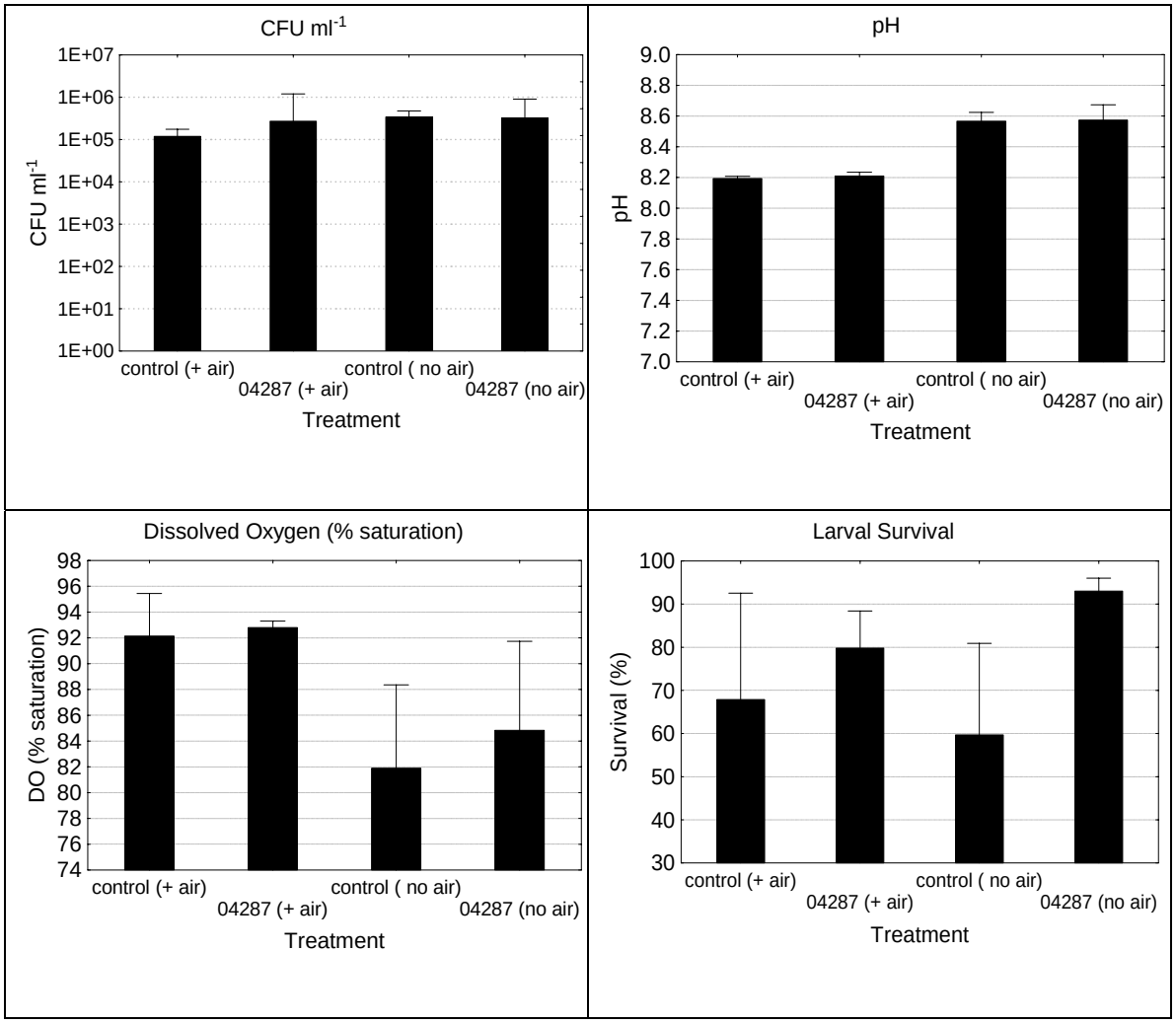


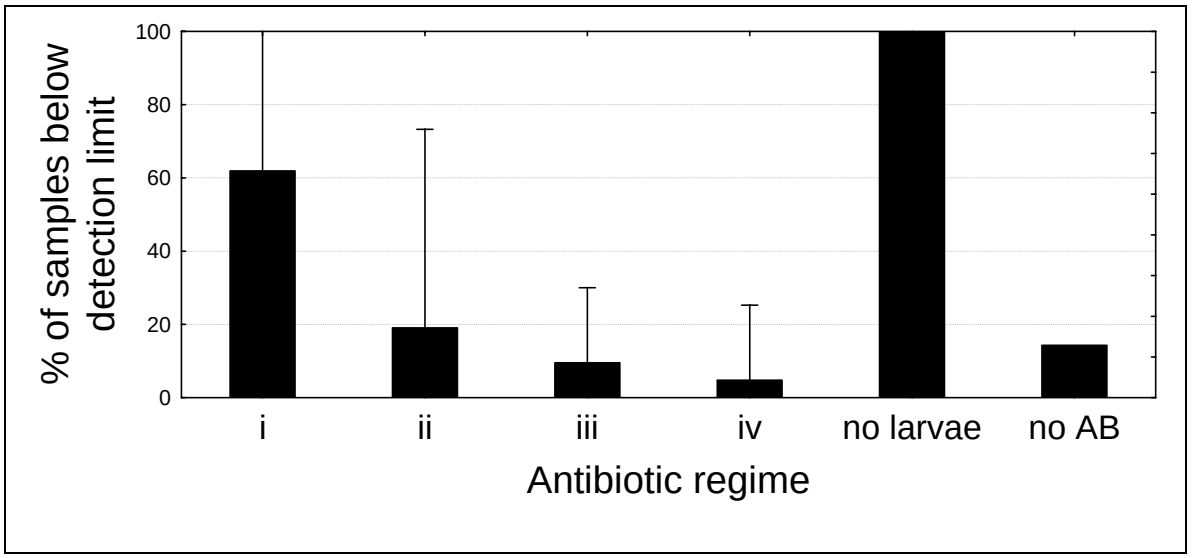
Table 2.1 Mean values (± 95% confidence intervals) of parameters measured upon day 7 of the flask experiment. Values not sharing the same superscript are statistically different ($p < 0.05$).

Parameter	n	Control (+ air)	04287 (+ air)	Control (no air)	04287 (no air)
Bacteria (CFU ml^{-1})	3	$1.2 \times 10^5 \pm 5.7 \times 10^4$	$2.7 \times 10^5 \pm 9.2 \times 10^5$	$3.4 \times 10^5 \pm 1.3 \times 10^5$	$3.2 \times 10^5 \pm 5.8 \times 10^5$
pH	3	8.19 ± 0.02^a	8.21 ± 0.02^a	8.57 ± 0.06^b	8.57 ± 0.10^b
DO (% saturation)	3	92.1 ± 3.3^a	92.8 ± 0.5^a	81.9 ± 6.5^b	84.8 ± 6.9^b
Larval survival (%)	9	67.8 ± 24.7^a	79.8 ± 8.6^a	59.7 ± 21.2^a	93.0 ± 3.0^b

2.3.3 Pre-treatment of larvae with antibiotics prior to experimentation

Figure 2.7 illustrates the reduction in bacterial numbers (expressed as the absence of bacterial colonies following surface-spread plating) after antibiotic exposure. In treatments without addition of larvae, no bacteria were detected. Regime number one (100 ml of antibiotic for 21 hrs) had the best effect in reducing bacterial numbers. It was statistically better than regime three and four ($p = 0.02$ and $p = 0.01$ respectively), but not the other treatments ($p > 0.05$). Day 7 larval survival was high in all treatments, however regime two was marginally better than regime one and four ($p = 0.044$ and 0.041 respectively) (Table 2.2).

Figure 2.7 Percentage of samples below the bacterial detection limit (10^1 CFU ml^{-1}) for samples taken from TCD bioassays over a seven-day period following antibiotic treatment of GSM larvae.



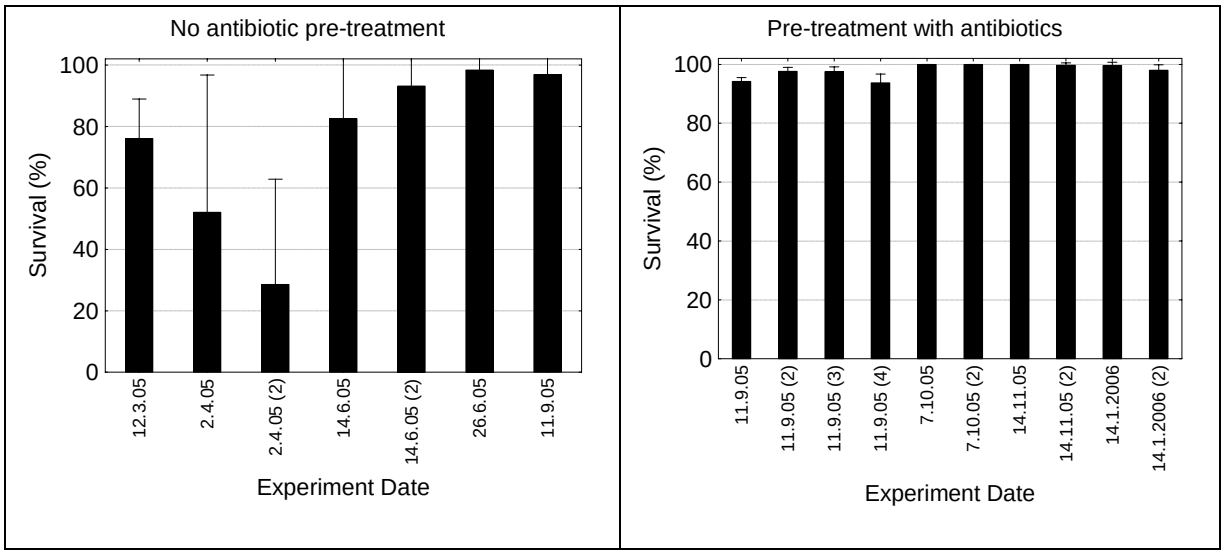
Note: Regimes: (i) larvae immersed in 100 ml of AB for 21 hours, (ii) larvae given two 3 ½ hour treatments of AB with 20 hours between treatments, (iii) larvae held for 24 hours, without feeding, before two 3 ½ hour treatments of AB with 20 hours between treatments, (iv) larvae held for 48 hours, without feeding, before two 3 ½ hour treatments of AB with 20 hours between treatments

Table 2.2 Day 7 larval survival (± 95% confidence intervals) following antibiotic treatment regimes. Values not sharing the same superscript are statistically different ($p < 0.05$).

	Regime i	Regime ii	Regime iii	Regime iv	No AB
Larval survival	94.2 ± 1.3^{a}	97.6 ± 1.3^{b}	97.6 ± 1.7^{ab}	93.7 ± 3.0^{a}	96.9 ± 8.5^{ab}
n	15	15	15	15	5

From a range of experiments not employing antibiotic pre-treatment, larval survival at day 7 in control treatments (no bacterial inoculum added) showed a large degree of variation (Fig. 2.8). In experiments conducted following AB pre-treatment of GSM larvae (regime one) high survival and marginal variation occurred.

Figure 2.8 Day 7 larval survival (%) in control treatments (no bacterial inoculum added) during a range of experiments in TCD bioassays with and without antibiotic pre-treatment of larvae. Error bars denote 95% confidence intervals.



2.4 Discussion

Tissue culture dishes offered a useful avenue in developing a larval bioassay for the purpose of screening for probiotic bacteria. Benefits of TCDs included: original sterile vessels; allowance of up to 12 replicates in small space; small and stackable to facilitate a large number of tests with ease; visualization of a whole population throughout an experiment; and ease in maintaining a constant experimental environment (they could be stacked and placed in an incubator). Tissue culture dishes have been used in the past for aquatic species larval bioassays (Lovko *et al*., 2003; Hjelm *et al*., 2004b; Unuma *et al*., 2004). Lovko *et al*. (2003) developed a bioassay to test the pathogenicity of several strains of *Pfiesteria* spp. on larval cyprinodontid fish. Hjelm *et al*. (2004b) employed TCDs to test the efficacy of a shortlist of probiotics on turbot larvae. This shortlist was derived from previous *in vitro* agar diffusion assays. Unuma *et al*. (2004) used TCDs to assess various parameters in larval eel culture; egg quality, fertilization rates, hatching and survival. Review of the literature suggests that TCD bioassays have not been employed during initial screening for aquatic probiotics.

GSM larvae were able to survive for over 23 days without food, aeration or water exchange in TCD bioassays. This was also witnessed when animals were provided an initial bacterial inoculum; this situation would occur during probiotic screening trials. Under conditions of added bacterial inoculum, larval survival was very good for at least seven days and better than survival in controls. Previously, GSM larval survival was shown to be better when the larvae were unfed, when compared with feed concentrations of 1 to 200 cells μl^{-1} *Isochrysis galbana* (T-Iso) (Buchanan, 1998). In those trials, tanks

were also non-aerated; however, water was exchanged three times weekly. Unfed mussel larvae, *Mytilus* spp., have also been reported to survive for 20-30 days and up to 150 days in sterile seawater (Lutz & Kennish, 1992).

Initially, within-treatment variation of larval survival was high, but AB pre-treatment corrected this. Use of ABs facilitated a reduction of extraneous larvae-associated bacteria entering the TCDs. High larval survival and minimal variation in experiments pre-treated with ABs implicated extraneous bacteria with mortalities seen in previous non-treated experiments. A reduction in number of extraneous bacteria was also seen as important for future probiotic trials, thereby increasing the likelihood of correct interpretation of the effect of test bacteria. Marques *et al.* (2006) discussed the importance of using axenic animals to observe potential positive effects in isolation; in a more complex system, specific effects of a test bacterium are harder to distinguish.

Based on larval survival, under conditions of no aeration, feed or water exchange with GSM larvae at a concentration of 10 larvae ml^{-1}, water quality degradation over seven days did not impact upon larvae. This was probably helped by the low stocking densities and the assumption that, without food, there would have been a reduced amount of excrement decomposition. Total culturable bacterial numbers did not differ between treatments with and without aeration. This was seen both in treatments supplied an initial bacterial inoculum and those without, indicating a maximum carrying capacity for bacteria (10^5 CFU ml^{-1}) that was always reached.

There was no effect caused by the addition of a bacterial inoculum on pH or dissolved oxygen, suggesting bacteria can be added to bioassays without detrimentally changing water quality compared with the control tanks. Statistical differences were seen, in pH and dissolved oxygen levels, to occur between flasks that were non-aerated and aerated. However, these differences did not appear biologically important as highest larval survival actually occurred in a non-aerated treatment. There is a lack of information on pH tolerance limits for GSM larvae. However, Brunner (2005) demonstrated, in GSM spat, behavioural responses began at pH 9.1, an EC_{50} value (50% shells closed) of pH 9.5, with animals capable of surviving five days at pH 9.5. In the freshwater zebra mussel, *Dreissena polymorpha*, veliger larvae require pH 7.4-9.4, with best performance at pH 8.4 (McMahon, 1996). In the present study, pH values of 8.2 and 8.6, seen in the aerated and non-aerated treatments respectively, fell safely below previous research on GSM spat, and compared favourably with that required by zebra mussel larvae.

Day 7 dissolved oxygen averaged 83% saturation (7.4 mg l^{-1}) for the non-aerated treatments and 92% (8.4 mg l^{-1}) for those with air. Although this was statistically different, it did not affect larval survival. Levels observed in both treatments are at the high end of the saturation scale (far from hypoxic) and can be considered safe as demonstrated by good larval survival. There is a lack of information on the DO tolerance limits for GSM. However, it can be assumed that the levels observed in this study were within those limits as judged by a lack of larval mortality over seven days. The observed levels were well above 60% saturation; the level below which *Mytilus* spp. cannot compensate by the auto-regulatory mechanism of increased extraction efficiency (Newell,

1989). They were also well above 4.5 mg l^{-1}; the level below which scallop survival and immune responses were negatively affected (Chen *et al*., 2007).

The results of this study demonstrated that *in vivo* screening using TCD bioassays is feasible with GSM larvae. Larval survival was good, water quality degradation not evident and a large number of treatments could be conducted over seven days in the controlled environment of an incubator. Potentially, this bioassay design could be used with other aquatic species for screening purposes. Studies of baseline effects would need to be performed for individual species, as shown in this study. Animals need to cope with a lack of food, water exchange and aeration. However, given negligible detriment to test animals over a timeframe long enough to perform screening tests, the bioassay offers an efficient and effective alternative method to *in vitro* screening.

Chapter 3

Two pathogens of Greenshell™ mussel larvae, *Perna canaliculus*: *Vibrio splendidus* and a *V. coralliilyticus/neptunius*-like isolate

3.1 Introduction

Although hatchery production of GSM larvae is being developed, wild caught stock still remains the dominant source of spat and the GACL hatchery often faces larval crashes. Larval problems typically begin around day 6 post-fertilization. Characteristic symptoms include: loss of lipids, deformed extended velum and erratic swimming. Appearance of these symptoms at GACL led to antibiotic management when required. The successful outcome resulting from antibiotic treatment of larvae indicated that bacterial agents might be implicated in larval crashes at the hatchery.

Past work has documented bacterial pathogens of molluscs (Lacoste *et al.*, 2001; Waechter *et al*., 2002; Paillard *et al*., 2006) and mollusc larvae (Elston & Leibovitz, 1980; Jeffries, 1982; Riquelme *et al*., 1995; Sugumar *et al*., 1998). All the aforementioned studies found *Vibrio* spp. to be the etiological agent, and most work concentrated on the Pacific oyster, *Crassostrea gigas*. Currently, there is no information in the published literature on bacterial pathogens of mussel larvae. This study investigated bacterial pathogens of GSM larvae in order to develop both management and prevention protocol systems during hatchery production.

3.2 Materials and methods

3.2.1 Isolation, storage and preparation of bacteria

A number of bacteria were isolated at the GACL during 2005. Bacteria were sourced from GSM larvae displaying the typical symptoms of infection, from tank water

containing symptomatic larvae and from microalgae cultures. Larvae were macerated using a Bosch homogenizer on No. 2 speed for 30 seconds.

Tryptone soy agar (TSA, Merck), a non-selective medium, made to 2% salinity using seawater (TSA-2%Sea) was used to isolate bacteria from the sources described above. Dominant colonies present during larval-crashing episodes were streaked for purity and stored at -70°C in Protect Bacterial Preservers (Technical Service Consultants Limited, Lancashire, UK). Prior to use in experiments, bacteria were revived in tryptone soy broth (TSB-2%Sea). Test isolates were streaked to ensure purity and sub-cultured on TSA-2%Sea three times before use in experiments.

3.2.2 Greenshell™ mussel larvae

Larvae used in experiments were obtained from the GACL (refer to Chapter 2.2.1). Three-day-old veliger larvae were transported to the Cawthron Institute for processing. Larvae were washed with sterile seawater and submitted to a treatment of antibiotics: 150µg ml^{-1} of both streptomycin sulphate and penicillin G sodium. The use of antibiotics was necessary to ensure larvae were in a healthy state at the start of experimentation. Health status of the larvae was confirmed as a result of survival of the larvae in the control treatments. Prior to experimentation, larvae were washed five times with sterile seawater and re-suspended in sterile seawater.

3.2.3 TCD bioassay screening for pathogens

Dominant bacterial colonies, isolated from unhealthy animals, were selected for a preliminary screen in order to determine pathogenicity. Twenty-two morphologically distinct bacterial strains were tested for pathogenesis in TCD bioassays. Of these, 18 were presumptive *Vibrio* spp. based on growth on thiosulphate citrate bile sucrose agar (TCBS, Acumedia, Michigan, USA) (0530, 0552-0569), one was *Vibrio anguillarum* (ATCC 19264, obtained from Environmental Science & Research New Zealand reference culture collection, Porirua, New Zealand), one was non-pathogenic (0444, based on previous bioassay development trials), and two were unidentified potential pathogens (DO1 and 0529, based on previous development trials). *Vibrio anguillarum* was tested as a pre-identified strain, while strain 0444 was included as a negative control.

Bacterial test isolates were cultured on TSA-2%Sea for 48 hours at 22°C prior to inoculation into TCDs containing larvae. Bacteria were suspended in 10 ml of 2% sterile seawater to a concentration of 10^9 CFU ml^{-1} using MacFarland standards. These estimates were verified via plate dilutions. The appropriate bacterial suspension (400 µl) was added to TCD wells to yield concentrations 10^2, 10^4 and 10^6 CFU ml^{-1}. To avoid aerosol contamination between treatments, each tested concentration of bacteria was allocated a separate 12-well TCD.

Larvae, at a concentration of approximately 10 larvae ml^{-1}, were added to the wells of a 12-well TCD containing 4 ml of sterile seawater without food. Test bacteria were added at the required concentrations (10^2, 10^4 and 10^6 CFU ml^{-1}). Percentage larval survival was

recorded daily for a period of seven days. Tissue culture dishes containing larvae without any bacterial inoculum served as survival controls. All treatments contained eight replicates. Larvae were maintained at 19°C throughout the experiment.

3.2.4 The effect of water parameters on larval mortality during bioassays

Potential pathogens identified from TCD bioassays were further investigated to determine whether a change in pH or dissolved oxygen (DO, % saturation) was a factor in the larval mortality. Three potential pathogens, DO1, 0529 and 05101 were tested by up-scaling experiments to 500 ml in flat-bottom Florence flasks (1000 ml), which allowed sampling for both pH and DO. Test larvae were prepared as described above. No food or aeration was provided during experiments.

Each test isolate was cultured in 12 ml TSB-2%Sea for 10 hours at 22°C. Bacterial concentration was determined via optical density at 600nm (Appendix 2) using a PharmaSpec UV-1700 spectrophotometer (Shimadzu) and verified by means of surface-spread plate counts on TSA-2%Sea. To obtain broth-free bacteria, cells were washed three times with sterile seawater at 2500g for 5 min (Heraeus Biofuge Primo, Thermo Scientific, Sweden) before re-suspension in sterile seawater. Test bacteria were added to flasks at a concentration of 10^3 CFU ml^{-1}. Flasks without addition of bacteria served as controls. Each treatment was run in triplicate. Final DO and pH values were recorded from each flask upon completion of the experiment at day 7. Larval survival and total bacterial counts were measured on day 4 and day 7.

3.2.5 Identification of bacterial pathogens

Isolates demonstrating potential pathogenicity in the bioassays were further identified using both phenotypic and genotypic characterisation. Gram stain, oxidase, motility and catalase tests, and growth on TCBS were initially carried out, followed by API 20NE (BioMérieux). Further profiling included growth temperature range and salt tolerance.

Genotypic characterization of strains entailed the amplification and sequencing of the 16S ribosomal RNA (rRNA) gene. Chromosomal DNA was extracted from bacterial cultures using a commercial kit (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany) following the manufacturer's instructions. Two alternative 'universal' bacterial 16S rRNA PCR primer pairs were used for 0529 and DO1 respectively:

forward: EUBB-F (5'- AGAGTTTGATCMTGGCTCAG -3'),
reverse: EUB-A-R (5'- AAGGAGGTGATCCANCCRCA -3') (Suzuki & Giovannoni 1996), and

forward: pA (5'- AGAGTTTGATCCTGGCTCAG -3'),
reverse: pH (5'- AAGGAGGTGATCCAGCCGCA -3') (Edwards *et al.*, 1989)

Final amplification reaction mixtures consisted of: 20 µl 2.5 x HotMasterMix (Eppendorf, Hamburg, Germany), 2.0 µl DNA template, primers (final concentration 0.8 µM) and water to final volume of 50.0 µl. Amplification reactions were performed using an iCycler (Bio-Rad, Carlsbad, USA) with the following thermocycling conditions: 94°C

/ 2 minutes, 52°C / 1 minute, 65°C / 1 minute, 1 cycle; 94°C / 30 seconds, 55°C / 30 seconds, 65°C / 2 minutes, 35 cycles; 65°C / 7 minutes, 1 cycle. Amplification products were electrophoresed through 1.0 % (w/v) agarose gels stained with ethidium bromide and visualized under UV light. Bands of the expected size (1.5 kb) were purified (QIAquick gel extraction kit, Qiagen) and used as templates for sequencing by an external contractor (Environmental Science & Research, Porirua, New Zealand). Assembled sequences were used as query strings for interrogation of the following databases: Ribosomal Database Project II (RDP, http://rdp.cme.msu.edu/) and GenBank (http://www.ncbi.nlm.nih.gov/).

3.2.6 Phylogenetic analysis

The 16S rRNA gene sequences of strains 0529 and DO1 and top matches from GenBank and RDP were aligned in BioEdit (Hall, 1999) using ClustalW. *Escherichia coli* ATCC 25922 (accession number DQ683069) was used as an outgroup. Aligned sequences were transported in Nexus format to MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001) to construct phylogenetic trees. MrBayes analysis comprised two simultaneous runs for three million generations, with four chains each. The trees were sampled every 100 generations, with the burn-in set at 20,000.

3.2.7 Histopathology

Bioassays using selected potential pathogens derived from the screening assays were conducted in order to process larvae for pathology. Larvae, treated as previously described, were placed into beakers containing 500 ml sterile seawater to give a final

concentration of 20 larvae ml^{-1}. Healthy larvae and larvae exposed to a known non-pathogenic bacterium were included as controls. A separate beaker was dedicated to each treatment at the following concentrations: 0529 10^4 CFU ml^{-1}, DO1 10^4 CFU ml^{-1}, 0548 10^4 CFU ml^{-1}, and a control. Sampling occurred daily for five days.

Samples of 15 ml were centrifuged at 1000g for 1 min (Heraeus Biofuge Primo, Thermo Scientific, Sweden) and the pellet of larvae transferred to 1.5 ml Eppendorf tubes for fixing. Larvae were fixed with 10% formalin for 48 hours at 4°C, the formalin was removed and larvae rinsed once with 70% ETOH by centrifugation at 4300g for 30 seconds (Eppendorf MiniSpin Plus). Larvae were re-suspended in 70% ETOH. Larvae were then centrifuged at 4300g for 30 seconds to pellet larvae, ETOH was removed via pipette and 50°C agar (4.5%) was added. Once solidified, each agar block containing larvae was removed and placed into a histology cassette for processing, sectioning and stained using haematoxylin eosin (H+E); performed at Aquatechnz, Wellington, New Zealand.

3.2.8 Testing Koch's postulates

The pathogenic status of isolates 0529 and DO1 was investigated by following Koch's criteria (Madigan & Martinko, 2006). Tests were performed under normal GSM hatchery conditions in 2.5 l flow-through tanks (King *et al*., 2005, Fig. 3.1). Water flow rate was 80 ml min^{-1}, larvae were held at a concentration of 200 larvae ml^{-1} and fed a 2:1 mix of *Chaetoceros calcitrans*:*Isochrysis galbana* to provide a final concentration in tanks of 40 cells μl^{-1}.

On the third day post-larval hatching, pathogen was added to larval tanks at concentrations of 10^{6-7} CFU ml^{-1} and water flow was stopped for 1-2 hours; aeration was continued. After such time, water flow was resumed and survival was monitored against control tanks containing non-inoculated larvae. The presence of symptoms and the percentage larval survival was recorded daily. The experiment was conducted in triplicate and concluded at seven days post-hatching. Isolates 0529 and DO1 were tested on two separate occasions, each using a separate cohort of larvae.

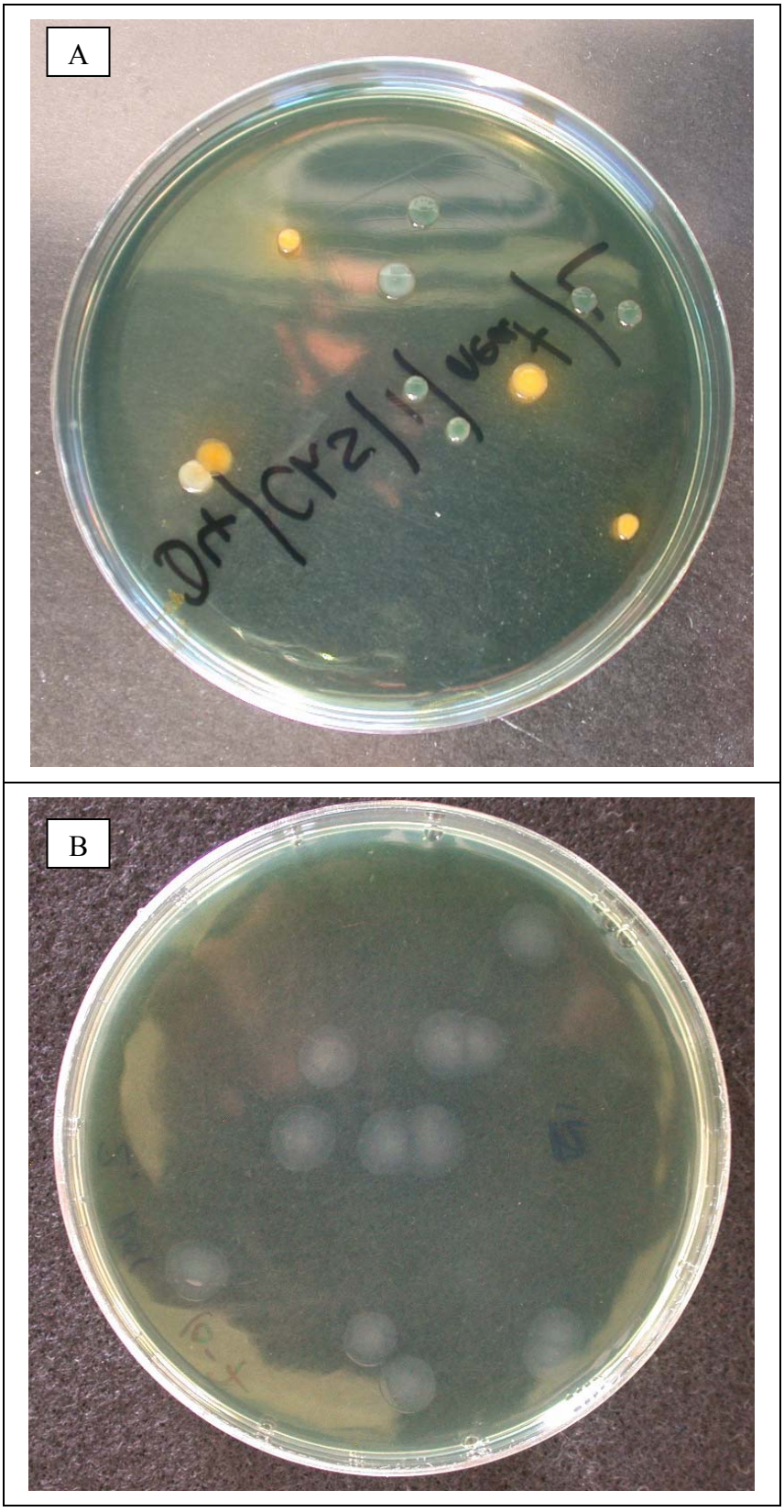
To confirm the presence of pathogens in the larvae, a 20 ml sample was taken from each tank and rinsed five times in a 45 μm screen with sterile seawater. The sample was then macerated and serially diluted in sterile 2% seawater before plating on the pre-determined selective media. Strain 0529 was recognised and identified by its green colony on TCBS agar (Acumedia, Michigan, USA) made to 2% salinity with natural seawater plus ampicillin, 5 μg ml^{-1} (Oxoid, Hampshire, England) (Fig 3.2), its oxidase positive reaction, gelatin hydrolysis and ONPG positive result. Strain DO1 formed 3 mm green swarming colonies on Ryans Ampicillin agar (Oxoid, Hampshire, England) after incubation at 25°C for 24 hours (Fig 3.2); it was identified by being oxidase positive, by its swarming growth and growth at 0.5% NaCl. Phenotypic characteristics were confirmed against control strains of 0529 and DO1. Each bacterium was streaked for purity and then stored at -70°C in Protect Bacterial Preservers (Technical Service Consultants Limited, Lancashire, UK).

The 16S rRNA gene was sequenced as described above. Once the phenotypic and molecular identity was confirmed, each isolate was re-inoculated into 2.5 l larval tanks,

as described above, for the second stage of Koch's postulates. Survival and bacterial presence was monitored for four days post-infection. Treatments were run in triplicate tanks and compared against test tanks inoculated with the original stored isolates and non-inoculated controls.

Figure 3.1 Greenshell™ mussel hatchery tank set-up at GACL

Figure 3.2 Colony appearance of (A) 0529, green colonies, and (B) DO1 on selective media.



3.2.9 Data analysis

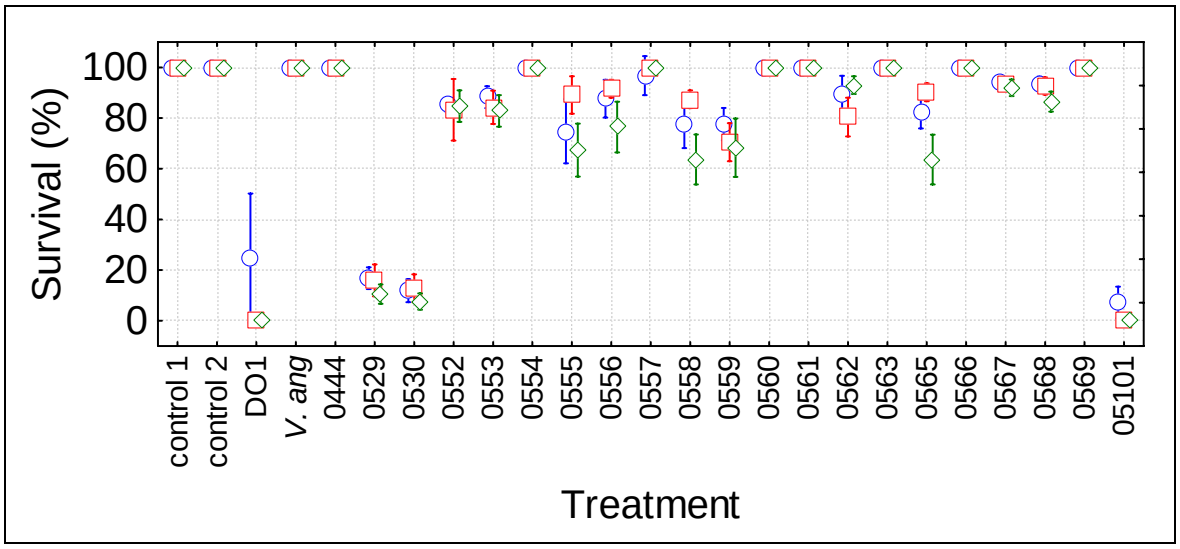
Percent survival data were arc sin square root transformed to approximate normality. Comparison followed multi factor ANOVA (p=0.05), treatment and inoculum concentration being the factors. Post hoc comparisons between survivals were compared using LSD test. Flask verification data were analysed using ANOVA. Post hoc differences were performed using LSD test.

3.3 Results

3.3.1 TCD bioassay screening for pathogens

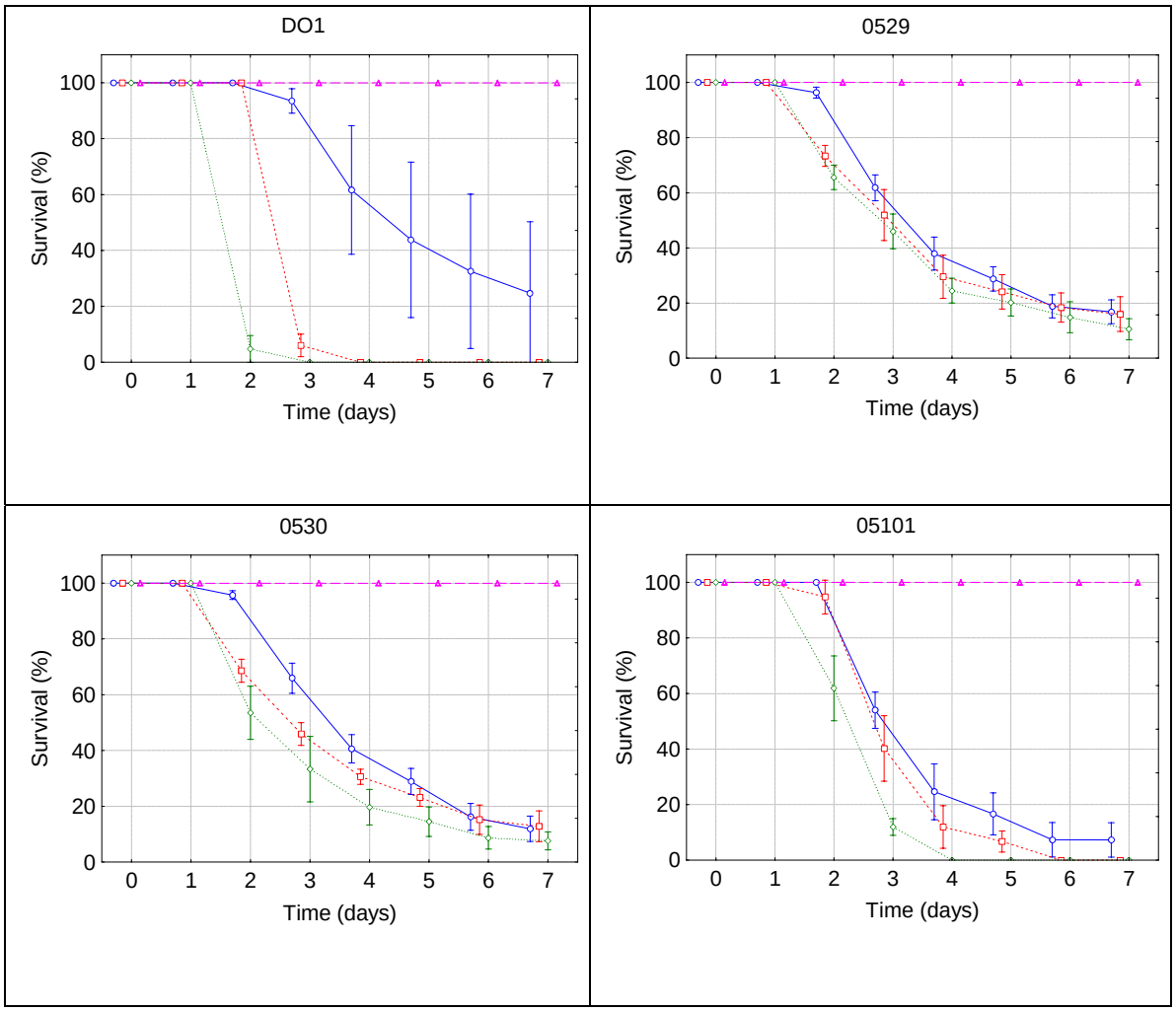
Covariate analysis demonstrated an effect caused by both isolate and inoculum size on larval survival (p = 0.00). Despite a statistical effect caused by inoculum concentration, Fig. 3.3 demonstrates that each isolate elicited a similar response regardless of inoculum concentration, i.e. it either caused large mortality or not. All treatments in which survival was under 93.8% were statistically lower than control treatments which had 100% survival over the duration of the experiment. The highest larval mortality occurred in treatments with the following isolates: DO1, 0529, 0530, and 05101. Mortalities in the presence of these four isolates were statistically higher than all other treatments regardless of inoculum size. The time-course of mortality occurring over seven days in treatments with the pathogens showed that regardless of initial inoculum size, mortality followed a similar pattern (Fig. 3.4). A lower concentration was sufficient to initiate infection under the static conditions of the bioassay.

Figure 3.3 Day 7 larval survival (%) in TCDs inoculated with various bacterial isolates. Bacteria were added at three concentrations: 10^2 CFU ml^{-1} (blue), 10^4 CFU ml^{-1} (red) and 10^6 CFU ml^{-1} (green). Non-inoculated larvae served as controls. Error bars denote 95% confidence intervals.



Note: control 1 and 2 = non-inoculated controls; *V. ang* = *Vibrio anguillarum* (ATCC 19264); DO1, 0444-05101 = bacterial isolates.

Figure 3.4 Percentage larval survival over seven days in isolates found to cause high mortality. Bacteria were added at three concentrations; 10^2 CFU ml^{-1} (blue), 10^4 CFU ml^{-1} (red) and 10^6 CFU ml^{-1} (green). Non-inoculated control larvae are represented in pink and maintained 100% survival. Error bars denote 95% confidence intervals.



3.3.2 The effect of water parameters on larval mortality during bioassays

Dissolved oxygen levels remained high for all treatments and were above 90% saturation (Fig. 3.5). Despite this, DO levels were statistically higher in 0529 and 05101 treatments (Table 3.1). No difference was found between pH levels of test treatments or controls ($p = 0.29$), which were approximately pH 8.17 (Table 3.1). Bacterial levels ranged from 10^5

to 10^6 CFU ml^{-1} in all treatments. On the fourth day, bacterial levels were statistically higher in 05101 than all other treatments (Table 3.1). On the seventh day, bacteria levels were higher in 05101 than DO1 and the control, but not 0529. Higher bacterial numbers did not manifest into higher mortality in 05101 (Fig. 3.5). Day 7 bacterial levels were not statistically different between DO1, 0529 and the control. Larval survival was very good in the control treatment during the experiment (91%, Table 3.1). When compared with the control, larval survival was significantly lower in DO1 and 0529 on both the fourth and seventh days (Table 3.1). There was no significant difference in survival between the control and 05101.

Figure 3.5 Dissolved oxygen (% saturation), pH, total bacterial numbers (CFU ml^{-1}) and larval survival (%) in flask cultures of larvae inoculated with potential pathogenic bacteria or not (control). Error bars denote 95% confidence intervals.

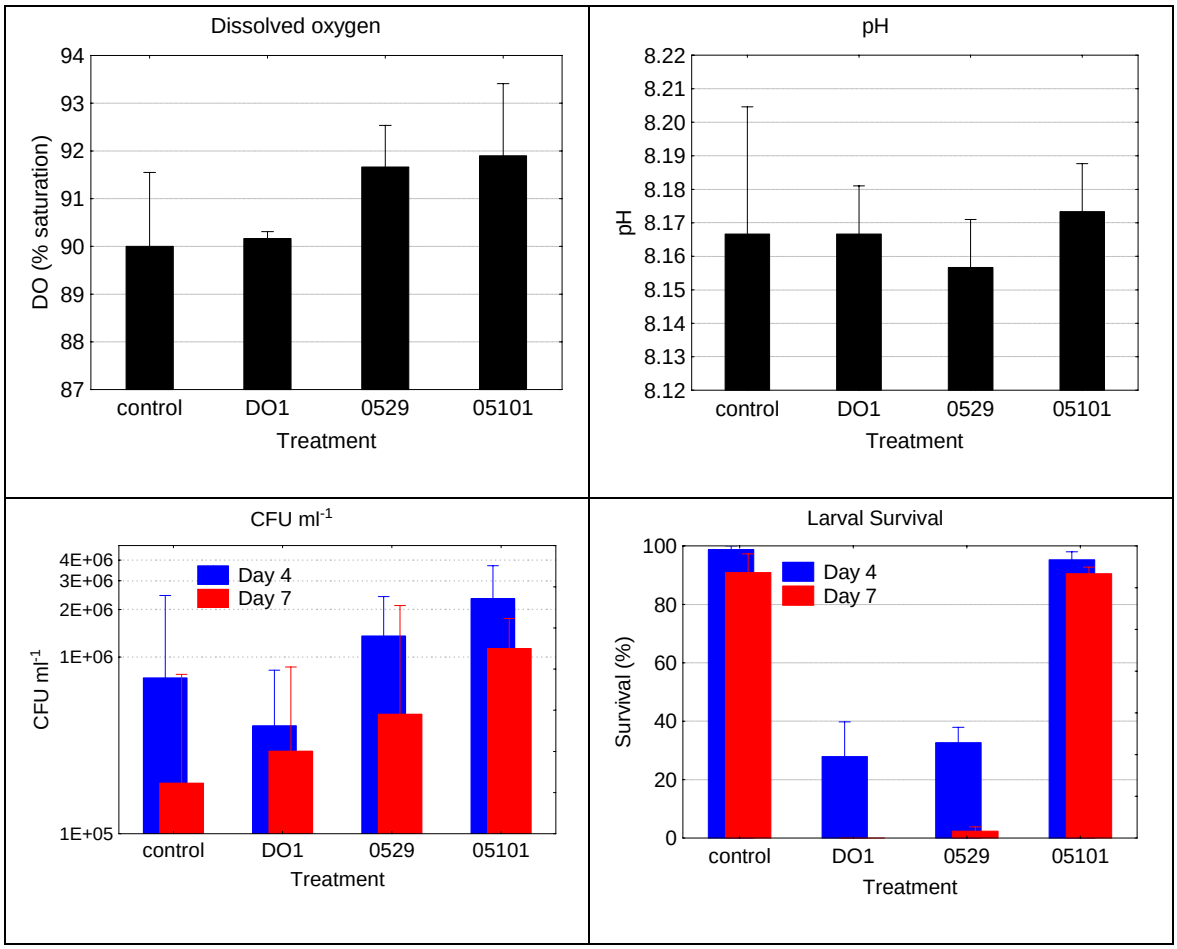


Table 3.1 Measurements of water parameters, bacterial levels and larval survival during larval challenge with potential pathogens (± 95% confidence intervals). Values not sharing the same superscript are statistically different ($p < 0.05$).

Parameter	Control	DO1	0529	05101
Day 7 DO	90.0 ± 1.6^{a}	90.2 ± 0.1^{a}	91.7 ± 0.9^{b}	91.9 ± 1.5^{b}
Day 7 pH	8.17 ± 0.04	8.17 ± 0.01	8.16 ± 0.01	8.17 ± 0.01
Day 4 CFU ml^{-1}	$8.3x10^{5} \pm 1.7x10^{6\,a}$	$4.3x10^{5} \pm 4.9x10^{5\,a}$	$1.5x10^{6} \pm 1.0x10^{6\,b}$	$2.4x10^{6} \pm 1.4x10^{6\,c}$
Day 7 CFU ml^{-1}	$2.0x10^{5} \pm 6.7x10^{5\,a}$	$3.1x10^{5} \pm 6.6x10^{5\,a}$	$5.1x10^{5} \pm 1.7x10^{6\,ab}$	$1.2x10^{6} \pm 6.3x10^{5\,b}$
Day 4 survival (%)	98.8 ± 1.2^{a}	27.9 ± 11.9^{b}	32.7 ± 5.3^{b}	95.3 ± 2.7^{a}
Day 7 survival (%)	90.9 ± 6.4^{a}	0.0^{b}	2.4 ± 1.5^{b}	90.5 ± 2.2^{a}

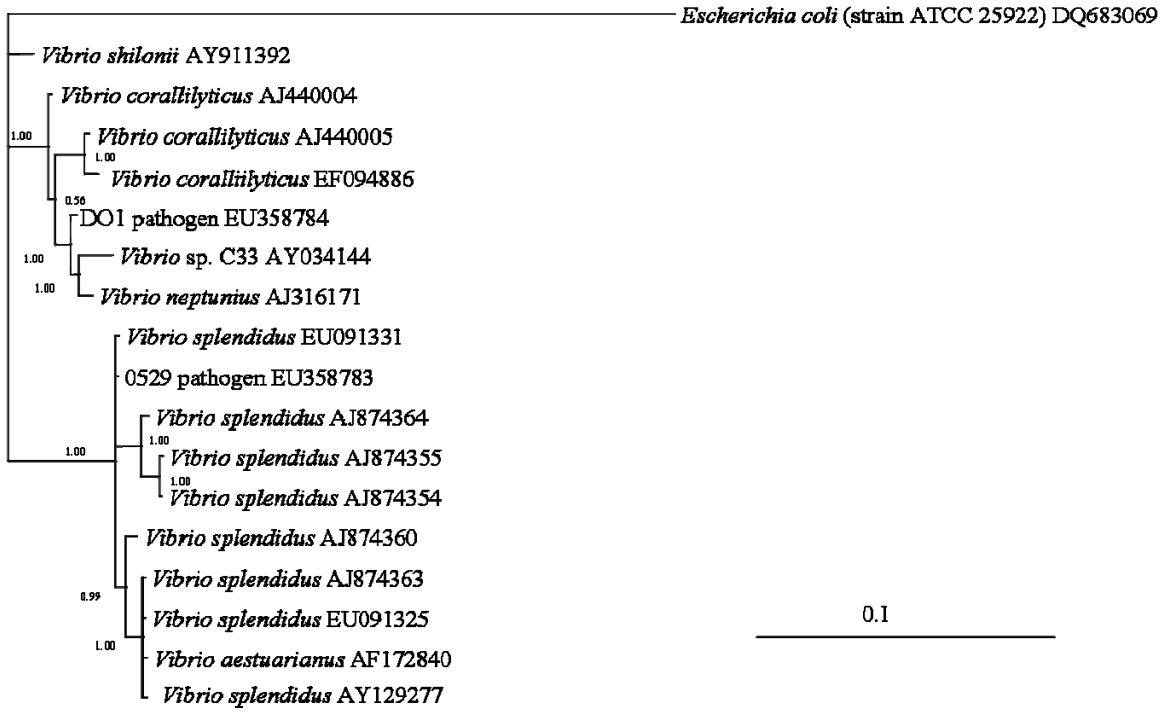
3.3.3 Identification of bacterial pathogens

Based on phenotypic characters and isolate origin, strains 0530 and 0529 appeared similar, while a separate experiment revealed that strain 05101 did not appear to reproduce the high larval mortality that was witnessed initially (Fig. 3.5). Hence, the study was pursued further with strains DO1 and 0529 only. Both strains were found to be motile Gram-negative rods, oxidase positive and catalase negative. Their biochemical activities are shown in Table 3.2. Phylogenetic trees were constructed for both pathogens following molecular identification (Fig. 3.6). Sequence data has been submitted to the GenBank database under the accession numbers EU358784 (DO1) and EU358783 (0529). Strain DO1 was identified as *Vibrio* sp., belonging to a clade that included three isolates of *V. coralliilyticus* amongst its members. It was 99.5% similar to one isolate of *V. coralliilyticus* and also *V. neptunius*. Strain 0529 was identified as *V. splendidus*, belonging to a clade including many isolates of *V. splendidus* and having 100% similarity to *V. splendidus*.

Table 3.2 Biochemical, morphological and physiological characteristics of GSM larvae pathogens: *Vibrio* sp. DO1 and *V. splendidus*.

Test	*Vibrio* sp. DO1	*V. splendidus* (0529)
Gram stain	-	-
Oxidase reaction	+	+
Catalase reaction	+	+
Motile	+	+
TCBS appearance	Yellow colonies	Green colonies
NaCl tolerance	0.5-5%	0.5-5%
Temperature growth range	17-37°C	17-30°C
Nitrate reduction	+	+
Indole production	-	-
Glucose fermentation	+	-
Arginine dihydrolase	+	-
Urea hydrolysis	-	-
Esculin hydrolysis	-	-
Gelatin liquefaction	+	+
ONPG	-	+
Glucose assimilation	-	-
Arabinose assimilation	-	-
Mannose assimilation	-	-
Mannitol assimilation	-	-
N-acetyl-Glucosamine assimilation	-	-
Maltose assimilation	-	-
Potassium gluconate assimilation	-	-
Capric acid assimilation	-	-
Adipic assimilation	-	-
Malate assimilation	-	-
Citrate assimilation	-	-
Phenylacetic acid assimilation	-	-

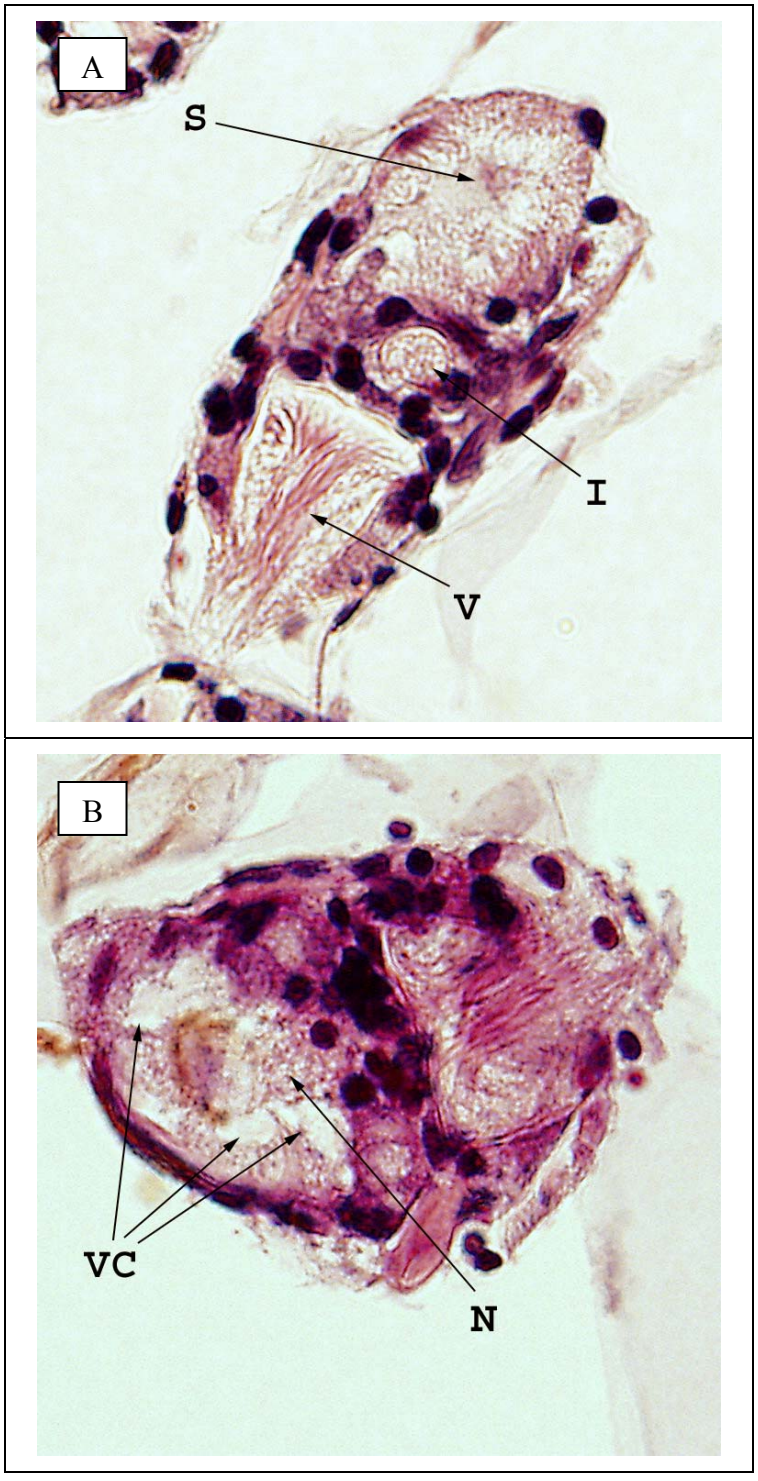
Figure 3.6 Phylogenetic trees for GSM larvae pathogens *V. splendidus* (0529) and *Vibrio* sp. DO1.

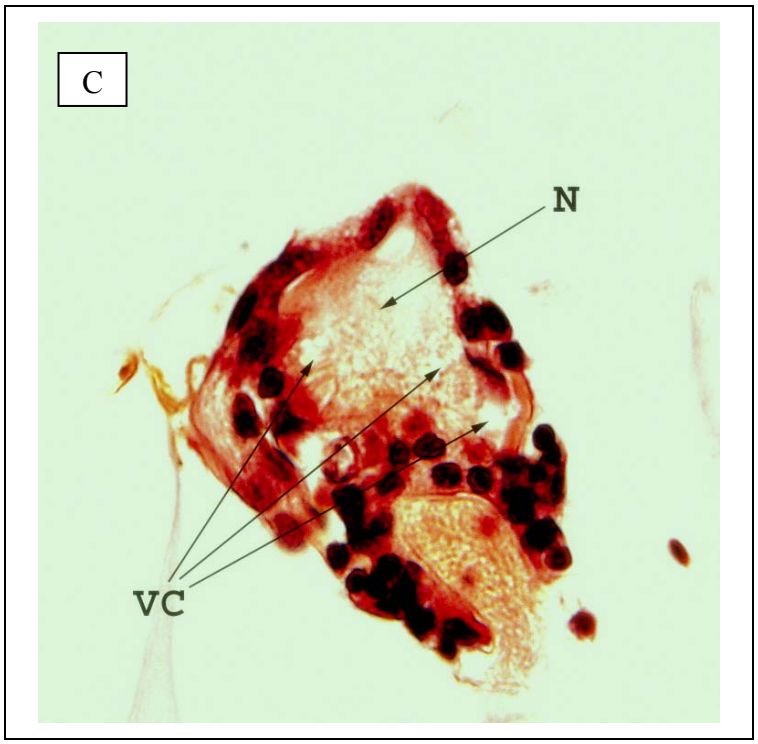


3.3.4 Histopathology

Gross symptoms of diseased larvae included irregular circular movements of larvae, detachment of cilia cells, aggregation of bacterial cells around larval velum, and deterioration of soft tissue until completely absent. Infection occurred largely in the digestive system (Fig. 3.7). The velum appeared not to be attacked, while necrosis and vacuolation in the digestive region was evident 24 hours post-inoculation of *Vibrio* sp. DO1 and 48 hours post-inoculation of *V. splendidus*.

Figure 3.7 Histopathology of GSM larvae: non-inoculated control (A), infected with *V. splendidus* (B) and infected with *Vibrio* sp. DO1 (C). S = stomach, I = intestine, V = velum, VC = vacuolation, N = necrosis. Magnification x 400, Stain H+E.

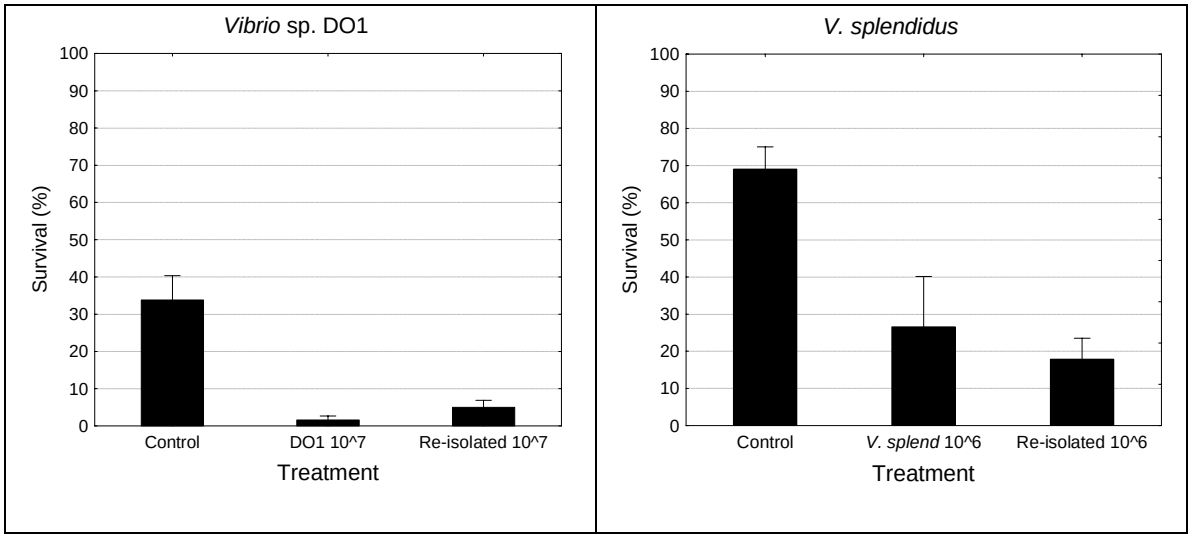




3.3.5 Testing Koch's postulates

Healthy larvae were infected successfully with the test pathogens as per Koch's postulates (Fig. 3.8). The identities of the pathogens re-isolated from infected larvae were confirmed against laboratory controls as the same strains inoculated initially. Inoculation of the re-isolated strain of *V. splendidus* caused 82% mortality. This was comparable to 74% mortality in larvae inoculated with the original strain, but significantly different from the 31% mortality in the controls ($p = 0.00$). Similarly, the re-isolated strain of *Vibrio* sp. DO1 caused 95% mortality, comparable with the initial DO1 strain at 98% mortality and statistically different from the control larvae 66% mortality ($p = 0.00$).

Figure 3.8 Survival of GSM larvae four days following inoculation with the initial pathogenic strain, the re-isolated pathogen strain, and the non-inoculated controls. Error bars denote 95% confidence intervals.



3.4 Discussion

Herein were isolated, identified and confirmed two bacterial pathogens of GSM larvae as per Koch's postulates. The similarities in gross pathology indicate that these pathogens were responsible for losses of larval batches at the GACL. Review of the published literature suggests that this is the first report on bacterial pathogens of marine mussel larvae. Anguiano-Beltrán *et al*. (2004) reported on a sub-lethal effect caused by *V. alginolyticus* on blue mussel larvae, *Mytilus galloprovincialis*, but did not describe virulent pathogenesis.

Isolate 0529, identified as *V. splendidus*, has previously been implicated in mortality of larval molluscs (Jeffries, 1982; Sugumar *et al.*, 1998; Gómez-León *et al.*, 2005). Further, *V. splendidus* has been shown to cause disease problems within New Zealand at a brill and turbot hatchery facility (Diggles *et al*., 2000). The mortality and pathology obtained

through infection with isolate 0529 demonstrated that the pathogenicity of *V. splendidus* extends also to marine mussels.

Identification of DO1 was less conclusive after database search of the 16S rRNA gene sequence. Molecular analysis designated DO1 to a clade including three strains of *V. coralliilyticus*, ranging from 99.3-99.5% similarity, and a *V. neptunius* strain, 99.5% similarity. *Vibrio coralliilyticus* is a known pathogen of coral, but has also been isolated from diseased oyster larvae in the United Kingdom (Ben-Haim *et al*., 2003). It is noteworthy that, in the present study, strain DO1 was also isolated from diseased oyster larvae at GACL hatchery indicating that, like *V. splendidus*, the host range for this strain encompasses other molluscs. The other close pairing, *V. neptunius*, has also been shown as a pathogen of the flat oyster, *Ostrea edulis* (Prado *et al*., 2005).

Whilst using API 20NE for *V. coralliilyticus* YB over two experiments, Ben-Haim & Rosenberg (2002) and Ben-Haim *et al*. (2003) obtained contrasting biochemical reactions for indole production, arginine dihydrolase, and urea. In addition, biochemical reactions for *V. splendidus* differed between this study and that of Gómez-León *et al*. (2005) using API 20E. Perhaps these identification systems are not appropriate for such marine organisms and it seems that sequencing of the 16S rRNA gene remains the best identification method.

Gross pathology observed in infected larvae was similar to that reported previously for a disease termed bacillary necrosis (Tubiash *et al*., 1965; Sugumar *et al*., 1998). However,

Elston (1999) suggested that the term bacillary necrosis should be replaced by a more descriptive name based on the type of infection; past work by Elston demonstrated a range of different bacterial diseases caused by vibrios (Elston & Leibovitz, 1980; Elston *et al*., 1981; Elston *et al*., 1999). In this study, the histopathology indicated that the presence of vibriosis, by both *V. splendidus* and *Vibrio* sp. DO1, was caused by an infection of the digestive system. Vacuolation of the tissues of the digestive tract was similar to that observed by Elston *et al*. (1981) in oyster larvae, *Crassostrea virginica*. Furthermore, necrotic tissue was observed in this region in the present study. Lane & Birkbeck (1999; 2000) have shown production of extracellular toxins to be a virulence mechanism in vibrios. This avenue warrants further investigation to assist management of vibriosis in GSM larvae.

The small scale bioassay methodology used in screening isolates for pathogenicity was a simple and effective tool for determining bacterial pathogens of GSM larvae. Similar approaches were utilized by Estes *et al*. (2004) who determined pathogens of Pacific oyster larvae and by Riquelme *et al*. (1996b) with scallop larvae. The ability of each pathogen to infect *in vitro* at low concentrations (10^2 CFU ml^{-1}) was, perhaps, a consequence of the static nature of the bioassay and the pre-treatment of larvae with antibiotics which removed most of the naturally occurring bacteria from the system. However, subsequent investigations, which involved infecting larvae under hatchery conditions without antibiotic pre-treatment, showed that both organisms were still pathogenic to larvae, albeit at higher doses (10^5 CFU ml^{-1}, refer to Chapter 5). Therefore, the higher bacterial level required for infection under natural conditions was probably due

to the natural resident flora in the water and the continuous renewal of this water in the hatchery flow-through system. By enhancing the pathogenic effect, the TCD bioassay used in this study is a sensitive tool to screen for pathogens, but the results need to be verified under normal hatchery conditions.

This work described for the first time bacterial pathogens of GSM larvae. The demonstration of *V. splendidus* pathogenicity further confirmed the species' status as a widespread pathogen of marine mollusc species, whilst the *V. coralliilyticus/V. neptunius* findings suggest that these bacteria are more of a potential problem for mollusc larvae than previously thought. These vibrio species represent a risk in GSM hatcheries and effective measures to prevent and/or manage these organisms are investigated in the following chapters.

Chapter 4

Infection of Greenshell™ mussel larvae, *Perna canaliculus*, using two *Vibrio* pathogens: a hatchery model

4.1 Introduction

Two pathogens of GSM larvae were previously demonstrated using a static TCD bioassay. They were identified as *V. splendidus* and *Vibrio* sp. DO1, a *Vibrio coralliilyticus/neptunius*-like isolate (refer to Chapter 3). However, in the hatchery production environment of GSM, conditions are markedly different from those of the TCD bioassay. A continuous water flow exists, food and aeration are provided and a milieu of ambient microflora is present. In order to understand the dynamics and facilitate effective management of microbial infections occurring at a hatchery facility, it is necessary to have an understanding of the conditions that initiate infection.

Many studies previously demonstrated experimental infections of aquatic animals: shrimp (Roque *et al*., 1998; Soto-Rodríguez, 2006), fish (Bergh *et al*., 1997; Itano *et al*., 2006), and molluscs (Sainz *et al*., 1998; Estes *et al*., 2004; Gay *et al*., 2004). Currently, there is a lack of literature on infection models during hatchery conditions, although Planas *et al*. (2005) developed an infection model under hatchery conditions where static water rather than flow-through systems prevailed. Likewise, there are few reports on infection models in flow-through water systems. Arkush *et al*. (2005) described an infection model in white sea bass incorporating flow-through water and intraperitoneal injection of the pathogen; however, this method of administration is not possible with GSM larvae. Additionally, both Nordmo *et al*. (1997) and Itano *et al*. (2006) utilized flow-through water systems in documenting experimental infections in Atlantic salmon and yellowtail fish respectively. In these studies, the animals were exposed to the pathogen by immersion periods of 45 min and 10 min (for Atlantic salmon and yellowtail

respectively) before being returned to the flow-through systems. Literature on experimental infection of molluscs has either described adult animals (Hervio *et al*., 1995; Gay *et al*., 2004), or larvae tested in static *in vitro* conditions (Sainz *et al*., 1998; Sugumar *et al*., 1998; Estes *et al*., 2004).

Herein, the effect of varying levels of *V. splendidus* and *Vibrio* sp. DO1 on GSM larvae during flow-through hatchery conditions was investigated, thereby allowing the construction of an experimental infection model. This model would permit future hatchery-based trials on treatments for bacterial infections. Additionally, it would also allow the introduction of routine monitoring of these pathogens in order to predict and, consequently, prevent impending larval crashes.

4.2 Materials and methods

4.2.1 Experimental animals

Refer to Chapter 3.2.2

4.2.2 Culture and harvest of bacteria

Two pathogens of GSM larvae, *Vibrio* sp. DO1 and *V. splendidus*, previously isolated from diseased larvae, were used. Each bacterium was revived from -70°C stores in TSB-2%Sea. Test isolates were streaked to ensure purity and sub-cultured on TSA-2%Sea three times.

Each pathogen was sub-cultured into separate 10 ml volumes of Marine Broth (MB, Difco) and incubated at 25°C for 10 hours. 0.1 ml of each culture was further sub-cultured into another 10 ml MB and further incubated for 10 hours at 25°C. The final 10 ml volume of each culture was aseptically transferred into 500 ml MB and incubated for 20 hours at 24°C-27°C on a New Brunswick G10 Gyrotory Shaker (N.J., USA) at 150 rpm.

The cell concentration of final broth cultures was determined by optical density at 600nm (Appendix 2) using a PharmaSpec UV-1700 spectrophotometer (Shimadzu). Broth cultures were then centrifuged (3700 rpm, 10 min, 15°C, Beckman J2-21M/E) and washed twice in sterile seawater. Supernatant-free cells were re-suspended in sterile seawater to their original concentrations (approximately 10^9 CFU ml^{-1}) and transported to the GACL for use in experiments. Prior to use in the experiments, 10-fold dilutions of the washed cultures were made and then surface-spread plated for enumeration on TSA-2%Sea to verify experimental concentrations.

4.2.3 Experimental design

Pathogens were added to day 3 post-hatching larvae housed in 2.5 l flow-through tanks (King *et al*., 2005). Pathogens were added at a range of concentrations: 10^4-10^8 CFU ml^{-1}. For exposure to the pathogens, water flow was switched off and static conditions maintained for one hour (*V. splendidus*) and two hours (*Vibrio* sp. DO1); after which times, water flow was resumed. Treatments were run in triplicate. Tanks without the addition of either bacterial species served as controls. Each day for the duration of the

experiment, triplicate aliquots (250 µl, containing approximately 50 larvae per aliquot) were removed from the tanks and the percentage larval survival determined. On alternate days, either outflow barrier-screens (nylon mesh size 40 µm) were cleaned, or full tank cleanings were performed (screens, tanks and larvae). During cleaning of the larvae, a 45 µm screen was used to ensure retention of the initial starting population, thereby allowing measurement of the cumulative larval survival for the duration of the larval period. Experimental duration was four days following pathogen inoculation. Four independent experiments were carried out for each pathogen.

The presence or absence of the pathogens was monitored daily in both the water column and larval samples. A 20 ml water sample (containing larvae) was taken from the tanks and passed through a 45 µm screen. The first 20 ml to pass through the screen were retained for assessing the presence of the pathogen in tank water. Larvae that were retained on the screen were rinsed five times with 50 ml sterile seawater and macerated using a Bosch homogenizer on speed No. 2 for 30 seconds. Samples were serially diluted in sterile 2% seawater before plating on selective media. The pathogens were isolated and identified as previously described (refer to Chapter 3).

4.2.4 Data analysis

Percent survival figures were arc sin square root transformed to approximate normality. Treatment differences were analysed using ANOVA ($p=0.05$). Post hoc comparisons between survivals were compared using Tukey's HSD test.

4.3 Results

4.3.1 Dose response

The concentrations of the pathogens tested resulted in various levels of larval response (Fig. 4.1). In *Vibrio* sp. DO1, levels of $\leq 10^5$ CFU ml^{-1} did not cause significant mortality ($p > 0.05$) by day 6, and 10^6 CFU ml^{-1} was the minimum dose required to initiate infection given the two-hour static period of water flow ($p = 0.00$). *V. splendidus* caused larval infection after lower exposure than that required by *Vibrio* sp. DO1; 10^5 CFU ml^{-1} at one-hour static exposure caused significant larval mortality ($p = 0.00$). With both pathogens, mortality rates further increased as a result of exposure to pathogen concentrations higher than the minimum infective dose.

In all repeat experiments, larval survival was significantly lower in the pathogen doses tested ($10^6/10^7$ CFU ml^{-1} for *Vibrio* sp. DO1, $10^5/10^6$ CFU ml^{-1} for *V. splendidus*) than in the controls (Tables 4.1 & 4.2). The average survival observed across all experiments with *Vibrio* sp. DO1 was 3.6% and 30.7% for the high and low dose of the pathogen respectively, compared with 65.2% in controls. For *V. splendidus*, mean survival across all repeat experiments was 27.3% and 42.1% for the high and low dose of pathogen respectively, compared with 77.3% in controls. Mean values of all pathogen treatments had significantly lower larval survival than controls.

Figure 4.1 Time course of GSM larval survival when exposed to varying levels of pathogens. Data points represent mean larval survival ± 95% confidence intervals.

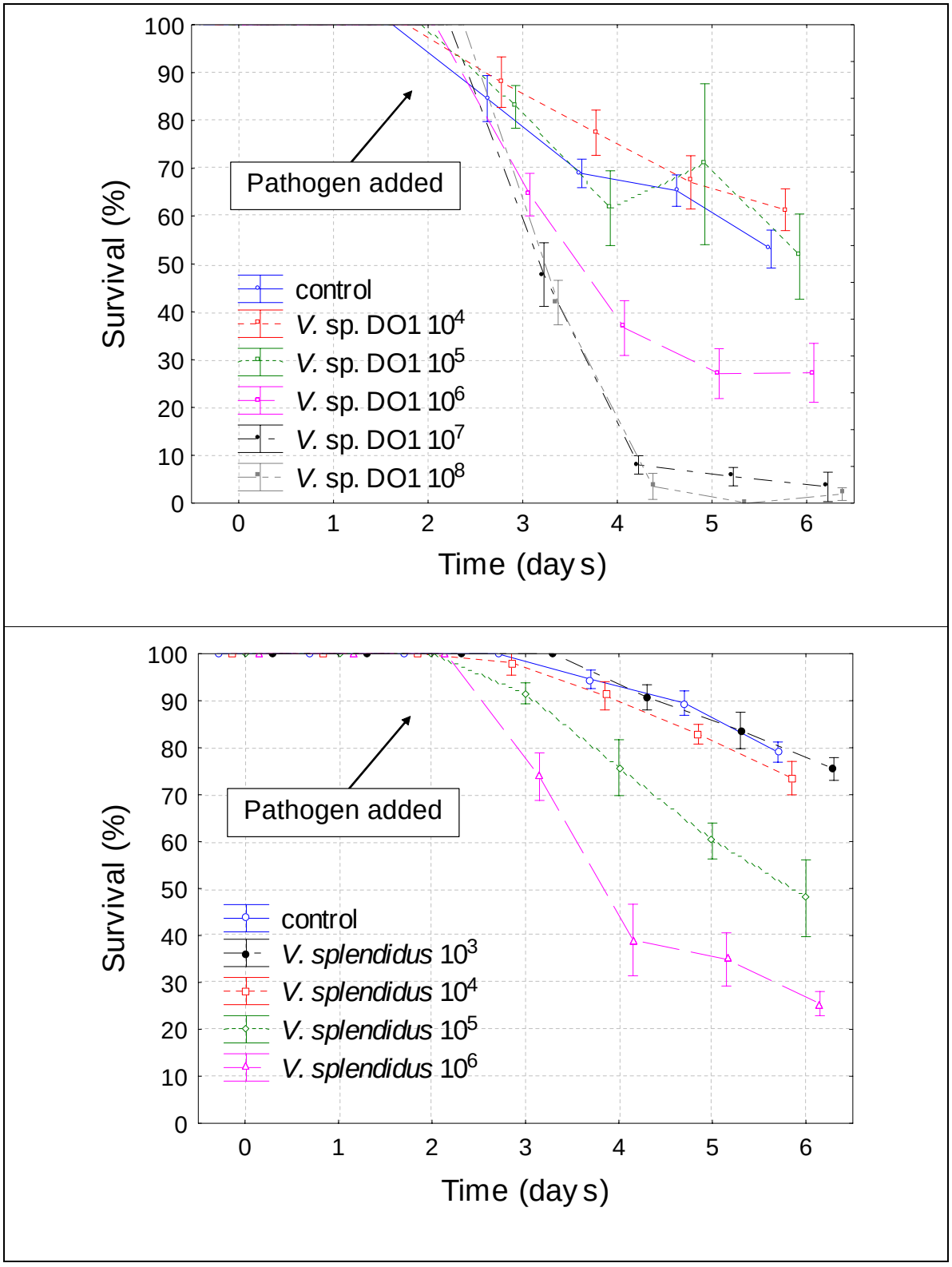


Table 4.1 GSM larval survival from four experiments involving pathogen-challenges with *Vibrio* sp. DO1 (day 4 post-infection). Values in each column not sharing the same superscript are statistically different ($p < 0.05$).

Treatment	Trial 1	Trial 2	Trial 3	Trial 4	Mean
Control	53.1 ± 4.0^{a}	33.8 ± 6.5^{a}	89.6 ± 3.4^{a}	84.2 ± 3.9^{a}	65.2 ± 8.1^{a}
Vibrio sp. DO1 10^6	27.3 ± 6.2^{b}	8.3 ± 3.0^{b}	56.5 ± 4.8^{b}	-	30.7 ± 8.3^{b}
Vibrio sp. DO1 10^7	3.1 ± 3.1^{c}	1.6 ± 1.1^{c}	2.5 ± 2.8^{c}	7.0 ± 4.8^{b}	3.6 ± 1.5^{c}

Table 4.2 GSM larval survival from four experiments involving pathogen-challenges with *V. splendidus* (day 4 post-infection). Values in each column not sharing the same superscript are statistically different ($p < 0.05$).

Treatment	Trial 5	Trial 6	Trial 7	Trial 4	Mean
Control	79.1 ± 2.2^{a}	69.0 ± 6.0^{a}	77.0 ± 4.6^{a}	84.2 ± 3.9^{a}	77.3 ± 2.6^{a}
V. splendidus 10^5	47.9 ± 8.2^{b}	20.8 ± 6.6^{b}	57.6 ± 10.1^{b}	-	42.1 ± 7.5^{b}
V. splendidus 10^6	25.5 ± 2.5^{c}	26.6 ± 13.6^{b}	24.8 ± 4.9^{c}	32.1 ± 5.9^{b}	27.3 ± 3.5^{b}

4.3.2 Bacteriology

Pathogens were detected in larvae and the surrounding water throughout the course of the experiment in tanks receiving the higher pathogen dosages, i.e. $\geq 10^6$ CFU ml^{-1} for *Vibrio* sp. DO1, and $\geq 10^5$ CFU ml^{-1} for *V. splendidus*. Test pathogens were absent in the water column and larvae in both the control tanks and in tanks with pathogen doses of $\leq 10^5$ CFU ml^{-1} *Vibrio* sp. DO1, and $\leq 10^4$ CFU ml^{-1} for *V. splendidus*.

4.4 Discussion

This study demonstrated a reproducible infectious dose of pathogens, *Vibrio* sp. DO1 and *V. splendidus*, to GSM larvae under flow-through hatchery conditions using a defined

protocol. Larval survival in control treatments varied between 33.8% and 89.6% (average 71.2% ± 4.4) across all experiments, illustrating that batch variability existed; possibly due to genetic differences of the larvae, potential health differences of the cohorts and differing environmental factors during experiments, such as extraneous bacteria. Despite this variation, dosages equal to or above 10^6 for *Vibrio* sp. DO1 and 10^5 CFU ml^{-1} for *V. splendidus*, consistently affected larval mortality significantly greater than that of the controls during four independent experiments (Tables 4.1 & 4.2), thereby confirming reproducibility of the infection model.

Previous mollusc larval infection models were conducted *in vitro*, without water exchange (Sainz *et al*., 1998; Sugumar *et al*., 1998; Estes *et al*., 2004). Such laboratory conditions are not representative of the naturally occurring hatchery situation and favour the effect of the pathogen. Indeed, in earlier work with *Vibrio* sp. DO1 and *V. splendidus* on GSM larvae, it was noted that levels as low as 10^2 CFU ml^{-1} initiated infection using *in vitro* static conditions (refer to Chapter 3). This is markedly lower than the minimum infectious dosages required in flow-through hatchery conditions that were witnessed in the present study.

Preliminary experiments showed that infection was more difficult to establish with *Vibrio* sp. DO1 and, hence, the protocol was designed to allow for the successful infection by both pathogens. This was achieved by turning off the water flow for two hours of static exposure with *Vibrio* sp. DO1 rather than one hour as with *V. splendidus*. The different ability of each pathogen to cause infection might have been due to different attachment

capabilities between the two; a mechanism previously shown to be important in *Vibrio* sp. infections. Grisez *et al*. (1996) suggested that, in *Vibrio anguillarum* infection of turbot larvae, the binding capabilities of *V. anguillarum* to brush border membranes of the intestinal epithelium might play an important role in its virulence. In the following chapter (Chapter 5), it is proposed that interference of pathogen attachment to GSM larvae, by probiotic bacteria, reduced the pathogenic effect, suggesting that attachment is important for the virulence of *Vibrio* sp. DO1 and *V. splendidus*.

Of interest is that, on occasion, it was noted that some of the larvae in the pathogen exposed tanks not only survived infection but appeared also to develop similar to the controls over time. This finding might be of considerable significance because the occurrence of similar larval development between pathogen exposed and pathogen non-exposed larvae could infer that the surviving larvae in the pathogen exposed tanks possessed an intrinsic resistance to these pathogens. Therefore, the infection model might allow for the selection of vibriosis resistant animals, similar to the study by Hervio *et al*. (1995).

This study demonstrated procedures and the effective pathogenic doses required to initiate infection of GSM larvae under hatchery production conditions. Study of the literature suggests this to be the first report where an infection model has been developed using mollusc larvae in a flow-through rearing process. The method is simple and reproducible, requiring that the water need be stopped only for a short period during pathogen exposure. With an infection model for GSM larvae developed, *in situ*

procedures to alleviate the effect of these were investigated and are presented in the following chapters.

Chapter 5

Screening for probiotics of Greenshell™ mussel larvae, *Perna canaliculus*, using a larval challenge bioassay

5.1 Introduction

Many studies have illustrated significant survival of animals challenged by bacterial pathogens as a result of probiotic use (Gibson *et al*., 1998; Rengpipat *et al*., 1998; Ruiz-Ponte *et al*., 1999; Kumar *et al*., 2006; Li *et al*., 2006; Taoka *et al*., 2006). Concerning probiotics for mollusc culture, much of the research investigated the Chilean scallop, *Argopecten pupuratus* (Riquelme *et al.*, 1997; Avendaño & Riquelme, 1999; Riquelme *et al.*, 2000; Riquelme *et al*., 2001). These researchers concluded that a *Vibrio* sp., *Pseudomonas* sp. and *Bacillus* sp. allowed completion of its larval cycle without the use of antibiotics. Other probiotic work has been published on the Pacific oyster, *Crassostrea gigas*, (Douillet & Langdon, 1993; 1994; Gibson *et al*., 1998; Elston *et al*., 2004), the great scallop, *Pecten maximus*, (Ruiz-Ponte *et al*., 1999) and the Manila clam, *Ruditapes philippinarum*, (Castro *et al*., 2002). These studies named *Alteromonas* sp., *Aeromonas media*, *Roseobacter* sp. and *Vibrio* sp. as probiotics. To date, there is no published literature on probiotics for mussels.

The aim of this study was to use a TCD bioassay to screen non-pathogenic, naturally-occurring bacterial strains from the hatchery environment as potential probiotics for GSM larval production. The study herein describes, for the first time, probiotic bacteria effective against pathogens of mussel larvae and demonstrates the benefits of a bioassay technique that includes GSM larvae in the first stage of probiotic screening.

5.2 Materials and methods

5.2.1 Isolation, culture and storage of bacteria

Bacteria were isolated from the GACL during 2005. Bacteria were obtained from three sources: (i) macerated healthy GSM larvae, (ii) hatchery seawater and (iii) microalgae cultures supporting the growth of healthy larvae. Isolation of bacteria was performed on TSA-2%Sea. Samples were serially diluted in sterile 2% seawater and plated out. Colonies were chosen on the basis of their dominance in cultures reflecting their dominance in the production system. Isolates were streaked for purity and stored at -70°C in Protect Bacterial Preservers (Technical Service Consultants Limited, Lancashire, UK).

Prior to use in experiments, bacteria were revived in broth (TSB-2%Sea), streaked and sub-cultured three times on TSA-2%Sea at 22ºC to ensure purity. For the experiments, bacteria were cultured on TSA-2%Sea for 48 hours at 22°C prior to inoculation of larvae. Bacteria were then suspended in 10 ml of 2% sterile seawater to a concentration of 10^9 CFU ml^{-1} using MacFarland standards. The turbidity estimates were verified by serial dilution and surface-spread plate counts.

5.2.2 Experimental animals

Refer to Chapter 3.2.2

5.2.3 Probiotic screens

5.2.3.1 Effect of probiotic pre-exposure time

Probiotic screens were performed using a pathogen-challenge bioassay developed in 12-well TCDs. Two pathogens of GSM larvae, *V. splendidus* and a *V. coralliilyticus/neptunius*-like isolate, *Vibrio* sp. DO1, were used. Before full-scale probiotic screening, an investigation was conducted to determine whether the time of larval exposure to probiotic, prior to pathogen challenge, influenced the protection afforded by the probiotic. Pre-exposure periods of both two and 20 hours were tested. Ten isolates were randomly chosen and tested for beneficial effect versus the pathogen. Larvae were transferred by pipette into TCDs containing 4 ml sterile seawater at approximately 10 larvae ml^{-1}. Each test isolate to be investigated was added to TCD wells at 10^5 CFU ml^{-1}. After periods of two or 20 hours, the pathogen was added at 10^3 CFU ml^{-1}, a concentration previously observed to initiate infection in the assays (refer to Chapter 3). Larval survival was monitored for seven days. Each treatment had six replicates. Each test bacterium was assigned to a separate 12-well TCD to avoid potential aerosol contamination between treatments. Control treatments included a TCD with non-inoculated larvae and TCDs containing pathogen-infected larvae alone.

5.2.3.2 Screening for probiotics in a TCD challenge bioassay

Dominant colonies from hatchery sources were chosen as test isolates. Additionally, a known probiotic strain to shellfish larvae and fish (Gibson *et al*., 1998; Lategan *et al*. 2004), *Aeromonas media* (ATCC 33907), was included as a control strain. About 40

larvae were added to each well of a TCD where each well contained 4 ml of sterile seawater without food. Only six wells per 12-well TCD were used, i.e. six replicates per treatment. A total of 69 test isolates were individually screened for activity against the two pathogens during three separate screenings (14/11/2005, 14/1/2006, 19/3/2006). Test isolates were inoculated at a concentration of 10^5 CFU ml^{-1} 20 hours prior to pathogen addition. Pathogens, *Vibrio* sp. DO1 and *V. splendidus*, were added at 10^3 CFU ml^{-1}. Larval survival was measured over seven days. Tissue culture dishes containing non-inoculated larvae served as survival controls, while those inoculated with the pathogen alone served as mortality controls. Larvae were maintained at 19°C throughout the experiment. A few isolates which were identified as potential probiotics during the first screening were repeated during the latter screenings to establish the reliability of the TCD bioassay and test isolate results.

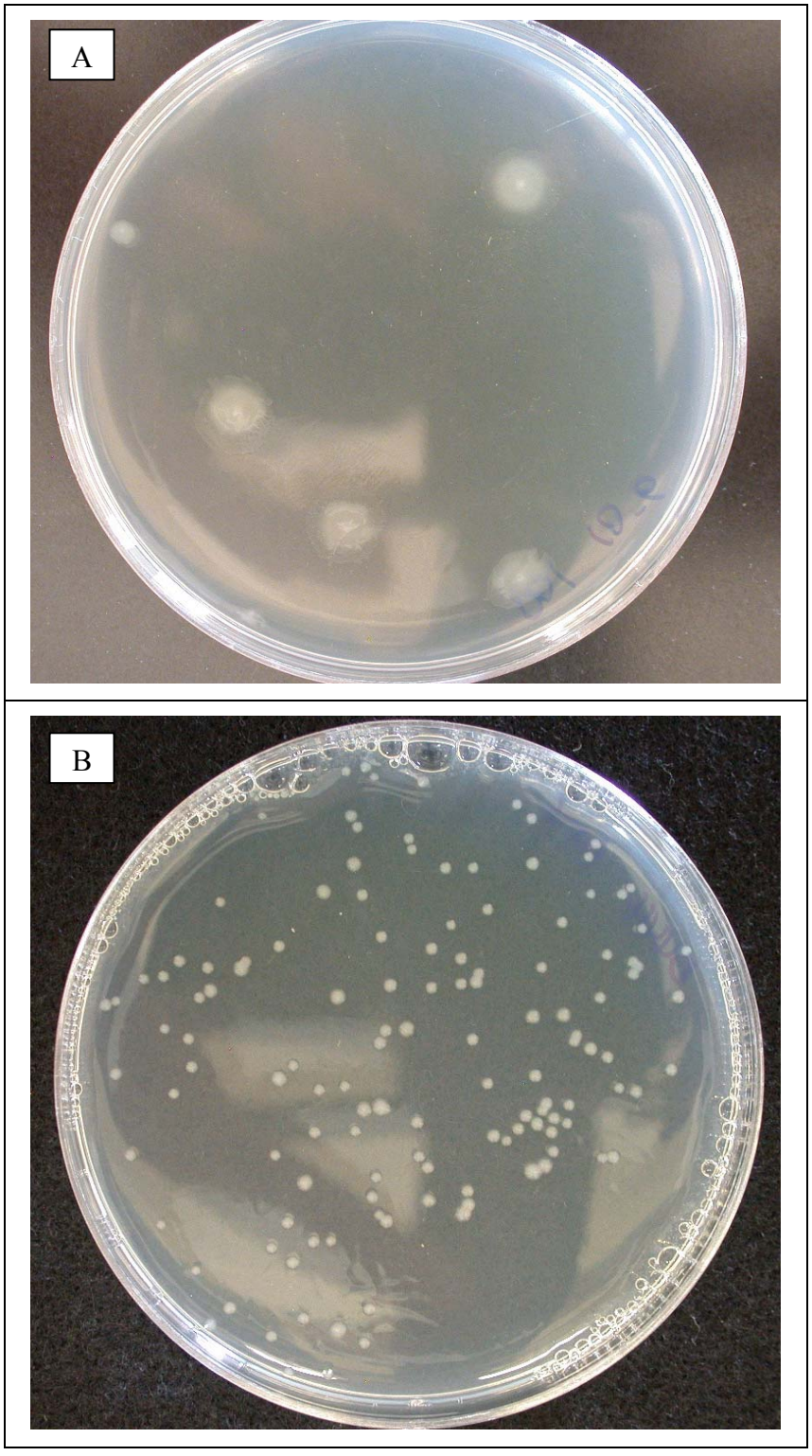
5.2.4 Bacterial levels during a challenge

In order to gain insight into the population dynamics occurring during a probiotic-pathogen challenge, the numbers of each bacterium were monitored during a bioassay incorporating the potential probiotic strain, 0444, and pathogen *Vibrio* sp. DO1. Strain 0444 was chosen as it had displayed protection against both pathogens. Larvae were set up in TCDs as described above. Strain 0444 was added to the wells at 10^6 CFU ml^{-1} and incubated for 20 hours at 19°C. *Vibrio* sp. DO1 was then added to separate TCDs at 10^2, 10^3 and 10^4 CFU ml^{-1}. Larvae inoculated with *Vibrio* sp. DO1 at concentrations of 10^2 and 10^4 CFU ml^{-1}, but without isolate 0444, served as pathogen controls. On days 1, 3, 5, and

7, water from a TCD well was serially diluted in sterile 2% seawater, plated onto TSA-2%Sea and incubated at 22°C.

Prior antibiotic treatment of the larvae reduced the level of extraneous bacteria, allowing both the pathogen, *Vibrio* sp. DO1, and the potential probiotic, 0444, to be differentiated on the basis of colony appearance (Fig. 5.1). *Vibrio* sp. DO1 was easily distinguished by characteristic 5 mm oxidase-positive, light-brown, spreading colonies within 24 hours, whilst 0444 was recognised by slow-growing, 1 mm oxidase-positive, white colonies (48-72 hours incubation). To aid with morphological comparisons, pure cultures of both pathogen and potential probiotic were set up simultaneously on TSA-2%Sea as controls and incubated alongside test sample enumeration plates.

Figure 5.1 Colony appearance of (A) *Vibrio* sp. DO1 and (B) isolate 0444 on TSA-2%Sea.



5.2.5 Probiotic pilot tests in the hatchery

Hatchery pilot trials were conducted to establish whether the probiotic benefit observed in the TCD bioassays could be transferred to the normal hatchery production conditions; eleven shortlisted isolates were chosen and tested. The shortlist was generated from larval survivals afforded during TCD bioassays and partial identifications using Vitek II; identifications were performed on those isolates which displayed beneficial properties in TCD bioassays. However, based on the Vitek II partial identifications, some isolates appeared to be potentially harmful bacteria and these were discarded from further testing. Experiments were conducted at the Glenhaven Aquaculture Centre. Larvae were contained in 2.5 l conical flow-through tanks at approximately 200 larvae ml^{-1} (King *et al.*, 2005). The water flow rate was 80 ml min^{-1}, aeration was provided, water temperature was 19°C and larvae were fed a 2:1 mix of *Chaetoceros calcitrans*:*Isochrysis galbana* to provide a final concentration in tanks of 40 cells μl^{-1}.

Treatments included larvae inoculated daily with each potential probiotic at 10^8 CFU ml^{-1} and challenged with *Vibrio* sp. DO1 and *V. splendidus* on the third day following hatching. Water flow was stopped for two hours during daily probiotic treatment, but aeration continued. Larvae were challenged at 10^6 CFU ml^{-1} with *Vibrio* sp. DO1 and at 10^5 CFU ml^{-1} with *V. splendidus*, in separate experiments (refer to Chapter 4). Pathogens were added after probiotic treatment on the third day and water remained static for a further two hours and one hour during challenge with *Vibrio* sp. DO1 and *V. splendidus* respectively. Control tanks included larvae with no additional inoculum, larvae with probiotics added alone each day and larvae challenged with the pathogen alone on the

third day. Treatments were non-replicated. Larval survival was measured on the sixth day following hatching, based on triplicate 250 µl aliquots from each tank.

5.2.6 Data analysis

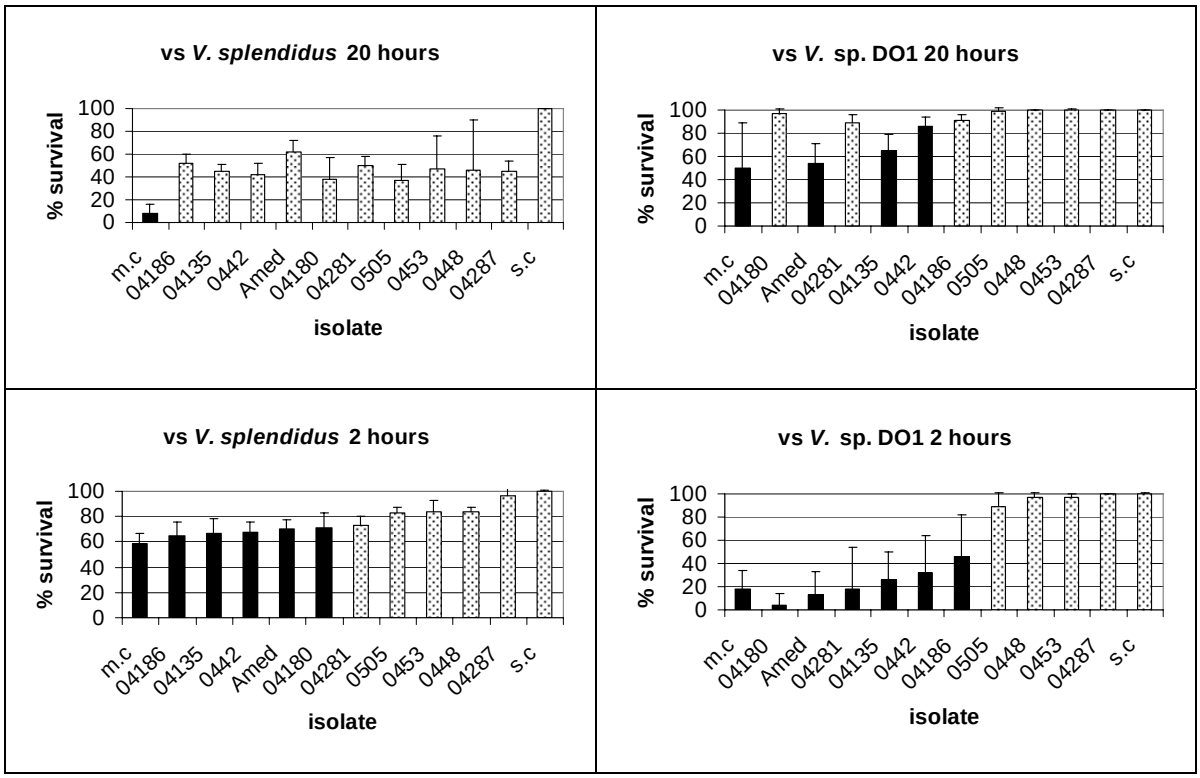
Percent survival figures were arc sin square root transformed to approximate normality. Treatment differences were analysed using ANOVA (p=0.05). Post hoc comparisons between survivals were compared using Tukey's HSD test. Since hatchery pilot studies were non-replicated, resulting in increased variance in the population mean estimates, post hoc comparisons were made using LSD test, the least conservative method.

5.3 Results

5.3.1 Probiotic screening: effect of probiotic pre-exposure

Bioassays utilizing 20 hours pre-exposure time of probiotic test isolates before challenge with *V. splendidus* showed a protective effect in all isolates (Fig. 5.2). In bioassays with a two-hour pre-exposure, five of these isolates did not provide a protective effect. Similarly, in bioassays challenged with *Vibrio* sp. DO1, seven isolates provided a benefit given 20 hours pre-exposure, while only four did after two hours pre-exposure (Fig. 5.2).

Figure 5.2 Day 7 larval survival in bioassays utilizing 20- or 2-hour pre-exposure of putative probiotic before pathogen addition. Black columns are not statistically different to the pathogen controls, whereas speckled columns are statistically different to the pathogen controls (p = 0.05).



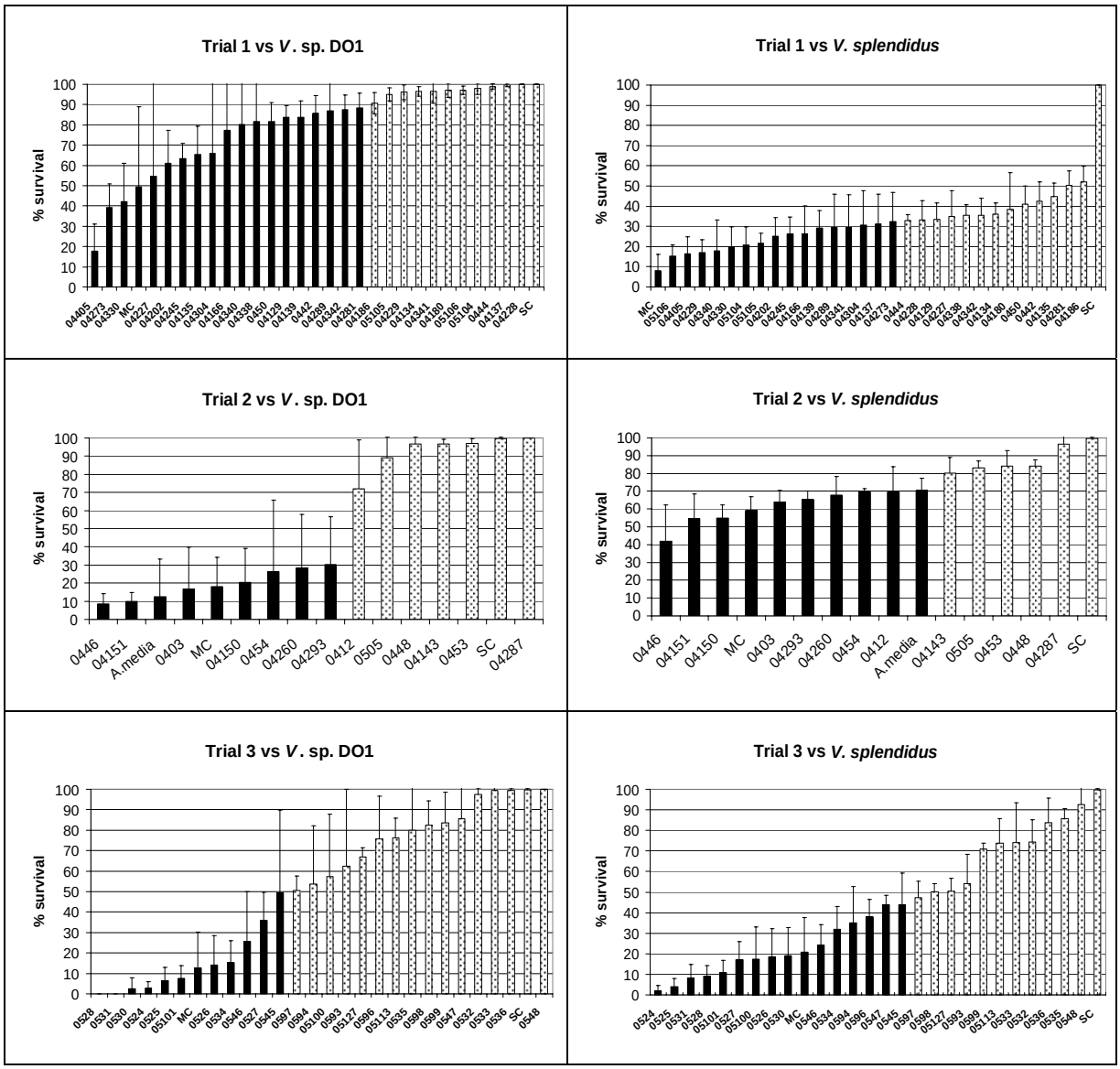
Note: sc = survival controls, mc = mortality controls.

5.3.2 Screening for probiotics in a TCD challenge bioassay

Based on larval survival significantly greater than pathogen controls, three separate screening trials highlighted many putative probiotics (Fig. 5.3). In total, 69 isolates were screened with 40 of these providing a statistically significant protective effect (58% success rate). Twenty-one of the putative probiotics provided a protective effect against both pathogens, while eight and 11 isolates, respectively, provided exclusive benefit to larvae challenged with *V. splendidus* and *Vibrio* sp. DO1 (Table 5.1). Across all experiments, benefits from isolates that were determined significant ranged from 21.3% to 87.4% improved larval survival. Furthermore, against challenge with *V. splendidus*,

24% of isolates offering significant protection provided >50% better larval survival compared with pathogen controls. Against challenge with *Vibrio* sp. DO1, 59% of the protective isolates offered >50% larval survival compared with pathogen controls. *Aeromonas media* control strain did not provide protection against either pathogen.

Figure 5.3 Mean GSM larval survival during three separate screenings for potential probiotic bacteria effective against two pathogen challenges. Values represent mean survival ± 95% confidence intervals. Black columns are not statistically different to pathogen controls, whereas the speckled columns are statistically different to pathogen controls (p = 0.05). For simplicity, the results for repeat-tested isolates have only been illustrated once.



Note: sc = survival controls, mc = mortality controls, *A. media* = *Aeromonas media* (ATCC 33907)

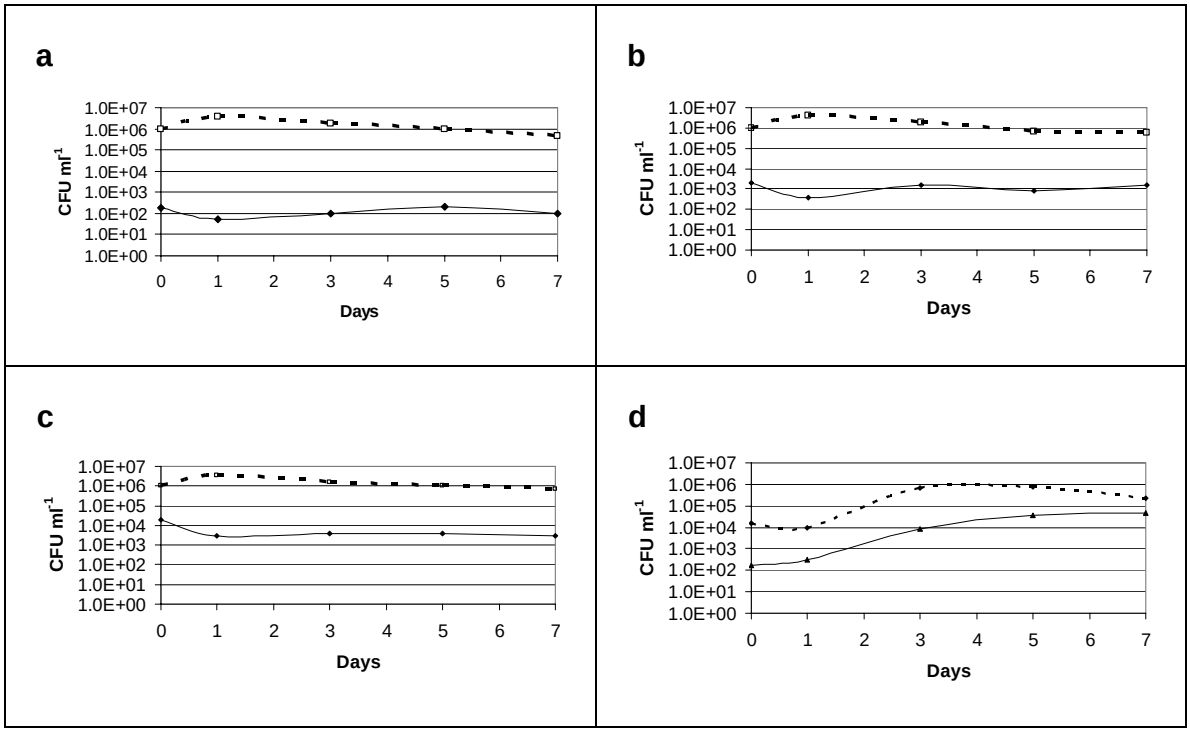
Table 5.1 Putative probiotic isolates identified in TCD bioassay screening experiments. Sixty-nine isolates were screened.

Pathogen	Isolates protecting against both pathogens					Isolates protecting against one pathogen			
V. splendidus	04134	04287	0505	0533	0593	04129	04281	04342	0450
	04143	0444	05113	0535	0597	04135	04338	0442	04227
	04180	0448	05127	0536	0598				
	04186	0453	0532	0548	0599				
	04228								
Vibrio sp. DO1	04134	04287	0505	0533	0593	0412	04341	05105	0594
	04143	0444	05113	0535	0597	04137	05100	05106	0596
	04180	0448	05127	0536	0598	04229	05104	0547	
	04186	0453	0532	0548	0599				
	04228								

5.3.3 Bacterial levels during a challenge

Bacterial levels of putative probiotic (0444) and, at pathogen-challenge concentrations of 10^2 and 10^3 CFU ml^{-1}, *Vibrio* sp. DO1, remained at initial inoculum levels over the seven days of the challenge (Fig. 5.4). In challenges with 10^4 CFU ml^{-1}, initial pathogen numbers dropped by an order of magnitude by the end of the experiment (Fig. 5.4c). In the absence of 0444, the pathogen was able to increase in numbers by two orders of magnitude.

Figure 5.4 Bacterial levels (CFU ml^{-1}) over seven days during larval challenge experiments with 10^6 CFU ml^{-1} putative probiotic, 0444 (broken line), and pathogen, *Vibrio sp*. DO1 (solid line), at concentrations of (a) 10^2, (b) 10^3 and (c) 10^4 CFU ml^{-1}. Pathogen control is shown in graph (d); whereby larvae where inoculated with pathogen alone at 10^4 CFU ml^{-1} (dotted line) and 10^2 CFU ml^{-1} (solid line). Inoculation of the pathogen signifies day 0; it should be noted that, on day 0, the probiotic was inoculated 20-hours before the pathogen despite their being plotted on the same axis.

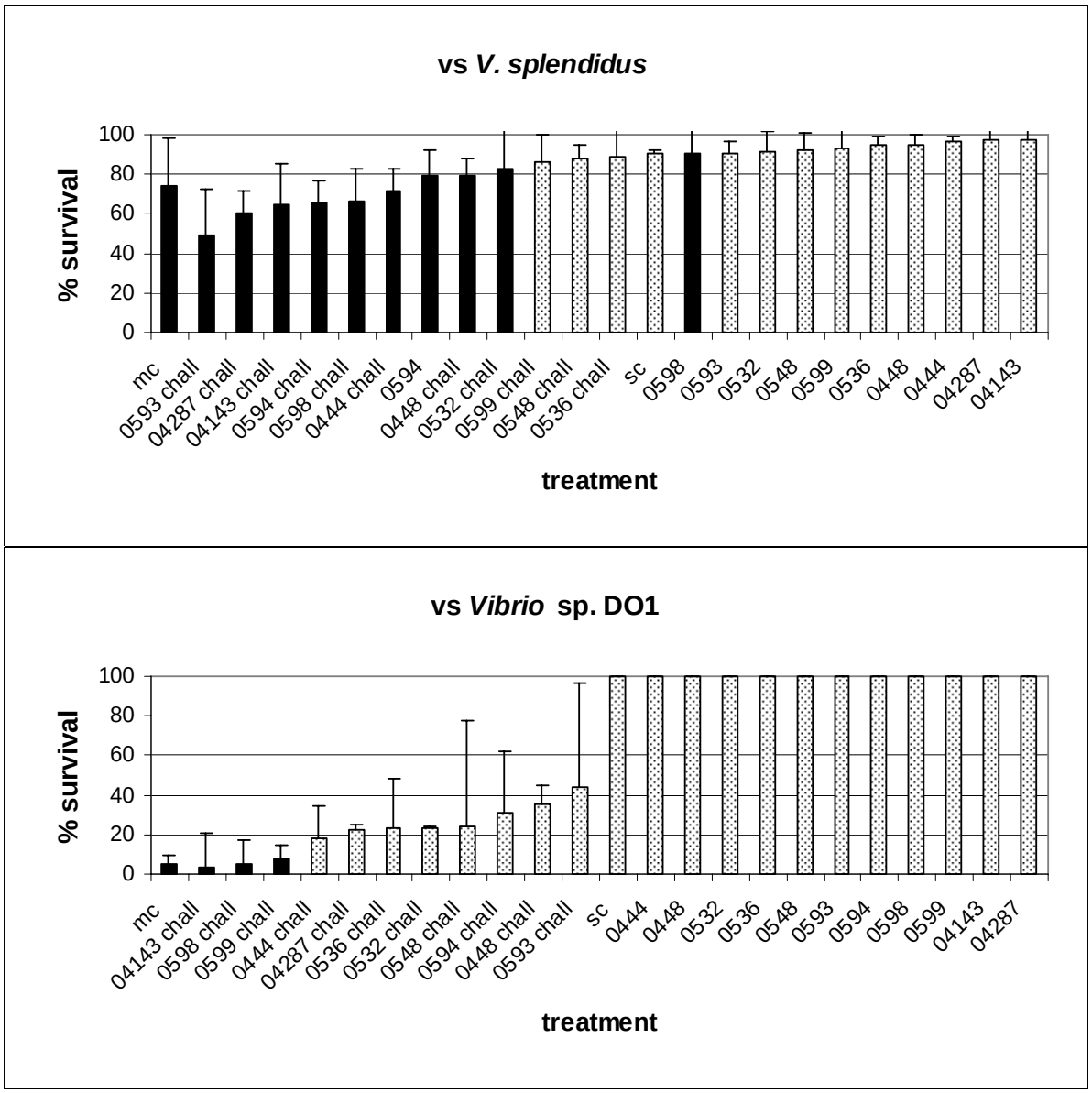


5.3.4 Probiotic pilot tests in the hatchery

Eleven strains showing a probiotic effect in the TCD bioassay were chosen for the experiments. Mortality observed in those larvae challenged with *V. splendidus* was minimal, e.g. 25% in the mortality control (Fig. 5.5). Comparisons against this control are tenuous. However, three isolates (0536, 0548, and 0599) did afford significantly greater larval survival. Apart from isolate 0594, larval survival with probiotic addition alone was no different or significantly greater than survival controls. Experiments with *Vibrio*. sp. DO1 produced high mortality in mortality controls, 95% (Fig. 5.5). With the exception of

04143, 0598 and 0599, all tested probiotics provided larval survival significantly greater than the mortality control. There were no differences in the larval survival rates between non-inoculated controls and probiotic controls.

Figure 5.5 Day 6 mean GSM larval survival during hatchery pilot tests of potential probiotic bacteria against two pathogen challenges. Values represent mean survival ± 95% confidence intervals. Black columns are not statistically different to pathogen controls, whereas the speckled columns are statistically different to pathogen controls (p = 0.05).



Note: sc = survival controls, mc = mortality controls, chall = challenge tests.

5.4 Discussion

The screening bioassay presented here demonstrated that 40 out of 69 tested isolates produced a protective effect in pathogen challenged GSM larvae. The success rate demonstrated the usefulness of this type of screening approach. It is unlikely the protection afforded by the bacterial isolates was a result of nutritional strengthening of larvae during the TCD bioassay, when compared with control larvae which did not receive test bacteria. This comment is based on the high virulence of the pathogens which was previously demonstrated in healthy, fed GSM larvae at the GACL hatchery (refer to Chapter 4). Therefore, the benefits observed against pathogen challenges in the present study were unlikely to be the result of a feeding effect. It is important to note that the screens were performed as a cursory evaluation of test isolates and, as such, screening experiments were not repeated for all isolates. Nonetheless, certain isolates which displayed potential benefits were repeatedly used in independent experiments; in two-hour and 20 hours trials and throughout the TCD bioassays (strains 0442, 0448, 0453, 04135, 04180, 04186, 04281, 04287, 0505). The constant performance of these isolates confirmed the reliability of the TCD method and supported the acceptance of results arising from single-screen experiments with six treatment replications.

In some cases, a probiotic pre-exposure time of two hours did not allow a protective effect to be seen. In fact, two isolates, 04180 and 04186, which would have been overlooked if the two-hour pre-exposure tests alone had been applied, actually provided dual protection against both *Vibrio* sp. DO1 and *V. splendidus* when inoculated 20 hours prior to the pathogen challenge. It was important to shortlist as many potential probiotics

in first stage screens and, as a consequence, the longer pre-exposure time was preferred. The longer period required for effective protection of the larvae could be as a result of competitive exclusion of the pathogen by the putative probiotics. Chabrillon (2005) demonstrated exclusion competition for attachment sites, but not a direct competition, meaning that the probiotic excluded the pathogen when the probiotic was present prior to the pathogen, but not when the two were added at the same time. Riquelme *et al*. (2000) determined that, in scallop larvae, a period of six hours was required for significant ingestion of two bacterial strains to occur, when administered at 10^6 CFU ml^{-1}. In the present bioassay, antibiotic pre-treatment of larvae resulted in each test bacterium becoming the dominant member of the TCD environment. However, it is possible that a period of time is still required for test isolates to be ingested and become associated with larval epithelium in amounts sufficient to exclude the pathogen by competition. Therefore, after a 20 hour pre-exposure to probiotic 0444 and, over a seven-day period (Fig. 5.4), a stasis of probiotic and pathogen, *Vibrio sp*. DO1, levels was obtained. *Vibrio* sp. DO1 was described as attacking the digestive system of GSM larvae (refer to Chapter 3). In the present study, the results suggest that the probiotic was not bactericidal to the pathogen, but, as a consequence of competitive gut colonisation, it appeared to have prevented the pathogen's ability to attach, infect and multiply in the larvae.

Incorporation of antibiotic treated larvae and sterile seawater in the bioassay design ensured larvae were healthy at the start of experimentation. However, it also reduced the effects of extraneous bacteria during experiments. Yet, antibiotic usage in the present study might have influenced the large number of putative probiotics displaying positive

effects during bioassays. Moreover, by reducing the levels of ambient competition, less biologically active probiotics, which would not be effective under normal situations, might display benefits by numerical attachment competition. This effect is likely to be enhanced by the reduced ambient bacteria and 20-hour pre-incubation time of probiotic to pathogen. This is supported by a separate study conducted on the inhibitory substance production, where only six out of the 11 putative probiotics appeared to produce a probiotic substance at very low levels (Appendix 3). It indicates that the main mechanism of probiotic action was something other than inhibitory substance production; competitive exclusion is a likely mode of action and, as previously stated, it is potentially aided by antibiotic pre-treatment of the larvae. However, the fact that not all test isolates demonstrated a beneficial effect in TCD bioassays indicated that the technique did not favour all test isolates and was, therefore, discriminatory.

Because a reduction of extraneous bacteria with antibiotic pre-treatment might have magnified a probiotic effect in the TCD bioassay, it was important to determine the effect of shortlisted isolates under hatchery conditions. The environment in the hatchery process differs markedly from that of the TCD bioassay (including: aeration, food, a milieu of background bacteria and flow-through water) and the test probiotic might not be the dominant organism regardless of how much is added. Pilot studies conducted at the GACL, under normal hatchery conditions, showed that a probiotic benefit was transferable from the TCD bioassay to the hatchery environment. Probiotic protection was not transferred with all shortlisted isolates, highlighting that conditions of the TCD bioassay did allow identification of probiotics which were not able to exert their benefit

in a more complex system. Nonetheless, the main aim of the pilot studies was to indicate whether potential probiotics identified using TCD bioassays were effective under normal rearing conditions. This was achieved, thereby confirming the usefulness of the bioassay. Additionally, potential isolates for future hatchery trials were determined and these were tested in full-scale, replicated experiments (refer to Chapter 6). It should be noted that during pilot studies with *V. splendidus* larval mortality was not high (25%), whereas high mortality occurred during challenge with *Vibrio* sp. DO1 (95%). Despite this, studies with *V. splendidus* did discern statistical differences. Furthermore, throughout independent trials with the pathogens (both TCD bioassays and pilot studies), differences in larval mortality were observed. These were potentially caused by genetic and health differences of the larval batches tested and, in the case of pilot studies, environmental differences at the time of each experiment, such as ambient bacteria and microalgae quality.

The success rate of the screening bioassay (58%) was notably high and probably aided because of the fact that this assay did not exclude any particular, specialised mode of probiotic activity. The limitations of screening trials employing agar diffusion tests and the benefits of including test animals into the first stage screens were discussed previously (refer to Chapter 1). Illustrating this point, Riquelme *et al*. (1997) found a mere 11 of 506 tested strains to be potential probiotics based on agar diffusion tests. In a separate study (Appendix 3), where only six out of 11 putative probiotics tested might have produced an inhibitory substance at very low levels, it is likely that all six isolates would not have been pursued for further testing because of their questionable pathogen

inhibition. This reinforces the notion that many candidate probiotic strains might be overlooked in screening trials employing agar diffusion tests.

This study is the first to screen for probiotics of mussels and has demonstrated the benefit of including test animals in the initial screening stages. Use of TCDs greatly facilitated the development of a larval bioassay for this purpose. Similar TCD bioassay approaches have been used in determining pathogens of larval fish and molluscs (Riquelme *et al*., 1995; Bergh *et al*., 1997; Lovko *et al*., 2003; Estes *et al*., 2004), in a study on eel fertilization (Unuma *et al*., 2004), in studying bacterial monoculture effects upon fish larvae and *Artemia* sp. (Verschuere *et al*., 1999; Makridis *et al*., 2005) and in later stage probiotic pathogen-challenge experiments using turbot larvae (Hjelm *et al*., 2004). Review of the literature suggests that this is the first study to include test animals into first stage probiotic screens under challenge experiment conditions. Given the possibility of readily available test subjects and size of bioassays, similar screening trials are likely to show great promise in identifying novel probiotics for aquaculture animals. It should also be considered that, when screening for effective probiotics, it is best to use bacteria associated with or existing in the natural environment of the host. Strains naturally adapted to the production environment would require less manipulation to ensure that their probiotic activity is maintained. In the present study, this was demonstrated through the discovery of novel probiotics which were effective in flow-through hatchery production conditions by switching the water flow off for just two hours following daily probiotic addition.

Chapter 6

Alteromonas macleodii and *Neptunomonas* sp. 0536, two novel probiotics for Greenshell™ mussel larvae, *Perna canaliculus*: protection in a hatchery facility during pathogen-challenge with *Vibrio splendidus* and a *Vibrio coralliilyticus/neptunius*-like isolate

6.1 Introduction

To date, the larval stages of GSM production intermittently encountered problems, which were often alleviated by administration of antibiotics. Isolation and demonstration of two pathogens of GSM larvae further implicated bacterial pathogenesis in larval problems. These pathogens were identified as *V. splendidus* and a *V. coralliilyticus/neptunius*-like isolate (refer to Chapter 3). Continued management of these pathogens with antibiotics was not preferred, prompting investigation into probiotic alternatives. Following a screening bioassay and hatchery pilot trials, two potential probiotic isolates emerged as being worthy of full-scale hatchery experimentation. Additionally, an experimental infection model was developed under hatchery conditions, allowing for field experimentation of these probiotics under standard larval rearing conditions and against a pathogen challenge.

In vivo trials have demonstrated the potential that probiotics hold for both disease prevention (Moriarty, 1998; Lategan *et al*., 2004a; 2004b; Chabrillon *et al*., 2006) and growth enhancement (Gatesoupe, 2002; Lara-Flores *et al*., 2003; Macey & Coyne, 2005). Riquelme *et al*. (2001) examined probiotic use at a commercial scallop hatchery. Although they did not examine protection against a specific disease challenge, they demonstrated that a *Vibrio* sp., *Pseudomonas* sp. and *Bacillus* sp. allowed completion of the larval period without the need for antibiotics previously thought to be indispensable.

At the final stage in the development of any new treatment, controlled trials are required in the environment where an effect is intended. In the search for probiotics in aquaculture

there have been few field trials under commercial aquaculture conditions. The aim of this study was to determine the benefits of two novel probiotics on GSM larvae reared under hatchery production conditions, when exposed to two separate pathogen challenges. The experiments were designed to determine the practical potential of the tested probiotics for GSM larval rearing.

6.2 Material and methods

6.2.1 Experimental animals

Experiments were conducted at the GACL. GSM larvae were obtained from the hatchery according to normal hatchery protocol (refer to Chapter 2). Newly hatched D-veliger larvae were screened on nylon mesh size 45 μm and then placed into 2.5 l tanks at a density of 200 larvae ml^{-1} for use in experiments. Water flow was continuous at 80 ml min^{-1}, aeration was provided, water temperature was 19°C and larvae were fed a 2:1 mix of *Chaetoceros calcitrans*:*Isochrysis galbana* to provide a final concentration in tanks of 40 algal cells μl^{-1}.

6.2.2 Culture and harvest of bacteria

Bacteria were grown and harvested as in Chapter 4.2.2

6.2.3 Identification of probiotic strains

Two potential probiotics, strains 0444 and 0536, were selected for hatchery trials, based on previous pilot studies conducted at GACL (refer to Chapter 5). Each was further

identified using both phenotypic and genotypic characterisation. Gram stain, oxidase reaction, motility and catalase tests were carried out initially, followed by inoculation of API 20NE (BioMérieux). Further profiling included growth temperature range, salt tolerance and antibiotic resistance (Etest®; AB BIODISK, Sweden). Prior to Etests, antibiotic resistance tests were conducted using the disc diffusion method (Appendix 4) in order to determine antibiotics suitable for further investigation. Using Etests, strain 0444 was tested for resistance to ampicillin and aztreonam, while 0536 was tested against erythromycin, trimethoprim and aztreonam. *E. coli* (ATCC 25922) was used as a control of known susceptibility for ampicillin and aztreonam, while *Staphylococcus aureus* (ATCC 29213) was a control for erythromycin and trimethoprim. Etests for 0444 and 0536 were carried out on TSA-2%Sea, with 24 hours incubation at 30°C, a temperature recommended by the Etest manufacturer and at which all organisms grew well. *E.coli* tests were performed on Mueller-Hinton (MH) agar (Oxoid) and those of *S. aureus* were performed on MH agar + 2% NaCl, according to the manufacturers' recommendations.

Genotypic characterization of strains entailed the amplification and sequencing of the 16S rRNA gene. Chromosomal DNA was extracted from bacterial cultures using a commercial kit (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany) following the manufacturer's instructions. The universal bacterial PCR primer pair used to amplify the 16S rRNA gene was (Suzuki & Giovannoni 1996):

forward: EUBB-F (5'- AGAGTTTGATCMTGGCTCAG -3'),
reverse: EUB-A-R (5'- AAGGAGGTGATCCANCCRCA -3').

Final amplification reaction mixtures consisted of: 20 µl 2.5 x HotMasterMix (Eppendorf, Hamburg, Germany), 2.0 µl DNA template, primers (final concentration 0.8 µM) and water to final volume of 50.0 µl. Amplification reactions were performed using an iCycler (Bio-Rad, Carlsbad, USA) with the following thermocycling conditions: 94°C / 2 minutes, 52°C / 1 minute, 65°C / 1 minute, 1 cycle; 94°C / 30 seconds, 55°C / 30 seconds, 65°C / 2 minutes, 35 cycles; 65°C / 7 minutes, 1 cycle. Amplification products were electrophoresed through 1.0 % (w/v) agarose gels stained with ethidium bromide and visualized under UV light. Bands of the expected size (1.5 kb) were purified (QIAquick gel extraction kit, Qiagen) and used as templates for sequencing by an external contractor (Environmental Science & Research, Porirua, New Zealand). Assembled sequences were used as query strings for interrogation of the following databases: Ribosomal Database Project II (RDP, http://rdp.cme.msu.edu/) and GenBank (http://www.ncbi.nlm.nih.gov/). The 16S rRNA sequences obtained in this study were deposited into GenBank with accession numbers FJ463597 and FJ463598.

6.2.4 Phylogenetic analysis

The 16S rRNA gene sequences of strains 0444 and 0536 and top matches from GenBank and RDP were aligned in BioEdit (Hall, 1999) using ClustalW. *Nitrosomonas europaea* (accession number M96399), a β-proteobacterium, was used as an outgroup. Aligned sequences were transported in Nexus format to MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001) to construct phylogenetic trees. MrBayes analysis comprised two simultaneous runs for three million generations, with four chains each. The trees were sampled every 100 generations, with the burn-in set at 20,000.

6.2.5 Experimental design

Pathogens, *V. splendidus* and *Vibrio* sp. DO1, were used to challenge the larvae after administration of strains 0444 and 0536. Challenge experiments were conducted on four separate occasions; each probiotic/pathogen combination being assigned a separate experiment. At a later stage, each probiotic/pathogen experiment was repeated.

During each experiment, larvae were inoculated daily with the test probiotic at 10^8 CFU ml^{-1} (final tank concentration), beginning on the first larval day post-hatching. During incubation of the probiotic, water flow was stopped and kept static for a two-hour period. Pathogen-challenge of the larvae occurred on the third day following hatching, immediately following the two-hour probiotic incubation on day 3, i.e. in challenge treatments, the probiotic remained in the static-water tank during pathogen challenge. Pathogens were inoculated at doses previously determined to cause infection (refer to Chapter 4) and at a higher dosage which was a concentration one order of magnitude higher than the minimum effective dose. These concentrations were represented by 10^5 and 10^6 CFU ml^{-1} for *V. splendidus*, and 10^6 and 10^7 CFU ml^{-1} for *Vibrio* sp. DO1. In order to allow the pathogens to establish infection, water flow remained static during pathogen exposure for one hour (*V. splendidus*) and two hours (*Vibrio* sp. DO1) (refer to Chapter 4). This resulted in the water flow remaining static, on the third day, for a total time of three hours and four hours respectively, for experiments involving *V. splendidus* and *Vibrio* sp. DO1. These conditions were also applied in the control treatments. Probiotic treatment continued daily after pathogen challenge. Experimental duration was seven days post-hatching (four days following pathogen inoculation).

Controls included: (i) larvae inoculated with the probiotic alone (10^8 CFU ml^{-1}), (ii) larvae inoculated with the two doses of pathogen alone, and (iii) non-inoculated larvae. All treatments were conducted in triplicate. Each day, triplicate aliquots (250 µl) were taken from tanks and larval survival determined (approximately 50 larvae per aliquot). On alternate days, either outflow barrier-screens (nylon mesh size 40 µm) were cleaned, or full tank cleanings were performed (screens, tanks and larvae). During cleaning of the larvae, a 45 µm screen was used to ensure retention of the initial starting population, thereby allowing measurement of the cumulative larval survival for the duration of the larval period.

Upon completion of several experiments, larval size (µm) was measured for each treatment. Larvae were placed into a 12-well TCD and rendered immobile by the addition of three drops of dilute Lugol's iodine solution. Larvae were then photographed (Olympus Camedia C7070) through microscope (Olympus CK-2) using Cam2Com software for image transferral to computer (Sabsik & Sergei Menchenin 2001-2006, www.sabsik.com/Cam2Com) and their size estimated using ImageJ 1.37v (Abramoff *et al*. 2004). Size analysis determined the greatest length for each larva. Each data point was traceable to photographic images, allowing data to be re-viewed in order to ensure that only live animals were included in the data sets (not empty shells).

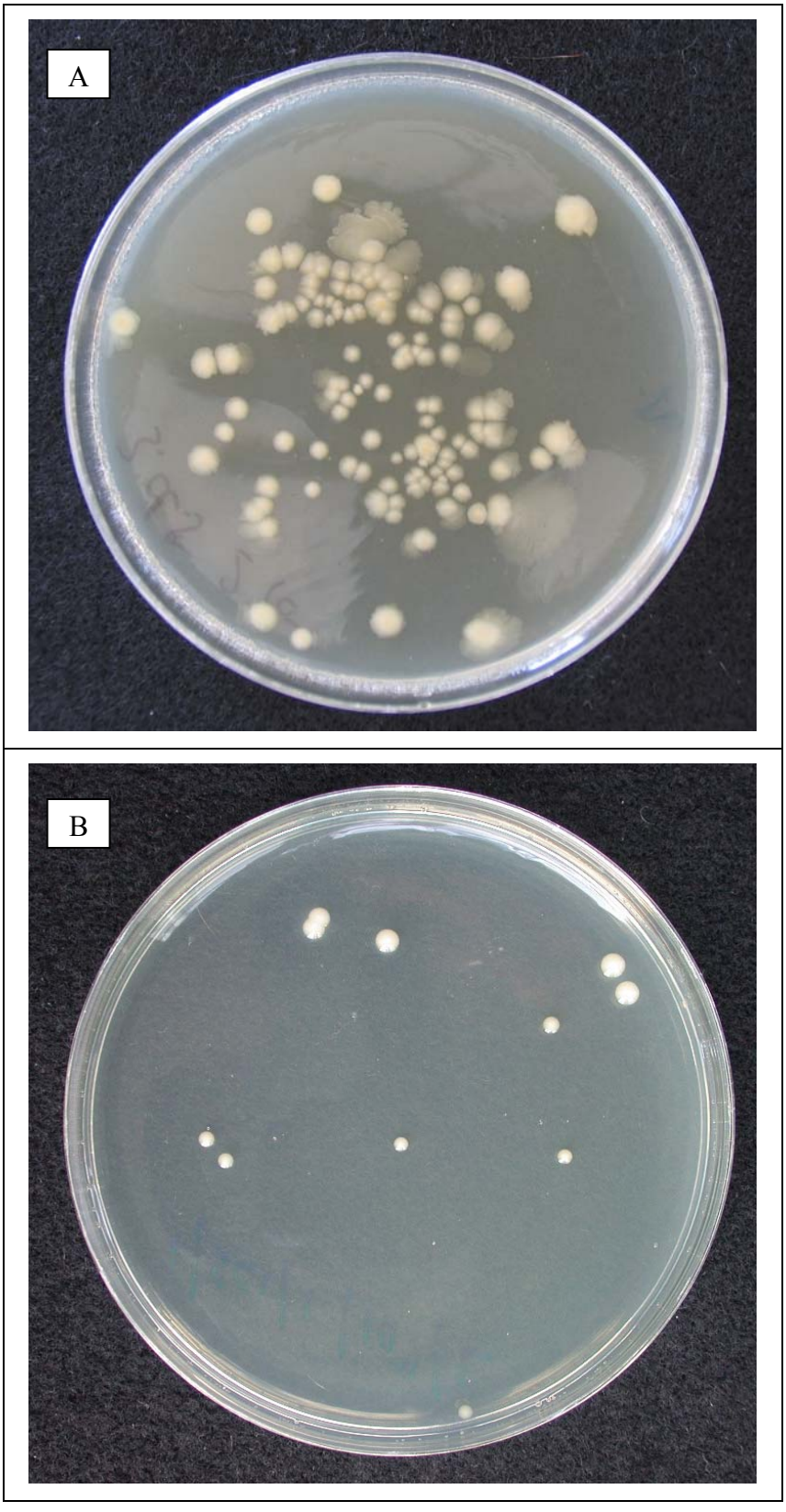
6.2.6 Bacterial monitoring for probiotics and pathogens

The presence or absence of the pathogens was monitored daily in both the water column and larval samples as described in Chapter 4.2.3. Selective conditions and confirmatory

tests for pathogens were developed previously after biochemical and physiological profiling (refer to Chapter 3). Those for the probiotic bacteria were developed based on the bacteriological profiling conducted herein.

To re-isolate probiotic 0444, spreading, creamy-white "daisy-head" colonies on TSA (9% NaCl), after five days incubation at 44°C, were used for selection (Fig. 6.1A). Confirmatory tests included: Gram-negative staining, oxidase positive reaction and gelatin hydrolysis. To re-isolate the probiotic 0536, raised floret colonies which slid with the push of a loop, after seven days incubation at 37°C on TSA-2%Sea incorporating 4 $\mu g\ ml^{-1}$ erythromycin and 2 $\mu g\ ml^{-1}$ trimethoprim, were used for selection (Fig. 6.1B). Confirmatory tests included: growth at 40°C, Gram-negative and oxidase positive reactions. Control cultures of each isolate were grown alongside test samples for comparison.

Figure 6.1 Colony appearance of (A) 0444, and (B) 0536 on selective media.



6.2.7 Data analysis

Percent survival figures were arc sin square root transformed to approximate normality. Treatment differences were compared using ANOVA (p=0.05). Post hoc comparisons between survivals were compared using Tukey's test.

6.3 Results

6.3.1 Identification of probiotic strains

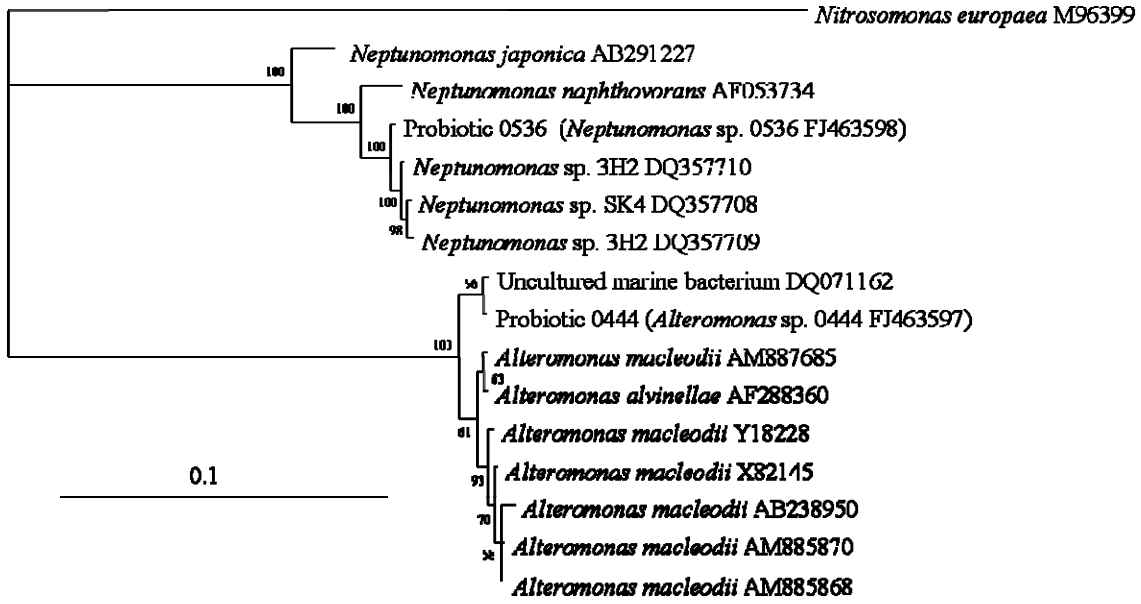
Biochemical tests revealed the probiotic strains to be fairly non-reactive, Gram-negative bacilli (Table 6.1). Although strain 0444 was sensitive to the antibiotics tested, it displayed a high tolerance for salinity and temperature allowing the development of selective conditions for the bacterium. Strain 0536 had moderate tolerance to salinity and temperature and had a resistance to levels of erythromycin which inhibited the growth of other bacteria in hatchery samples. Additionally, although sensitive to trimethoprim, growth did occur in the presence of 4 μg ml^{-1} of the bacteriostatic antibiotic after 24 hours. Hence, selective conditions for the re-isolation of 0536 incorporated a combination of erythromycin/trimethoprim plus elevated temperature. Genotypic and phylogenetic identification placed isolate 0444 in a clade dominated by *Alteromonas* spp. (Fig. 6.2). It had highest similarity to an uncultured bacterium and it exhibited 98% similarity to numerous strains of *A. macleodii* (Fig. 6.2). This strain has been entered into GenBank as *Alteromonas* sp. 0444, accession number FJ463597. Phylogeny of strain 0536 identified it as a *Neptunomonas* sp. It was 98% similar to three cloned *Neptunomonas* strains and 97% similar to *N. naphthovorans* (accession number AF053734). Isolate 0536 has been

entered into GenBank under the description *Neptunomonas* sp. 0536, accession number FJ463598.

Table 6.1 Biochemical, morphological and physiological characteristics of GSM larvae probiotics; 0444 and 0536. Values for Etests are minimum inhibitory concentrations after 24 hr at 30°C.

Test	Strain 0444	Strain 0536
Gram stain	-	-
Oxidase reaction	+	+
Catalase reaction	+	+
Motile	+	+
NaCl tolerance	0.5-10%	0.5-7%
Temperature growth range	17-44°C	17-40°C
Etest antibiotic susceptibility. Control values: Ampicill (*E. coli*) 4 μg ml^{-1}; Aztr (*E. coli*) 0.19 μg ml^{-1}; Eryth (*S. aureus*) 0.25 μg ml^{-1}; Trimeth (*S. aureus*) 1 μg ml^{-1}	Ampicillin 0.064 μg ml^{-1} Aztreonam 0.25 μg ml^{-1}	Erythromycin 16 μg ml^{-1} Trimethoprim 4 μg ml^{-1} Aztreonam 0.38 μg ml^{-1}
Nitrate reduction	+	-
Indole production	-	-
Glucose fermentation	-	+
Arginine dihydrolase	-	-
Urea hydrolysis	-	-
Esculin hydrolysis	-	-
Gelatin liquefaction	+	+
ONPG	-	-
Glucose assimilation	-	-
Arabinose assimilation	-	-
Mannose assimilation	-	-
Mannitol assimilation	-	-
N-acetyl-Glucosamine assimilation	-	-
Maltose assimilation	-	+
Potassium gluconate assimilation	-	-
Capric acid assimilation	-	-
Adipic assimilation	-	-
Malate assimilation	-	-
Citrate assimilation	-	-
Phenylacetic acid assimilation	-	-

Figure 6.2 Phylogenetic tree for GSM larvae probiotics *Alteromonas* sp. (0444) and *Neptunomonas* sp. (0536).



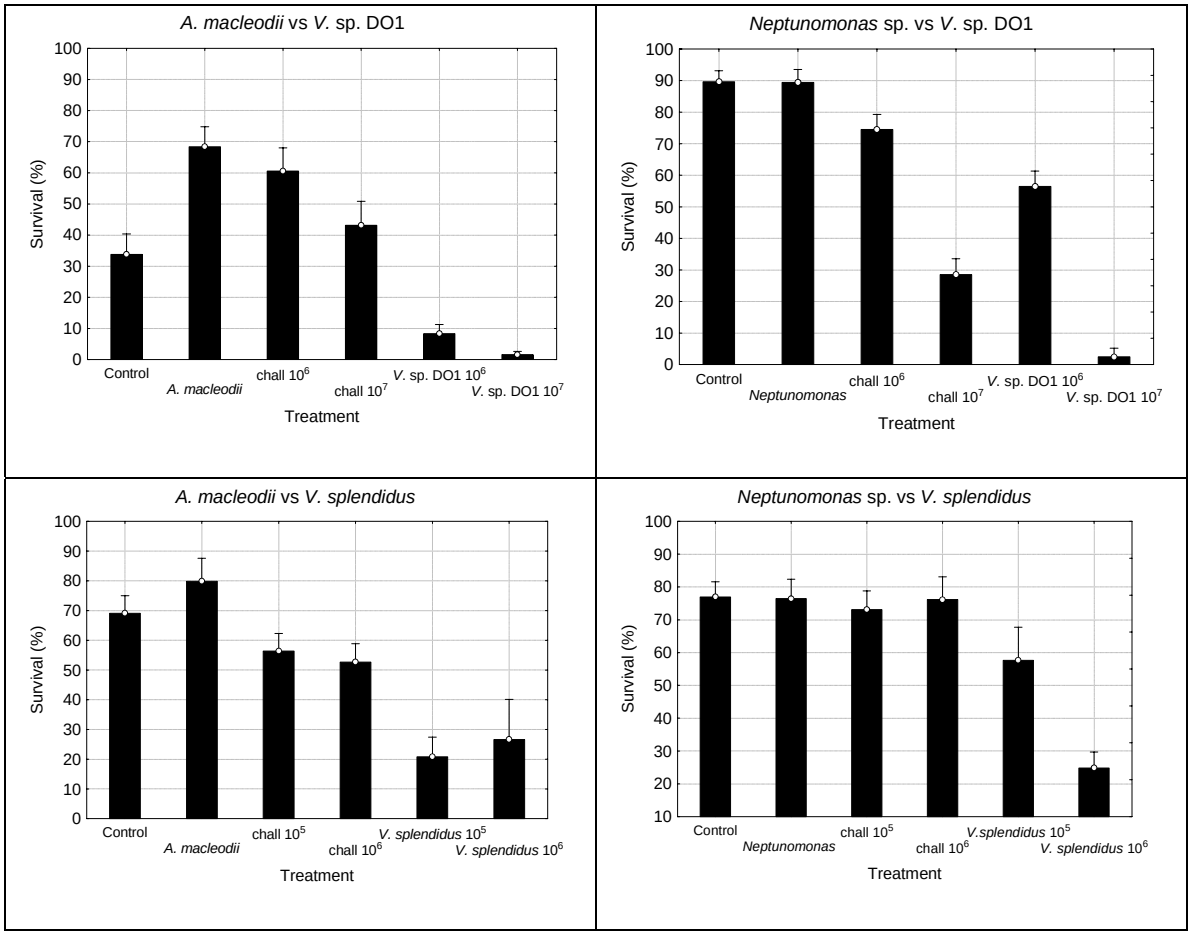
6.3.2 Pathogen-challenges of GSM larvae with/-out probiotics

When probiotics were administered to GSM larvae prior to pathogen challenge, both *A. macleodii* 0444 and *Neptunomonas* sp. 0536 afforded survival of larvae significantly greater than larvae challenged without probiotics ($p < 0.05$); increases ranging from 18% to 52% on the fourth day following pathogen exposure were observed (Fig. 6.3, Table 6.2). In all cases, the probiotic was effective at both the minimum effective pathogenic dose and the higher level of pathogen. When the probiotic alone was administered to larvae, *Neptunomonas* sp. 0536 did not affect the larvae compared with controls to which bacteria had not been added. However, administration of *A. macleodii* 0444 led to better larval survival, when compared with controls without bacterial addition, in both experiments utilizing the bacterium; this was statistically significant in the experiment conducted with *Vibrio* sp. DO1 challenge. Across all experiments, the average survival of

larvae exposed only to pathogens at the high and low dosages was 13.9 ± 5.1% and 35.8 ± 8.0% respectively. If probiotics were used, averaged larval survival was improved to 50.1 ± 6.5% and 66.1 ± 3.7% respectively. Survival rates of control larvae and those administered probiotic alone were 67.4 ± 7.4% and 78.5 ± 3.7% respectively.

In repeat challenge experiments utilizing the higher pathogen dose, the probiotic benefit was duplicated with *Neptunomonas* sp. 0536 against both pathogens (Table 6.3). Increased larval survival from use of *A. macleodii* 0444 was only duplicated against *Vibrio* sp. DO1, and not *V. splendidus*. Additionally, unlike the initial trials, sole administration of *A. macleodii* 0444 did not produce larval survival greater than non-inoculated controls. Neither probiotic adversely affected larvae survival to any significant degree when compared with the non-inoculated larval controls.

Figure 6.3 Mean GSM larval survival (± 95% confidence intervals) on the fourth day following pathogen exposure. Each graph displays the results of a separate challenge experiment.



Note: Chall = challenge treatment

Table 6.2 Statistical analysis of GSM larval survival on the fourth day following pathogen exposure. Probiotic concentrations are 10^8 CFU ml^{-1} in all experiments. In experiments challenged with *Vibrio*. sp. DO1, low pathogen = 10^6 CFU ml^{-1}, high pathogen = 10^7 CFU ml^{-1}. In experiments challenged with *V. splendidus*, low pathogen = 10^5 CFU ml^{-1}, high pathogen = 10^6 CFU ml^{-1}. Values represent mean survival ± 95% confidence intervals. Values in a column not sharing the same superscript are statistically different ($p < 0.05$).

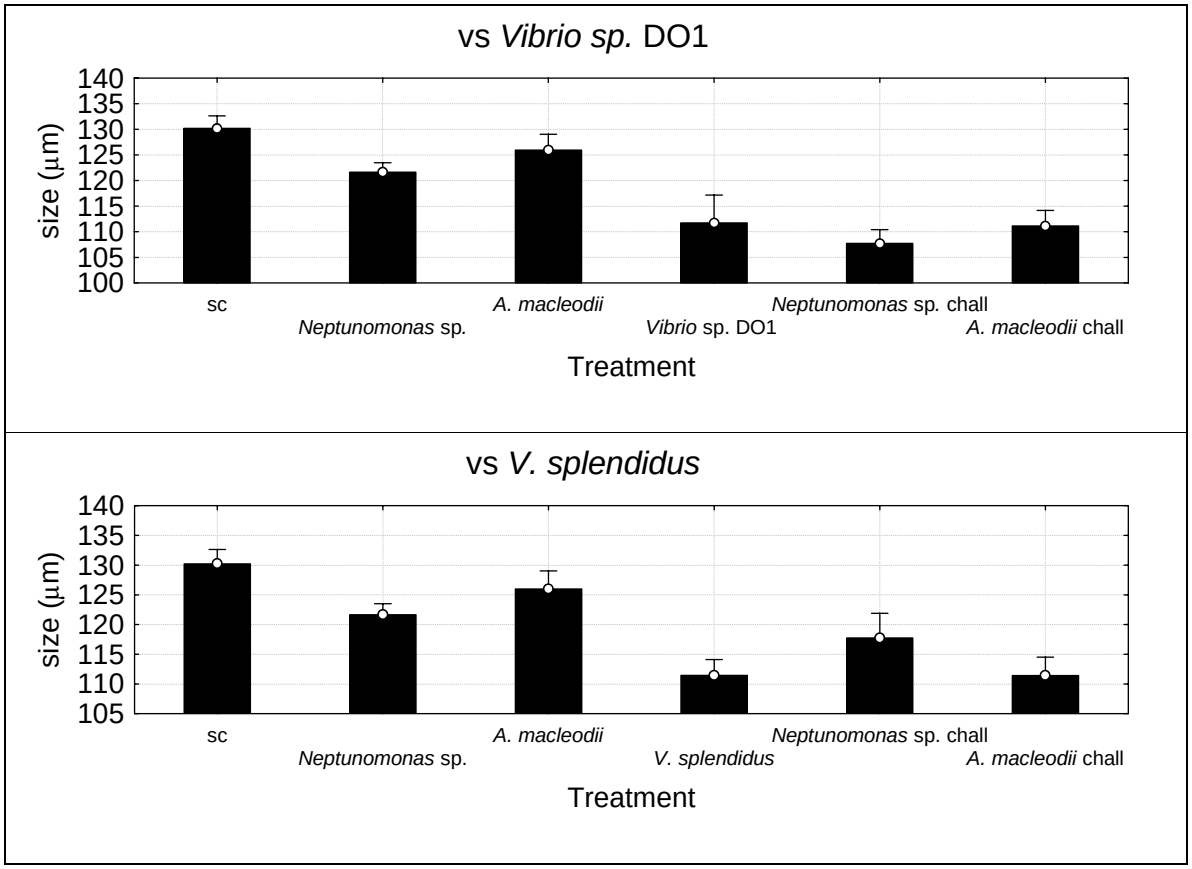
Treatment	*A. macleodii* vs *Vibrio* sp. DO1	*A. macleodii* vs *V. splendidus*	*Neptunomonas* sp. vs *Vibrio* sp. DO1	*Neptunomonas* sp. vs *V. splendidus*
Survival control	33.8 ± 6.5^{b}	69.0 ± 6.0^{ab}	89.6 ± 3.5^{a}	77.0 ± 4.6^{a}
Probiotic	68.4 ± 6.4^{a}	79.9 ± 7.7^{a}	89.4 ± 4.1^{a}	76.5 ± 5.9^{a}
Low pathogen	8.3 ± 3.0^{c}	20.8 ± 6.6^{d}	56.5 ± 4.8^{b}	57.6 ± 10.1^{b}
Low pathogen/probiotic	60.6 ± 7.4^{a}	56.3 ± 5.9^{bc}	74.5 ± 4.8^{c}	73.1 ± 5.7^{a}

Table 6.3 Day 6 survival of GSM larvae during probiotic/pathogen challenge repeat experiments; pathogen challenge was on day 2. High doses of the pathogen were tested; 10^6 CFU ml^{-1} for *V. splendidus*, 10^7 CFU ml^{-1} for *Vibrio sp*. DO1. Values represent mean survival ± 95% confidence intervals. Values in a column not sharing the same superscript are statistically different ($p < 0.05$).

Treatment	*A. macleodii* vs *V. splendidus*	*A. macleodii* vs *Vibrio sp*. DO1	*Neptunomonas* sp. vs *V. splendidus*	*Neptunomonas* sp. vs *Vibrio sp*. DO1
Survival control	84.15 ± 3.91^a	84.15 ± 3.91^a	84.15 ± 3.91^a	84.15 ± 3.91^a
Probiotic (10^8 CFU ml^{-1})	72.98 ± 7.32^a	72.98 ± 7.32^a	80.96 ± 3.24^a	80.96 ± 3.24^a
High pathogen	32.11 ± 5.87^b	7.01 ± 4.84^b	32.11 ± 5.87^b	7.01 ± 4.84^b
High pathogen/probiotic	34.96 ± 11.69^b	22.87 ± 7.07^c	55.08 ± 7.65^c	38.27 ± 7.22^c

The average size of six-day-old GSM larvae ranged between 107.74 and 130.22 μm across all treatments (Fig 6.4). Statistically, there was no difference between the size of control larvae and those provided *A. macleodii* 0444, but larvae treated with *Neptunomonas* sp. 0536 were smaller. In all cases, probiotic treated larvae that were challenged with a pathogen were no different to pathogen controls, except for challenges involving *Neptunomonas* sp. 0536 and *V. splendidus.* Larval sizes obtained from separate repeat experiments showed minimal larval size fluctuation across all treatments during a challenge involving *Neptunomonas* sp. 0536 and *V. splendidus* (Table 6.4). However, in a challenge with *Neptunomonas* sp. 0536 and *Vibrio* sp. DO1, probiotic pathogen-challenged larvae were significantly larger than the pathogen controls.

Figure 6.4 Mean day 6 GSM larval size (µm) of different treatments during a probiotic pathogen-challenge experiment. High doses of the pathogen were tested; 10^6 CFU ml^{-1} for *V. splendidus*, 10^7 CFU ml^{-1} for *Vibrio sp*. DO1. Probiotics were administered at 10^8 CFU ml^{-1}.Values represent mean length ± 95% confidence intervals.



Note: sc = survival control, chall = challenge experiment.

Table 6.4 Mean day 6 GSM larval size (µm) of different treatments during two separate probiotic pathogen-challenge experiments. Values represent mean length ± 95% confidence intervals. Values not sharing the same superscript are statistically different ($p < 0.05$).

Treatment	*Neptunomonas* sp. vs *V. splendidus*	*Neptunomonas* sp. vs *Vibrio sp*. DO1
Survival control	127.41 ± 1.53^{ab}	133.99 ± 2.23^{a}
Probiotic	123.65 ± 1.87^{b}	126.24 ± 1.39^{b}
Low pathogen	126.04 ± 2.64^{bc}	116.22 ± 1.84^{c}
Low pathogen/probiotic	127.78 ± 2.33^{ac}	125.92 ± 2.10^{b}
High pathogen	126.56 ± 5.90^{bc}	Nil
High pathogen/probiotic	128.38 ± 2.37^{ac}	118.11 ± 3.64^{c}

6.3.3 Bacterial examination of challenged larvae and tank water

Pathogens were detected in both the larvae and water samples of the probiotic/pathogen-challenge treatments during all experiments, except when *Neptunomonas* sp. 0536 was tested with *V. splendidus* (Table 6.5). In that experiment, the pathogen was not observed if the probiotic was administered prior to challenge. Furthermore, the administration of *Neptunomonas* sp. 0536 excluded not only *V. splendidus*, but also most of the naturally-occurring putative vibrios from the hatchery water environment (Fig. 6.5). Test isolates were not detected in the control treatments during any experiment. Probiotics and pathogens were detected in the probiotic and pathogen controls treatments respectively, during all experiments (Table 6.5).

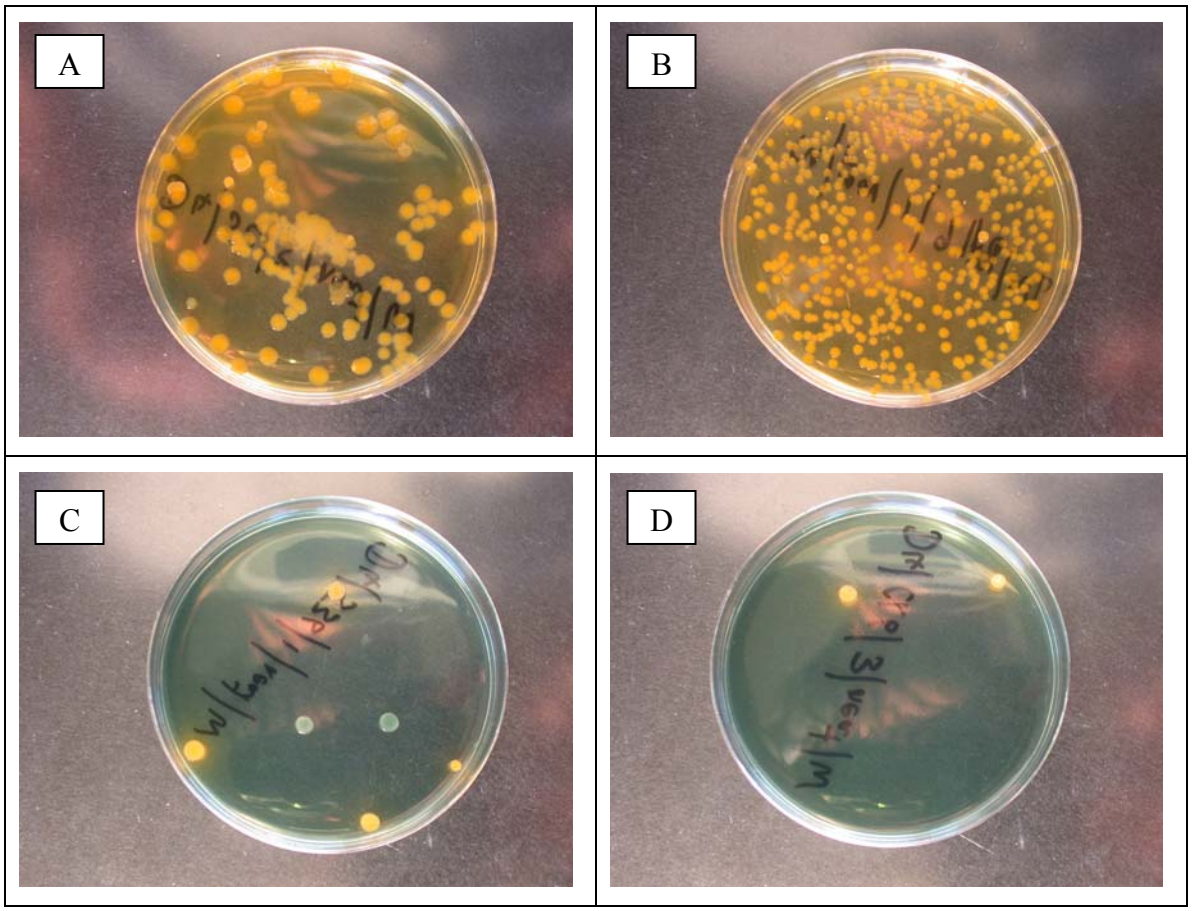
In one experiment, testing *A. macleodii* 0444 against *Vibrio* sp. DO1, larvae from the probiotic pathogen-challenge and control treatments were cultivated until settlement (16 days), although probiotic addition ceased on the fifth day. Probiotic and pathogen were continually monitored. The pathogen was detected in the larvae and water of pathogen-challenge treatments until the ninth day post-hatching (i.e. six days after pathogen exposure) but was undetectable by day 11 post-hatching (Appendix 5). Despite probiotic addition ceasing on the fifth day following hatching, probiotics were still detected in both larvae and water until settlement.

Table 6.5 Detection of test isolates from treatments during probiotic pathogen-challenge experiments. Data represents a summary of presence/absence in each treatment during each experiment, although test isolates were monitored daily for the six day duration of each experiment.

Treatment		*A. macleodii* vs *V. splendidus*		*A. macleodii* vs *Vibrio sp*. DO1		*Neptunomonas* sp. vs *V. splendidus*		*Neptunomonas* sp. vs *Vibrio sp*. DO1	
		Water	Larvae	Water	Larvae	Water	Larvae	Water	Larvae
Survival control	Prob	-	-	-	-	-	-	-	-
	Path	-	-	-	-	-	-	-	-
Probiotic control	Prob	+	+	+	+	+	+	+	+
	Path	-	-	-	-	-	-	-	-
Low pathogen challenge	Prob	+	+	+	+	+	+	+	+
	Path	+	+	+	+	-	-	+	+
High pathogen challenge	Prob	+	+	+	+	+	+	+	+
	Path	+	+	+	+	-	-	+	+
Low pathogen control	Prob	-	-	-	-	-	-	-	-
	Path	+	+	+	+	+	+	+	+
High pathogen control	Prob	-	-	-	-	-	-	-	-
	Path	+	+	+	+	+	+	+	+

Note: Prob = probiotic, Path = pathogen.

Figure 6.5 Growth of putative *Vibrio* spp. on TCBS selective agar during a probiotic pathogen-challenge experiment involving *Neptunomonas* sp. 0536 and *V. splendidus*. A = normal control larvae, B = pathogen challenged larvae, C = control larvae plus probiotic, D = pathogen challenged larvae plus probiotic.



6.4 Discussion

6.4.1 Probiotic identities

Two novel probiotic γ-proteobacteria demonstrated benefits for GSM larvae subjected to pathogen challenges. The phylogenetic positioning of probiotic strain 0444 clearly placed it in the *Alteromonas* genus, and indicated it to be *A. macleodii* (98% similarity to

numerous strains), while strain 0536 was shown to be closely related to members of the genus *Neptunomonas* (98% similarity).

Neptunomonas is a relatively new genus, being first proposed by Hedlund *et al*. (1999). This is the fist time it has been documented as a probiotic bacterium. However it was isolated previously from healthy microalgae cultures in a hatchery facility (Schulze *et al*., 2006). Moreover, *N. naphthavorans* previously showed bioremediation potential following degradation of many polycyclic aromatic hydrocarbons (Hedlund *et al*., 1999). In contrast to *N. naphthavorans*, the strain investigated in the present study was able to hydrolyze gelatin and had a considerably higher temperature tolerance, namely 40°C compared with 24°C.

Although numerous strains of *Alteromonas* were documented previously as probiotics in aquaculture environments (Imada *et al*., 1985; Dopazo *et al*., 1988; Gil-Turnes *et al*., 1989; Lemos *et al*., 1991; Douillet & Langdon, 1993; 1994; Riquelme *et al*., 1996a; Abraham *et al*., 2001; Wang *et al*., 2002; Abraham, 2004; Junfeng, 2008), this is the first report to describe *A. macleodii* as a probiotic. Other work on the genus began with purification of a glycoprotein from *Alteromonas* sp., which was capable of inhibiting the proteases of the fish pathogens *Aeromonas hydrophila* and *V. anguillarum* (Imada *et al*., 1985). Since then, strains within the genus were shown to possess beneficial antibacterial abilities in shrimp embryos and larvae (Gil-Turnes, 1989; Abraham *et al*., 2001; Abraham, 2004), scallop larvae (Riquelme *et al*., 1996a; Wang *et al*., 2002) and Pacific oyster larvae (Douillet & Langdon, 1993; 1994). It is worth pointing out the fact that, in

the aforementioned literature, *Alteromonas* spp. previously exhibited probiotic properties in the larval culture of three other mollusc species; *Argopecten irradians*, *Argopecten purpuratus*, *Crassostrea gigas*. *Alteromonas* have also been described as opportunistic bacteria under nutrient enriched conditions (Pinhassi & Berman, 2003). This is important because the intensive practice of aquaculture corresponds with an environment of high nutrient levels, thereby making conditions excellent for the probiotic. *Alteromonas* are well known also for the production of extracellular substances such as antibiotics (McCarthy *et al*., 1994), siderophores (Junfeng, 2008) and protease inhibitors (Imada *et al*., 1985); each of which has probiotic connotations. Further work on extracellular substance production is intended for *A. macleodii* 0444. However, pathogen inhibition testing of strain 0444, utilising agar diffusion cross-streak methods, as described by Gibson *et al*. (1998), did not indicate substance production (Appendix 3). One possible reason for this could be that, if produced, the antibacterial substance is bound to the outer surface of the cell and, therefore, does not diffuse freely into the agar, as suggested by Abraham (2004) for other *Alteromonas* strains. Nicolas *et al*. (2004) and Schulze *et al*. (2006) isolated *A. macleodii* from a range of aquaculture-hatchery sources; the strain in the present study was isolated also from a hatchery.

6.4.2 Probiotic effects

At both the low and high dosages of both pathogens, probiotic use offered significant protection for GSM larvae against pathogen challenge. During individual trials, the benefit ranged from 18% to 52% increased larval survival four days following pathogen exposure. Across all experiments, the average survival of larvae exposed only to

pathogens at the high and low dosages was 13.9% and 35.8% respectively on the fourth day post-exposure. When probiotic was administered before challenge, larval survival was improved to 50.1% and 66.1% respectively. Comparing these data with control larvae and those administered probiotic alone, 67.4% and 78.5% respectively, it was evident that, not only did the probiotics offer protection against pathogens, but they could also be beneficial in normal larval rearing. Potentially this situation would avoid large monetary losses in GSM larval rearing, since approximate hatchery costs run at US$600 day^{-1} = US$12,000 per larval batch lost (D. McCall, personal communication: hatchery spat project operations manager, Marlborough Mussel Co. Ltd.).

Protection against a pathogen-challenge was reproducible in all cases with one exception, namely *A. macleodii* 0444 vs *V. splendidus*, where a higher dose of the pathogen was used. It is possible that a benefit from use of *A. macleodii* 0444 would have occurred still at the minimum effective pathogenic dose of *V. splendidus* (10^5 CFU ml^{-1}), although other environmental factors (for example, health status of the larval batch) might have influenced the outcome. Interestingly, a study on microbial community changes in environmental samples, mentioned both *A. macleodii* and *V. splendidus* (Pinhassi & Berman, 2003). The study determined both as opportunistic γ-proteobacteria utilising nutrient enriched sources. In one experiment, *V. splendidus* became the total dominant member in a community. Yet, when the test was repeated six days later, *A. macleodii* became the dominant member. While this showed that both strains revelled in an enriched environment, it also highlighted that there are other factors contributing to the growth responses of the two strains. Perhaps in the repeat experiment with *A. macleodii*

0444, conditions were more favourable to the opportunistic nature of *V. splendidus*, exacerbated by the high dose exposure.

Sizes of larvae administered probiotic alone were generally no different to those of control animals, indicating that no nutritional benefit is afforded by the probiotics when compared with fed animals. It would be interesting to investigate whether size differences exist between unfed GSM larvae and larvae which are also unfed but administered the probiotic. In one experiment, animals administered probiotics before pathogen challenge were, on average, smaller than non-challenged larvae, perhaps reflecting that some members of the population were infected and displayed stunted growth. This is of minor importance compared to the probiotic effect because, more importantly, a significant proportion of the population survived the pathogen challenge.

The experiments presented herein were concluded on the sixth day of the larval period. The results do not imply larval rearing success because the larval period takes approximately 18 days, but they do demonstrate protection against a pathogen challenge in the early stages of GSM larval rearing (which is when most larval mortality incidents have occurred at GACL). It cannot be assumed that protection during the early stages will allow successful completion of the GSM larval period. However, one experiment which continued until settlement, observed that pathogen-challenged larvae receiving probiotic reached settlement similar to non-exposed control larvae (Appendix 5). In addition, the pathogen was not detected in larvae by day 9 (six days after pathogen-exposure). This

strongly suggests that larvae given the probiotic have a good likelihood of continued normal larval rearing to settlement despite exposure to pathogen in the early larval stages.

Despite the better survival rates achieved when probiotics were used before pathogen-challenge of the larvae, in most cases, the pathogen was still detected in the samples during the experiments (Table 6.4). This might be expected because some larvae in these treatments were infected and subsequently sampled. It was noted previously that some larvae were able to develop normally in the presence of sub-lethal levels of the pathogen (refer to Chapter 4). In this study, it was likely that the probiotic protected a large proportion of the larvae against the initial elevated pathogen challenge and, once the water flow resumed, pathogen levels declined to sub-lethal levels, aided by daily probiotic addition for a further three days. The most encouraging observation during bacterial detection was that *Neptunomonas* sp. 0536 was able to limit both *V. splendidus* to non-detectable levels during a challenge and also largely inhibit naturally-occurring vibrios in the hatchery environment (Fig. 6.5A). This offers a potentially wider application for this probiotic in the aquaculture field, where vibriosis continues to be a major cause of disease.

Having demonstrated that the probiotics can allow larval survival during the unfavourable conditions of an induced pathogen challenge, the potential benefits to routine larval rearing (whereby conditions might not be as detrimental) are exciting. In fact, in both challenge experiments involving *A. macleodii* 0444, larvae treated with the probiotic performed better than normal control larvae in terms of survival. Advantageously, *A.*

macleodii 0444 was detected still in the larvae 11 days after its final addition to the tanks. This was observed despite the continual water flow during rearing and indicated that the probiotic had a high affinity for GSM larvae, likely contributing to the probiotic protection afforded. The beneficial effect from the probiotic is likely to stem from inhibiting pathogen attachment to the GSM gut (refer to Chapter 5).

The need for alternative approaches to disease management has fuelled interest in probiotics within aquaculture. New agents are constantly required to compete in the ongoing battle between pathogens and antimicrobials. This study has documented two novel probiotics which displayed beneficial effects against pathogen attack in GSM larval rearing. This benefit offers stability and cost savings to GSM larval rearing. Following this work, the effect of probiotic administration during normal larval rearing, but not including pathogen challenge, was investigated.

Chapter 7

Effects of administering a probiotic bacterium, *Alteromonas macleodii* 0444, during routine hatchery production of Greenshell™ mussel larvae, *Perna canaliculus*

7.1 Introduction

While many studies previously documented the use of probiotics against pathogen attack (Lategan *et al*., 2004a; 2004b; Chabrillon *et al*., 2006), few proceeded to document probiotic utilisation during routine conditions of aquaculture production. One report described the administration of strains of *Vibrio*, *Pseudomonas* and *Bacillus* during routine larval rearing of the Chilean scallop, *Argopecten purpuratus* (Riquelme *et al*., 2001).

In earlier studies, *Alteromonas macleodii* 0444 was isolated and demonstrated as a probiotic bacterium for GSM larvae (refer to Chapter 6). Although it was shown to protect GSM larvae from pathogen attack by *V. splendidus* and a *V. coralliilyticus/neptunius*-like isolate, certain parameters of larval performance were not investigated including feed consumption, larval appearance and settlement success of animals administered the probiotic. Additionally, animals were not reared past the larval period. The aim of the present study was to evaluate the effects of administering *A. macleodii* 0444 during routine GSM larval production in the absence of a pathogen attack when compared with larvae not treated with the probiotic. Parameters of larval performance were monitored in the hatchery and the on-going presence of the bacterium was determined in test animals after their placement on a mussel farm for continued cultivation as per normal industry practices.

7.2 Materials and methods

7.2.1 Culture of probiotic

Probiotic bacterium, *Alteromonas macleodii* 0444, was grown and harvested as in Chapter 4.2.2

7.2.2 Experimental animals

Refer to Chapter 6.2.1

7.2.3 Experimental design

At daily intervals during the first 11 days of culture, larvae were administered the probiotic, *A. macleodii* 0444. The probiotic was added at two concentrations: 10^7 CFU ml^{-1} and 10^8 CFU ml^{-1}. During incubation of the inoculum, water flow was stopped for two hours and then resumed. Larvae, not inoculated with the bacterium, were included as controls. Treatments were run in triplicate.

On alternate days, either outflow barrier-screens (nylon mesh size 40 μm) were cleaned, or full tank cleanings were performed (screens, tanks and larvae). GSM were reared through the larval stage, settled after metamorphosis and transferred to the Marlborough Sounds, New Zealand, where they were on-grown at a commercial mussel operation. For settlement, coir (roped coconut husk) was used as the settlement substrate. Initial settlement occurred under static conditions for 48 hours, before water flow was resumed.

7.2.4 Probiotic effects on larval performance and settlement

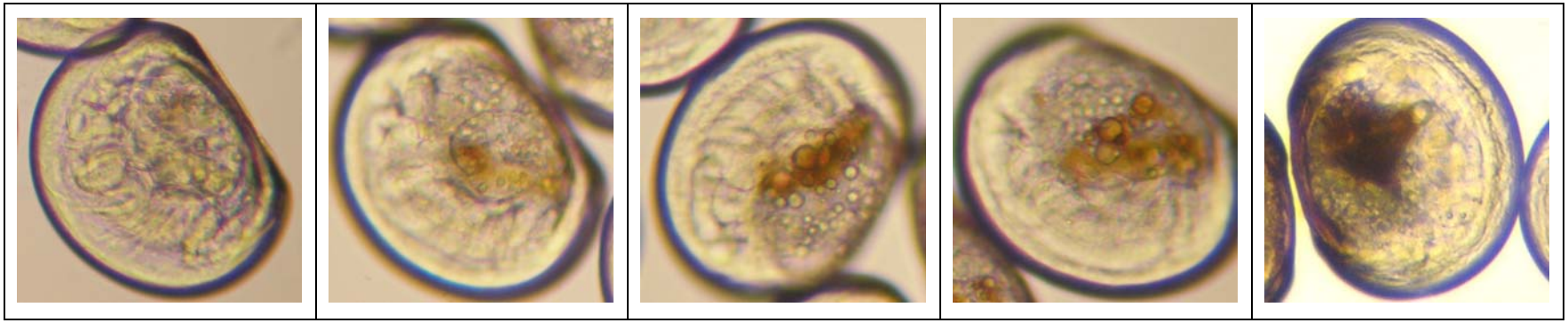
Microscopic observations were made daily throughout the larval period upon larval swimming behaviour, gut colour and lipid levels. About 100 larvae in each treatment were visualised and a score, from one to five, assigned to represent the average for each parameter in the treatments (Fig. 7.1 & 7.2). A score of five was judged as being very healthy. Scores for consecutive days were grouped and averaged to provide two-day data blocks.

Three times per week larval survival (%), size (µm) and feed consumption (cells larvae^{-1} day^{-1}) were measured. Survival was calculated as a percentage for each tank after taking triplicate aliquots from tanks (250 µl, approximately 50 larvae per aliquot). Larval size was measured as described in Chapter 6.2.5. To measure feed consumption, chlorophyll levels were determined in the inflow water and outflows for each tank using a fluorometer (CYCLOPS-7, Turner Designs, CA, USA) connected to a multimeter, thereby providing mV readings. Cell concentrations of the microalgae cultures fed to the larvae were determined by counting on a haemocytometer and the cultures were measured also in the fluorometer, allowing translation of mV readings into microalgae cell values. Microalgae consumption was calculated using the following equation:

Consumption = (inflow cells – outflow cells) / number alive larvae in the tank

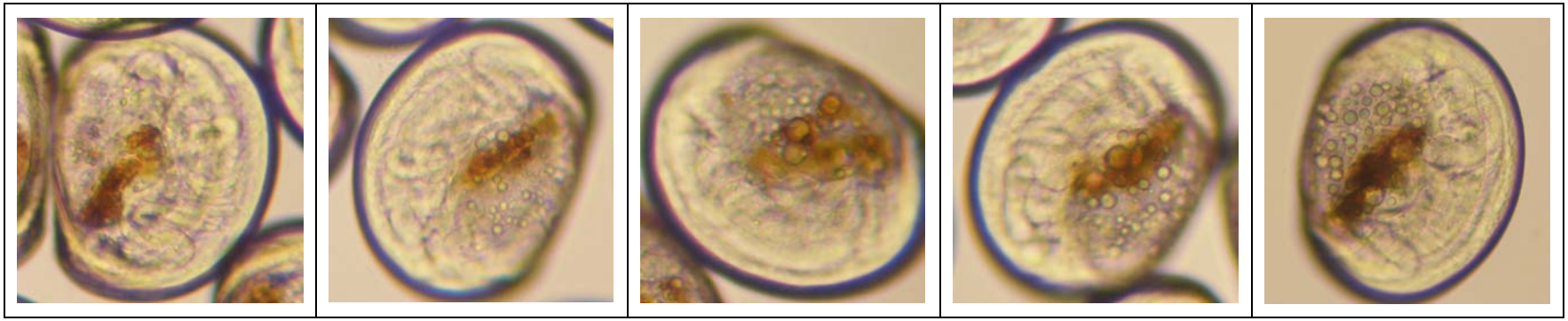
Where: 1 mV = 0.28 microalgae cells

Figure 7.1 Guide for visual assessment of larval GSM gut colour.



Note: Left to right = categories one to five, five being very good.

Figure 7.2 Guide for visual assessment of larval GSM lipid levels. The lipids are the globules inside the mussels.



Note: Left to right = categories one to five, five being very good.

Larvae were screened on the 17th and 19th days post-hatching using nylon mesh size 178 μm. This coincided with larval eye spots indicating that larvae were ready to settle. Those larvae retained on the screen were used to determine the proportion of GSM reaching settlement capacity. In addition, a probiotic effect on larval settlement success was investigated. Five thousand animals from each tank were settled on coir; each replicate was settled onto coir in a separate tank. Settlement utilised larvae from the day 17 screening. Following initial settlement in static conditions for 48 hours, water flow was resumed for eight days. Upon the 10th day, the coir was removed and treated with chlorine to remove settled animals. Larval settlement was determined as a percentage of the initial five thousand.

7.2.5 Probiotic persistence in GSM larvae and on-grown mussels

Probiotic treatment of GSM larvae ended on the 11th day of the larval period. The presence/absence of *A. macleodii* 0444 in the larvae and tank water was determined on the 17th and 18th days of larval culture; this was one week after final probiotic addition and just prior to settlement. Water samples (containing larvae) from tanks were processed as described in Chapter 4.2.3. Re-isolation of *A. macleodii* 0444 was performed following the scheme described in Chapter 6.2.6.

Test animals were cultivated in the field for seven months in order to determine the persistence of the probiotic in GSM prior to reaching the consumer. Fifty thousand larvae from each tank were settled onto coir for on-growing in the Marlborough Sounds, New Zealand. Animals from each treatment and replicate were kept separate. On the fourth

and seventh months following hatching, 20 mussels from each replicate were sampled for presence/absence of *A. macleodii* 0444. Animal meat was removed from the shells and placed into a stomacher for four minutes. Ten-fold dilution schemes were made in sterile 2% seawater and plated onto selective media.

7.2.6 Data analysis

Percent survival figures were arc sin square root transformed to approximate normality. Treatment differences with regard to larval survival, microalgae consumption, larval size, and the proportion of larvae retained on nylon mesh size 178 μm during the 17^{th} and 19^{th} days were compared using ANCOVA ($p=0.05$); day of the larval period and treatment being the co-variables. Data for settlement success were analysed using ANOVA. Post hoc comparisons were made using Tukey's test.

7.3 Results

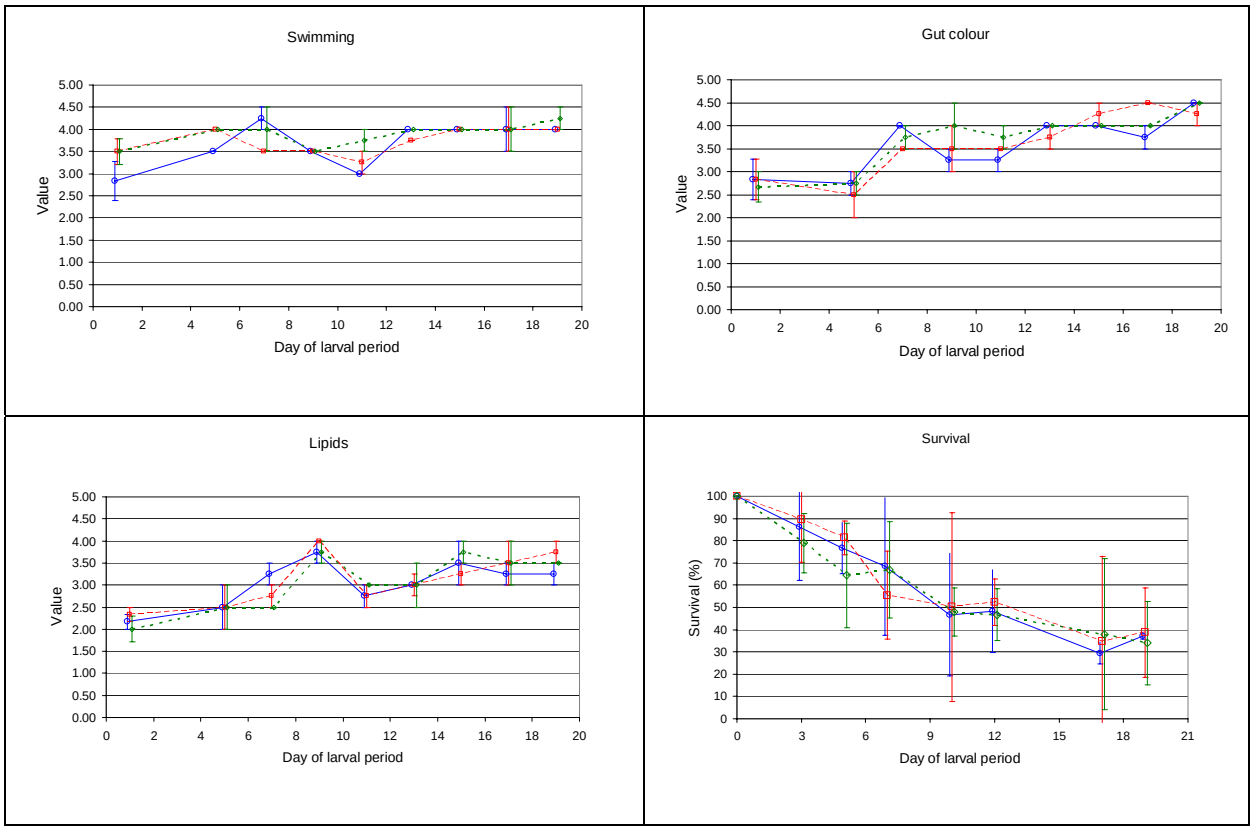
7.3.1 Visual observations

Apart from days seven to ten, larvae administered *A. macleodii* 0444 were swimming more actively than control larvae until the 13^{th} day, (Fig. 7.3). Gut colouration of larvae in probiotic treatments was better than control larvae throughout the second half of the larval period. There were minor differences in lipid levels of the three treatments. However, animals administered probiotics did have better lipid stores just prior to metamorphosis at the end of the larval period.

7.3.2 Larval survival

There was no difference in the survival of larvae in any treatment (Fig. 7.3). By the end of the larval period, larval survivals (± 95% confidence intervals) were 37.2 % ± 1.5 (control), 38.8% ± 20.1 (*A. macleodii* 0444 10^7 CFU ml^{-1}) and 34% ± 18.9 (*A. macleodii* 0444 10^8 CFU ml^{-1}).

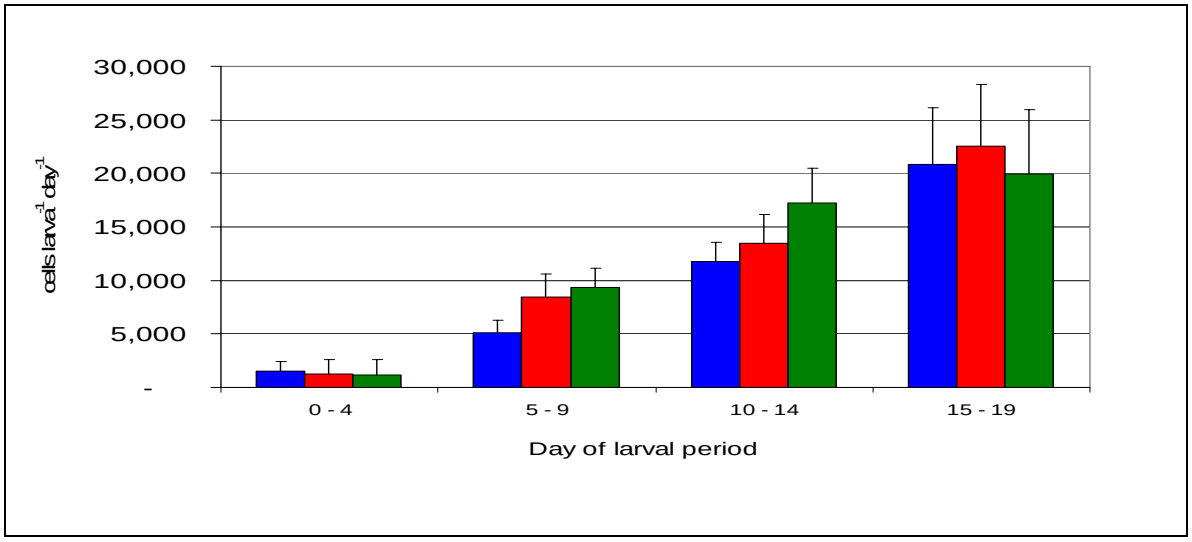
Figure 7.3 Observations of GSM larvae throughout the larval period. Parameters: swimming behaviour, gut colour and lipid levels were scored one to five; five being best. Observational data (swimming behaviour, gut colour and lipid) are displayed as two-day averages (± standard error), while survival data represents average larval survival ± 95% confidence intervals. Blue line = control larvae, red line = *A. macleodii* 10^7 CFU ml^{-1}, green line = *A. macleodii* 10^8 CFU ml^{-1}.



7.3.3 Larval feed consumption

During the first four days of the larval period, larvae in the probiotic treatments fed slightly less than control larvae (Fig. 7.4). For the remainder of the larval period, probiotic treatments generally consumed more algae than control larvae. None of the differences were statistically significant ($P > 0.05$).

Figure 7.4 Microalgae consumption (cells larva^{-1} day^{-1}) of GSM larvae administered probiotic, *A. macleodii* 0444, and non-treated larvae throughout the duration of the larval period. Data points are mean consumption (± 95% confidence intervals) from triplicate tanks over a five-day period.



Note: Blue columns = control larvae, red columns = *A. macleodii* 10^7 CFU ml^{-1}, green columns = *A. macleodii* 10^8 CFU ml^{-1}.

7.3.4 Larval size

Larval size did not differ ($p > 0.05$) between the treatments throughout the course of the larval period (Table 7.1). During two larval screenings on a mesh size 178 µm, the proportion of larvae retained and ready for settlement did not differ significantly between

the treatments (Fig. 7.5). Additionally, the success of larval settlement onto coir showed no difference between the treatments (Fig. 7.6).

Table 7.1 Mean size (µm ± 95% confidence intervals) of GSM larvae in different treatments throughout the larval period.

Treatment	Day 0	Day 3	Day 5	Day 7	Day 10	Day 12	Day 17	Day 19
Control	88.8 ± 0.7	104.9 ± 0.6	115.0 ± 0.5	128.6 ± 0.8	142.9 ± 1.1	159.6 ± 2.0	201.4 ± 5.0	219.7 ± 3.8
A. macleodii 10^7	88.8 ± 0.7	104.1 ± 0.6	114.8 ± 0.6	125.5 ± 0.9	146.5 ± 1.2	166.3 ± 2.6	209.8 ± 5.1	217.3 ± 3.8
A. macleodii 10^8	88.8 ± 0.7	103.7 ± 0.6	112.9 ± 0.7	126.1 ± 0.8	149.1 ± 1.3	167.4 ± 2.8	206.2 ± 6.1	214.4 ± 5.3

Figure 7.5 Proportion (%) of GSM larvae which were retained on a 178 µm screen during the first and second settlement screening. Values represent mean size ± 95% confidence intervals.

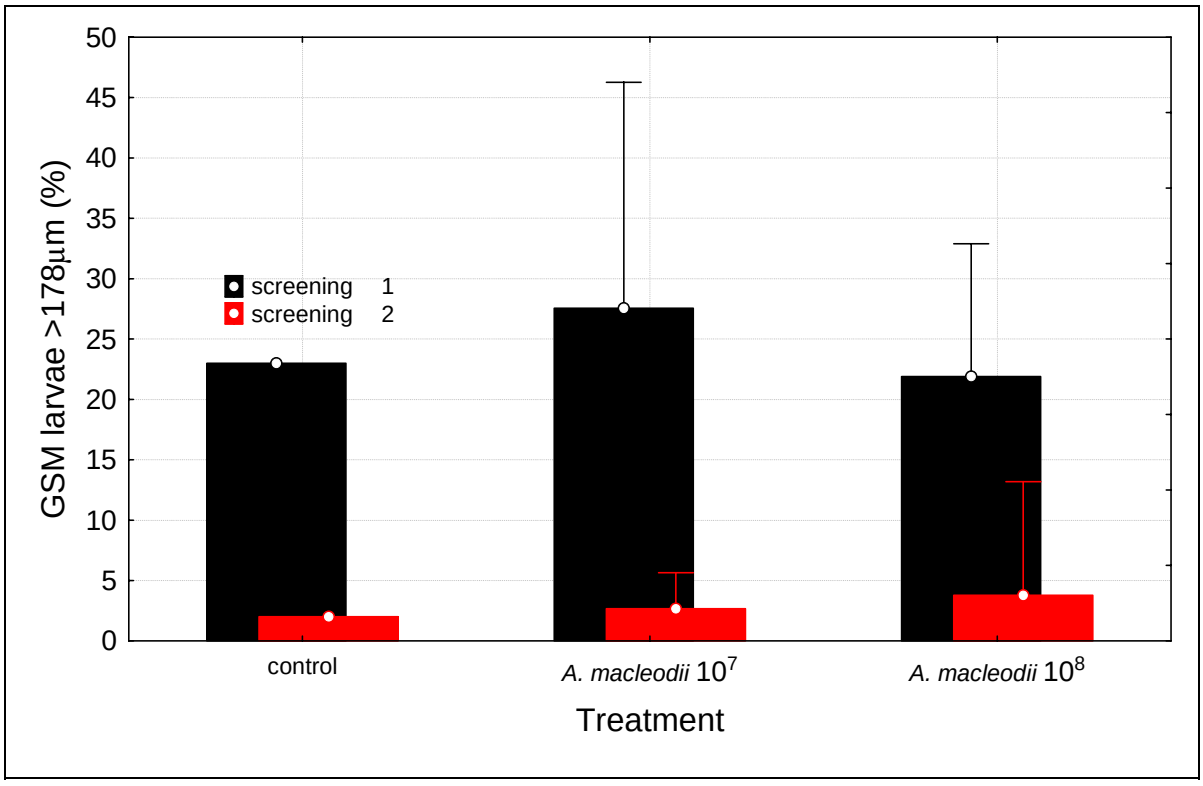
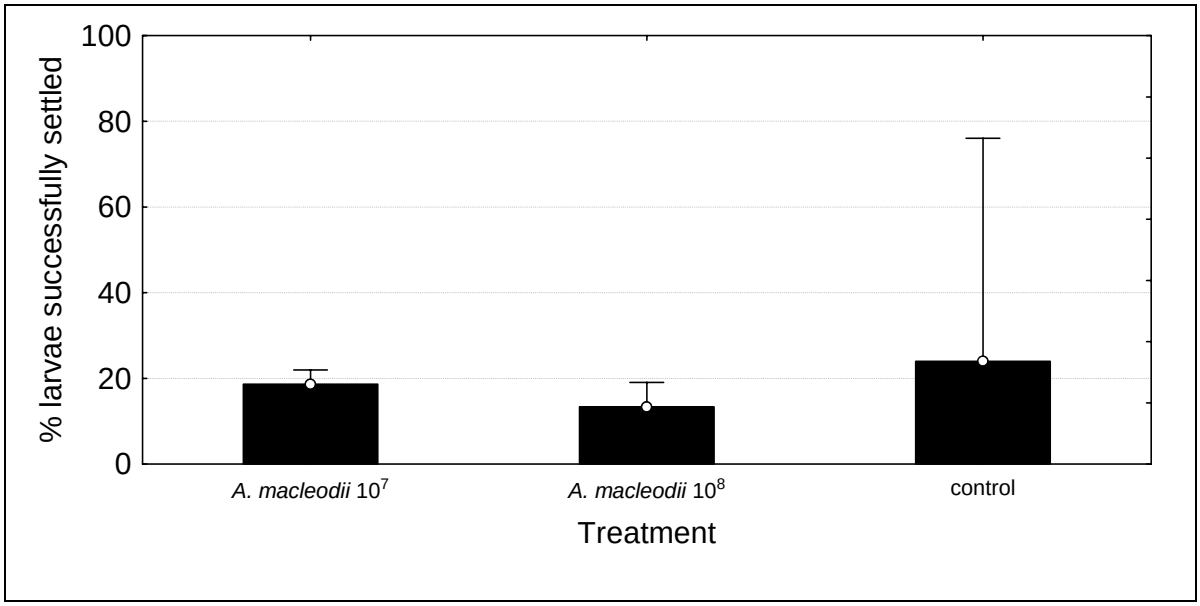


Figure 7.6 Mean percentage of larvae successfully settled on coir after the larval period. Values represent mean success from three replicate tanks ± 95% confidence intervals.



7.3.5 On-going probiotic presence

A. macleodii 0444 was re-isolated from larvae and tank water in the 10^7 and 10^8 treatments on the 17th and 18th days post-hatching, but it was not detected in larvae or water from the control treatment (Table 7.2). *A. macleodii* 0444 was not detected in on-grown GSM after four or seven months cultivation (Table 7.2).

Table 7.2 Detection of probiotic, *A. macleodii* 0444, in GSM larvae and mussels on-grown in the Marlborough Sounds.

Treatment	Age* of mussels			
	Day 17	Day 18	4 months	7 months
Non-inoculated control (1)	-	-	-	-
Non-inoculated control (2)	-	-	-	-
Non-inoculated control (3)	-	-	-	-
A. macleodii 0444 10^7 CFU ml^{-1} (1)	+	+	-	-
A. macleodii 0444 10^7 CFU ml^{-1} (2)	+	+	-	-
A. macleodii 0444 10^7 CFU ml^{-1} (3)	+	+	-	-
A. macleodii 0444 10^8 CFU ml^{-1} (1)	+	+	-	-
A. macleodii 0444 10^8 CFU ml^{-1} (2)	+	+	-	-
A. macleodii 0444 10^8 CFU ml^{-1} (3)	+	+	-	-

Note*: The duration represents days or months post-hatching.

7.4 Discussion

This study demonstrated that GSM larvae, administered the probiotic bacterium, *A. macleodii* 0444, performed neither better nor worse than a healthy and successful batch of normal GSM larvae. Certain probiotics have been shown to be beneficial for aquaculture in terms of growth advantages; benefits which, by nature, were apparent when compared with normal controls (Gatesoupe, 2002; Lara-Flores *et al*., 2003; Macey & Coyne, 2005; ten Doeschate & Coyne, 2008). However, the majority of probiotic research focused on health benefits against detrimental conditions such as pathogen attack (Lategan *et al*., 2004a; 2004b; Chabrillon *et al*., 2006). The benefits of *A. macleodii* 0444 against pathogen attacks were previously demonstrated (refer to Chapters 5 & 6). In the absence of detrimental conditions, a beneficial effect caused by administration of *A. macleodii* 0444 was not demonstrable. This does not lessen the

probiotic's effectiveness, but rather confirms the contention that *A. macleodii* 0444 is suitable as a prophylactic treatment for bacterial disease prevention in GSM larviculture.

In the presence of microalgal feed, *A. macleodii* 0444 did not offer significant additional nutritional aid to GSM larvae since similar larval sizes were obtained throughout the larval period (Table 7.1). However, for most of the larval period, larvae administered the probiotic consumed more food and they also contained better lipid levels just prior to metamorphosis and settlement. These attributes favour successful settlement; lipid levels prior to settlement were shown to correlate with settlement success in Pacific oyster larvae (Laing & Earl, 1998). However, in the present study, there were no differences in the settlement success rates of GSM (Fig. 7.4). It is interesting that larvae administered the probiotic had a feed consumption approximately 20% less than controls during the first four days post-hatching. It is possible that GSM larvae fed upon *A. macleodii* 0444 in these early stages, as was shown to occur in great scallop larvae, *Pecten maximus* (Moal *et al*., 1996). Probiotic addition had a noticeable effect on GSM swimming behaviour which was most evident in the first few days following hatching. This was manifest as more actively swimming larvae compared with control larvae (Fig. 7.3). Moreover, this coincided with lower microalgae consumption in the more active animals. More active larvae and less microalgae consumption in the early stages possibly indicate an advantageous role for bacteria in the first feeding stages of GSM larvae.

Detection of *A. macleodii* 0444 in GSM larvae, seven days after the last addition, illustrated its ability for retention to the larvae. This is in agreement with the previous

observation of its presence in GSM larvae 11 days after final addition into larval tanks (Appendix 5). Persistence of the probiotic in the larvae could be an added benefit by extending the protective effect as a consequence of attachment exclusion of pathogens from the larvae. This mechanism was proposed previously as aiding in the probiotic properties of *A. macleodii* 0444 (refer to Chapter 6).

In the presence of *A. macleodii* 0444, GSM larvae could be reared through to settlement in a manner similar to a batch of healthy larvae. This is an encouraging complement to the benefits *A. macleodii* 0444 demonstrated previously against pathogenic encounters (refer to Chapter 6). Additionally, *A. macleodii* 0444 could not be detected in treated mussels four months after their transfer to the farm, which indicates that the probiotic does not persist in the animals until they are harvested a further 12 months later. Based on the results presented herein and those which previously documented the benefits against pathogen attack, the routine administration of *A. macleodii* 0444 in GSM larval rearing is recommended. Such use will reduce the influence of opportunistic bacteria and protect against pathogenesis, thereby facilitating more reliable larval rearing.

Chapter 8

Performance of two probiotic strains, administered in combination, during hatchery production of Greenshell™ mussel larvae, *Perna canaliculus*, including exposure to pathogen challenges

8.1 Introduction

Application of probiotics as multi-strain or multi-species preparations has been shown to provide improved benefits when compared with single-strain application in human models (Zoppi *et al*., 2001; Collado *et al*., 2007) and animal models (Lema *et al*., 2001). The argument for improved protection implies that a combination of probiotics allows for the incorporation of different mechanisms of probiotic action into the one treatment. This was demonstrated when administration of six strains of lactic acid bacteria in combination, each possessing different benefits, provided additive protection compared with the protection offered by the individual bacteria alone (Timmerman *et al*., 2007). Such mechanisms that probiotics may act by include: production of substances inhibitory to pathogenic bacteria, competition for attachment sites on the host, production of beneficial enzymes, out-competition for nutrients and immunostimulatory effects (refer to Chapter 1). Another potential benefit of probiotic combinations is the enhancement of a particular trait, as was demonstrated when *Bifidobacterium lactis* adhesion to mucus more than doubled in the presence of two strains of *Lactobacillus* (Ouwehand *et al*., 2000).

Within aquaculture almost all probiotic research has involved single-strain probiotic application. There has been a lack of studies evaluating probiotic combinations compared with single-strain administration. However, a few reports do exist. Irianto & Austin (2002b) included a combination treatment alongside testing four strains of probiotic bacteria individually against *Aeromonas salmonicida*. They found that, although each treatment improved the survival of rainbow trout against the pathogen, there was no

advantage of combination administration. Similarly, Díaz-Rosales *et al*. (2006) found no additional benefit from the combined use of two heat-inactivated probiotic strains of *Vibrio* sp. in the immune response of gilthead sea bream. On the other hand, strains of *Lactobacillus* and *Bacillus*, used in combination, improved the immune response of gilthead sea bream compared with single-strain administration, in either the live or heat-inactivated form (Salinas *et al*., 2005; 2008). In a recent study of single-strain and combination use of *Bacillus subtilis* and *Lactobacillus acidophilus* on Nile tilapia (Aly *et al*., 2008), all probiotic treatments performed better than controls. Although there was little advantage in immunological parameters with combination use, survival after pathogen attack was improved with the combination.

Previously, two bacterial strains, *Alteromonas macleodii* 0444 and *Neptunomonas* sp. 0536, were shown to be effective against vibriosis in GSM larviculture. The aim of this study was to determine whether an additional benefit was afforded to GSM larvae by using the two probiotics in combination during normal larval rearing and against pathogen attack. Combination use of the probiotics was compared with single-strain administration of each and with non-inoculated control larvae. Two dosages of probiotic administration, 10^7 and 10^8 CFU ml^{-1}, both singly and in combination, were tested.

8.2 Materials and methods

8.2.1 Experimental animals

Refer to Chapter 6.2.1

8.2.2 Culture and harvest of bacteria

Bacteria were grown and harvested as in Chapter 4.2.2

8.2.3 Experimental design

Two probiotics of GSM larvae, *Alteromonas macleodii* 0444 and *Neptunomonas* sp. 0536, were tested against two pathogens of GSM, *V. splendidus* and *Vibrio* sp. DO1. GSM larvae were inoculated with the probiotics at daily intervals for the first six days of the experiment. Probiotics were administered at two concentrations in separate treatments; 10^7 and 10^8 CFU ml^{-1}. During incubation of the probiotic, water flow was stopped and kept static for a two-hour period. Pathogen-challenge of the larvae occurred on the third day following hatching, immediately following the two-hour probiotic incubation on day 3, i.e. in challenge treatments, the probiotic remained in the static-water tank during pathogen challenge. Pathogens were inoculated at doses previously determined to cause infection (refer to Chapter 4). These concentrations were 10^6 CFU ml^{-1} for *V. splendidus* and 10^7 CFU ml^{-1} for *Vibrio* sp. DO1. Water flow remained static during pathogen exposure for one hour (*V. splendidus*) and two hours (*Vibrio* sp. DO1). Consequently, water flow on the third day remained static for a total time of three hours and four hours respectively, for experiments involving *V. splendidus* and *Vibrio* sp. DO1. This condition was applied also in the control treatments. Larval rearing and measurements continued until settlement (17 days following hatching). On alternate days, either outflow barrier-screens (nylon mesh size 40 μm) were cleaned, or full tank cleaning were performed (screens, tanks and larvae).

A set of treatments (Table 8.1) was designed to allow comparisons between:

1. Non-inoculated larvae
2. Larvae administered a single-strain of each probiotic at 10^7 and 10^8 CFU ml^{-1}
3. Larvae administered dual combination (1:1) of each probiotic at 10^7 and 10^8 CFU ml^{-1} $probiotic^{-1}$
4. Larvae from (2), exposed to pathogen challenges with each pathogen separately
5. Larvae from (3), exposed to pathogen challenges with each pathogen separately
6. Larvae challenged with the pathogens alone

Table 8.1 Experimental treatments for trialling the use of dual-probiotic combinations against pathogen challenges.

Treatment	*A. macleodii* 0444 (daily)	*Neptunomonas* sp. 0536 (daily)	*V. splendidus* (3^{rd} day chall)	*Vibrio* sp. DO1 (3^{rd} day chall)
Non-inoculated Control	-	-	-	-
Prob control (low)	10^7 CFU ml^{-1}	-	-	-
Prob control (high)	10^8 CFU ml^{-1}	-	-	-
Prob control (low)	-	10^7 CFU ml^{-1}	-	-
Prob control (high)	-	10^8 CFU ml^{-1}	-	-
Path control	-	-	10^6 CFU ml^{-1}	-
Path control	-	-	-	10^7 CFU ml^{-1}
Prob (low) path-chall	10^7 CFU ml^{-1}	-	10^6 CFU ml^{-1}	-
Prob (high) path-chall	10^8 CFU ml^{-1}	-	10^6 CFU ml^{-1}	-
Prob (low) path-chall	10^7 CFU ml^{-1}	-	-	10^7 CFU ml^{-1}
Prob (high) path-chall	10^8 CFU ml^{-1}	-	-	10^7 CFU ml^{-1}
Prob (low) path-chall	-	10^7 CFU ml^{-1}	10^6 CFU ml^{-1}	-
Prob (high) path-chall	-	10^8 CFU ml^{-1}	10^6 CFU ml^{-1}	-
Prob (low) path-chall	-	10^7 CFU ml^{-1}	-	10^7 CFU ml^{-1}
Prob (high) path-chall	-	10^8 CFU ml^{-1}	-	10^7 CFU ml^{-1}
Combo control (low)	10^7 CFU ml^{-1}	10^7 CFU ml^{-1}	-	-
Combo control (high)	10^8 CFU ml^{-1}	10^8 CFU ml^{-1}	-	-
Combo (low) path-chall	10^7 CFU ml^{-1}	10^7 CFU ml^{-1}	10^6 CFU ml^{-1}	-
Combo (high) path-chall	10^8 CFU ml^{-1}	10^8 CFU ml^{-1}	10^6 CFU ml^{-1}	-
Combo (low) path-chall	10^7 CFU ml^{-1}	10^7 CFU ml^{-1}	-	10^7 CFU ml^{-1}
Combo (high) path-chall	10^8 CFU ml^{-1}	10^8 CFU ml^{-1}	-	10^7 CFU ml^{-1}

Note: Prob = probiotic, Path = pathogen, chall = pathogen-challenge, Combo = combination.

All treatments were conducted in triplicate. Daily for the first six days, plus on days 9, 12 and 17, triplicate aliquots (250 µl) were taken from tanks and larval survival determined (approximately 50 larvae per aliquot). On day 17, larvae were screened for the first time on nylon mesh size 178 µm. This coincided with eye spots in the larvae indicating that larvae were ready to settle. As per normal hatchery procedure, the larvae were initially screened to remove those larvae which were fast-growing and ready to settle. The remaining larvae were reared two days further in the flow-through system, after which they were re-screened on nylon mesh size 178 µm to settle. The proportion of larvae in each tank that reached settlement capability was determined.

8.2.4 Larval feed consumption

Microalgae feed consumption (cells larvae^{-1} day^{-1}) was measured for each tank on days 1-6, 9, 12-17 as described in Chapter 7.2.4.

8.2.5 Larval size

Upon completion of the larval cycle (day 17), larval size (µm) was measured for each treatment as in Chapter 6.2.5

8.2.6 Bacterial examination of challenged larvae and tank water

During the first six days, the presence or absence of the pathogens was monitored daily in both the water column and larval samples as per Chapter 6.2.6

8.2.7 Data analysis

Percent survival figures were arc sin square root transformed to approximate normality. Treatment differences were compared using ANOVA (p=0.05). Post hoc comparisons between survivals were compared using Tukey's test. Size data were compared by non-parametric Kruskal-Wallis test.

8.3 Results

8.3.1 Larval survival

Regardless of whether a probiotic was administered or not, all treatments exposed to a pathogen attack had significantly lower survival than those unchallenged, namely the control larvae, or larvae exposed to single-strain or combination probiotics alone (Table 8.2). Larvae from treatments administered either single-strain or combination probiotics, in the absence of a pathogen attack, achieved the same survivals as non-inoculated control larvae, except on the 17^{th} day in the 10^8 CFU ml^{-1} combination treatment where survival was lower (Fig. 8.1).

When larvae were challenged with *V. splendidus*, both 10^7 and 10^8 CFU ml^{-1} dosages of *Neptunomonas* sp. 0536 and the probiotic combination treatments afforded significantly better larval survivals. Administration of *A. macleodii* 0444, as a single strain probiotic, also afforded better survival against *V. splendidus*; however this was not statistically significant except on the 17^{th} day at the high dosage of the probiotic (Table 8.2). Throughout the experiment, there were no statistically significant differences between the

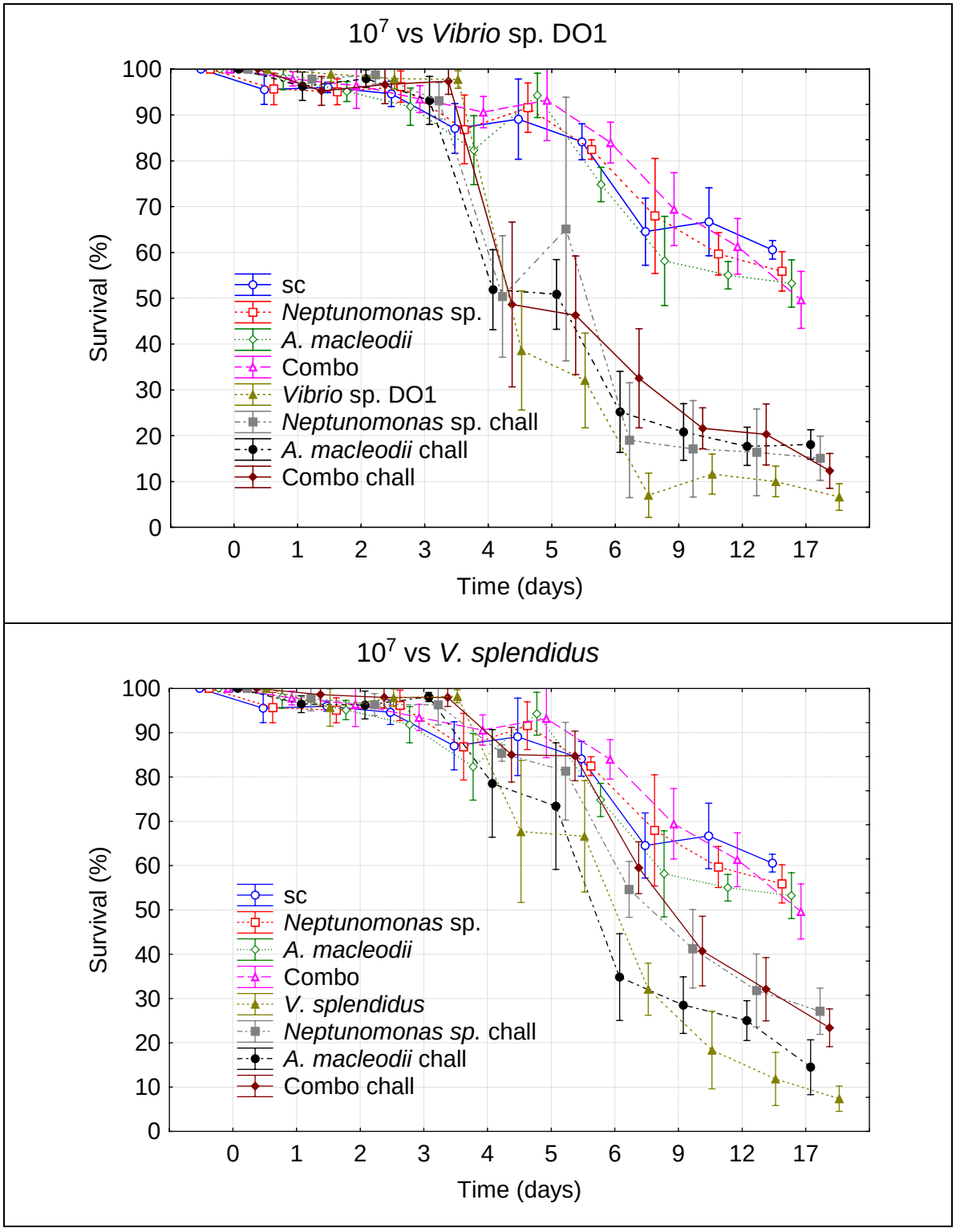
use of probiotic combinations or single strain *Neptunomonas* sp. 0536, at either the high or low dosages, against *V. splendidus* challenge.

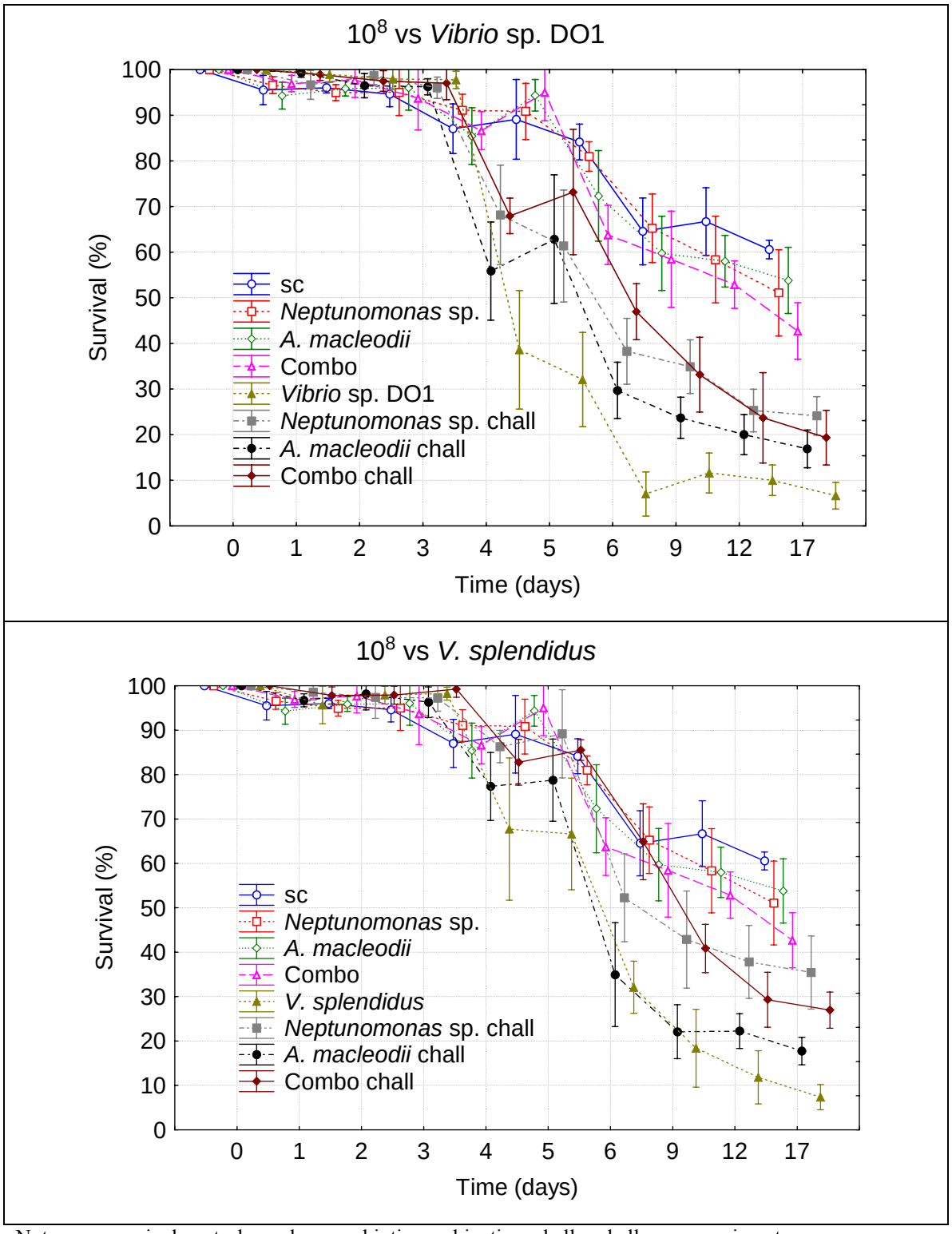
When challenged with *Vibrio* sp. DO1, survival of larvae administered a low dose of *Neptunomonas* sp. 0536 was statistically no different to pathogen controls. However, on the 17th day their survival rate was significantly better. Moreover, a high dose of *Neptunomonas* sp. 0536 did afford significantly greater larval survival when compared with pathogen controls. Similarly, when a combination of probiotics was applied against *Vibrio* sp. DO1, the low combination dose was ineffective, while the high dosage provided significantly improved larval survival compared with the pathogen controls. When tested against *Vibrio* sp. DO1, no difference in survival was observed between the high dose of *Neptunomonas* sp. 0536, used alone, and the high dose of the combination probiotics. Administration of *A. macleodii* 0444, as a single-strain probiotic, provided improved survival against pathogen attack by *Vibrio* sp. DO1; however this was statistically significant only on the 6th and 17th days. There were no statistical differences between either probiotic as a single strain protection against *Vibrio* sp. DO1 at both high and low concentrations.

Table 8.2 Cumulative GSM larval survival in different treatments of a dual-probiotic administration experiment. Values represent mean survival ± 95% confidence intervals. Values not sharing the same superscript are statistically different ($p < 0.05$).

Treatment	Day 6	Day 9	Day 12	Day 17
Non-inoculated Control	84.2 ± 3.9 ^{a}	64.6 ± 7.3 ^{a}	66.7 ± 7.4 ^{a}	60.6 ± 2.0 ^{a}
Neptunomonas sp. 0536 (low)	82.5 ± 2.1 ^{a}	68.0 ± 12.5 ^{a}	59.7 ± 4.6 ^{a}	55.9 ± 4.3 ^{ab}
Neptunomonas sp. 0536 (high)	81.0 ± 3.2 ^{a}	65.2 ± 7.5 ^{a}	58.4 ± 9.5 ^{a}	51.1 ± 9.5 ^{ab}
A. macleodii 0444 (low)	74.8 ± 3.8 ^{ab}	58.2 ± 9.7 ^{abc}	55.0 ± 3.0 ^{a}	53.2 ± 5.2 ^{ab}
A. macleodii 0444 (high)	72.3 ± 9.9 ^{abc}	59.7 ± 8.1 ^{ab}	58.0 ± 5.7 ^{a}	53.8 ± 7.2 ^{ab}
Combo control (low)	84.0 ± 4.4 ^{a}	69.5 ± 8.0 ^{a}	61.3 ± 6.1 ^{a}	49.7 ± 6.2 ^{ab}
Combo control (high)	63.8 ± 6.5 ^{abcd}	58.5 ± 10.5 ^{abc}	52.9 ± 5.2 ^{ab}	42.7 ± 6.2 ^{bc}
Neptunomonas sp. 0536 (low) vs *V. splendidus*	54.7 ± 6.3 ^{cdf}	30.2 ± 13.9 ^{bcde}	31.8 ± 8.2 ^{cde}	27.1 ± 5.2 ^{def}
Neptunomonas sp. 0536 (high) vs *V. splendidus*	52.3 ± 9.9 ^{cdef}	42.9 ± 10.9 ^{bcde}	37.8 ± 8.2 ^{bc}	35.4 ± 8.2 ^{cd}
A. macleodii 0444 (low) vs *V. splendidus*	34.8 ± 9.8 ^{egh}	23.0 ± 7.4 ^{defg}	25.0 ± 4.5 ^{cdefg}	14.5 ± 6.2 ^{ghij}
A. macleodii 0444 (high) vs *V. splendidus*	35.0 ± 11.7 ^{gh}	22.1 ± 6.1 ^{fgh}	22.3 ± 3.9 ^{cdefg}	17.8 ± 3.1 ^{efgh}
Combo (low) vs *V. splendidus*	59.5 ± 5.8 ^{df}	40.7 ± 7.9 ^{cde}	32.1 ± 7.1 ^{cd}	23.4 ± 4.3 ^{defg}
Combo (high) vs *V. splendidus*	64.9 ± 8.5 ^{bcd}	40.8 ± 5.4 ^{cd}	29.3 ± 6.2 ^{cde}	27.0 ± 4.1 ^{df}
V. splendidus	32.1 ± 5.9 ^{gh}	18.4 ± 8.8 ^{gh}	11.8 ± 6.0 ^{gh}	7.4 ± 2.8 ^{ij}
Neptunomonas sp. 0536 (low) vs *Vibrio* sp. DO1	19.0 ± 12.5 ^{ij}	14.2 ± 7.5 ^{gh}	16.4 ± 9.5 ^{efgh}	15.1 ± 4.8 ^{ghi}
Neptunomonas sp. 0536 (high) vs *Vibrio* sp. DO1	38.3 ± 7.2e ^{fgh}	34.9 ± 5.9 ^{def}	25.3 ± 4.7 ^{cdef}	24.1 ± 4.2 ^{defg}
A. macleodii 0444 (low) vs *Vibrio* sp. DO1	25.2 ± 8.8 ^{hi}	20.8 ± 6.2 ^{fgh}	17.7 ± 4.2 ^{efgh}	18.1 ± 3.2 ^{efgh}
A. macleodii 0444 (high) vs *Vibrio* sp. DO1	29.7 ± 6.2 ^{hi}	23.7 ± 4.5 ^{efgh}	20.0 ± 4.4 ^{defgh}	16.9 ± 4.1 ^{egh}
Combo (low) vs *Vibrio* sp. DO1	32.5 ± 10.8 ^{hi}	21.6 ± 4.5 ^{fgh}	15.2 ± 7.1 ^{fgh}	12.3 ± 3.8 ^{hij}
Combo (high) vs *Vibrio* sp. DO1	47.0 ± 6.1 ^{efg}	33.1 ± 8.2 ^{defg}	23.7 ± 9.9 ^{cdefg}	19.3 ± 6.0 ^{efgh}
Vibrio sp. DO1	7.0 ± 4.8 ^{j}	11.6 ± 4.4 ^{h}	10.0 ± 3.3 ^{h}	6.6 ± 2.9 ^{j}

Figure 8.1 GSM larval survival (%) during a probiotic pathogen-challenge experiment utilising probiotics in single-strain administration or in dual-combination. Each graph represents either a different pathogen or a different test concentration of the probiotics. Values represent mean survival ± 95% confidence intervals.



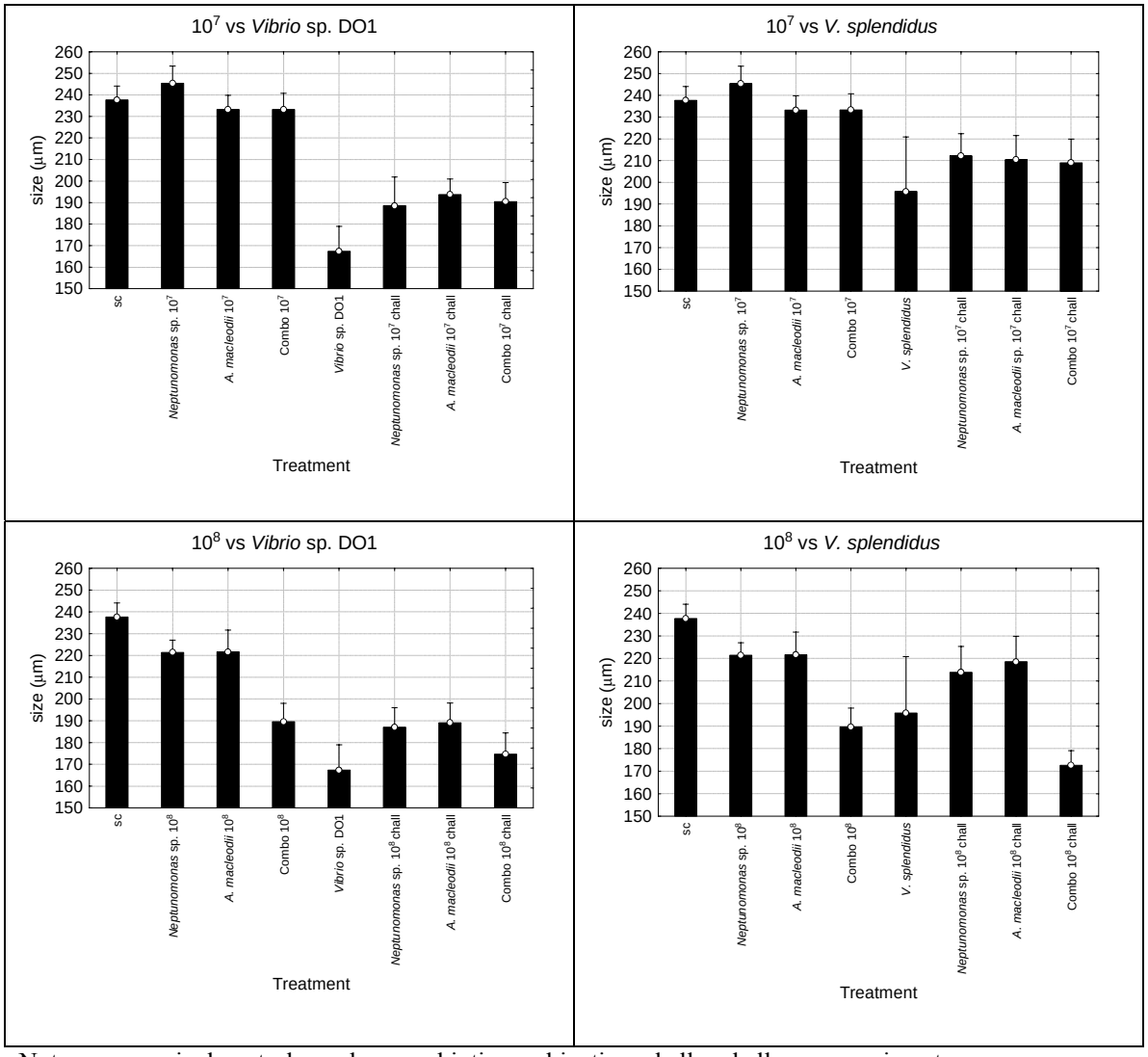


Note: sc = survival control, combo = probiotic combination, chall = challenge experiment.

8.3.2 Larval size

At day 17, non-inoculated control larvae and those in probiotic controls at 10^7 CFU ml^{-1} (both combination and single-strain), were significantly larger than larvae challenged with either pathogen, but were no different with respect to each other (Fig. 8.2). This occurred also at the 10^8 CFU ml^{-1} level of administration, except in the combination probiotic controls, which were statistically no different to challenged larvae. Sizes were greater for challenged larvae if they had received probiotic treatment, either 10^7 or 10^8 CFU ml^{-1}, although they were not statistically larger than pathogen controls. This occurred against both pathogens.

Figure 8.2 Mean day 17 GSM larval size (µm) of different treatments during a probiotic pathogen-challenge experiment utilising probiotics in single-strain administration or in dual-combination. Each graph represents either a different pathogen or a different test concentration of the probiotics. Values represent mean size ± 95% confidence intervals.

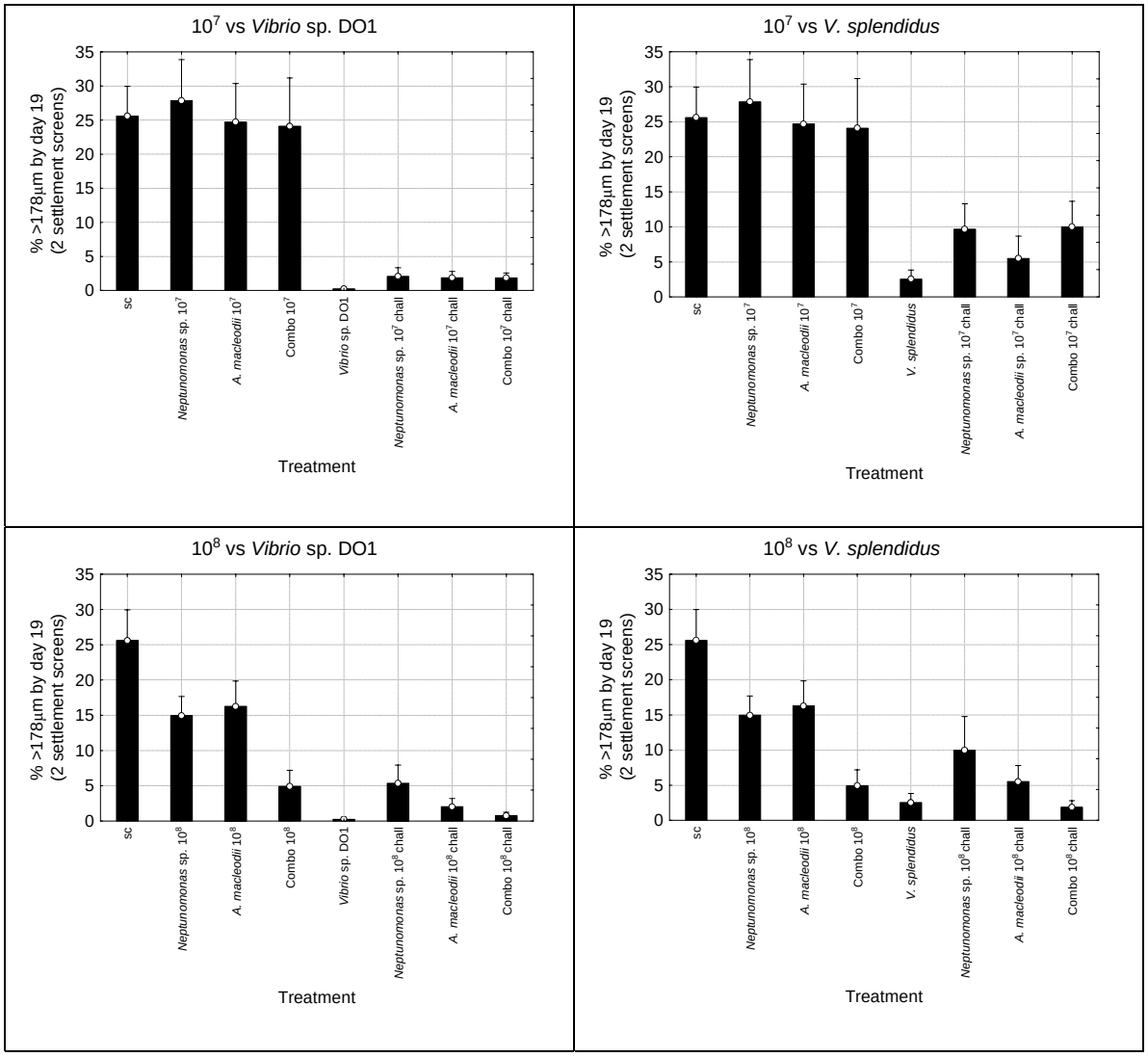


Note: sc = survival control, combo = probiotic combination, chall = challenge experiment.

8.3.3 Larvae to reach settlement

By day 19, proportions of each larval population which were retained on a 178 μm mesh, showed that probiotic administration at 10^7 CFU ml^{-1} levels, either singly or in combination, in the absence of pathogen challenge, were no different to the non-inoculated controls (Fig. 8.3). However, when probiotics were administered at 10^8 CFU ml^{-1} concentrations, significantly fewer larvae were retained on the screen when compared with the non-inoculated controls. Furthermore, in the 10^8 CFU ml^{-1} combination probiotic controls, the proportion of larvae retained on the mesh was significantly lower than with single-strain probiotics alone and statistically no different to pathogen controls. Compared with the pathogen controls, a larger proportion of larvae were retained on the mesh if probiotics had been administered prior to challenge. This observation was statistically insignificant in all cases except one, namely, the pathogen *V. splendidus* using combination 10^7 CFU ml^{-1} treatment (10.0% ± 3.6) compared with pathogen controls (2.3% ± 1.3).

Figure 8.3 Proportion (%) of GSM larvae which were retained on a 178 µm screen by the second settlement screening (day 19). Each graph represents either a different pathogen or a different test concentration of the probiotics. Values represent mean size ± 95% confidence intervals.



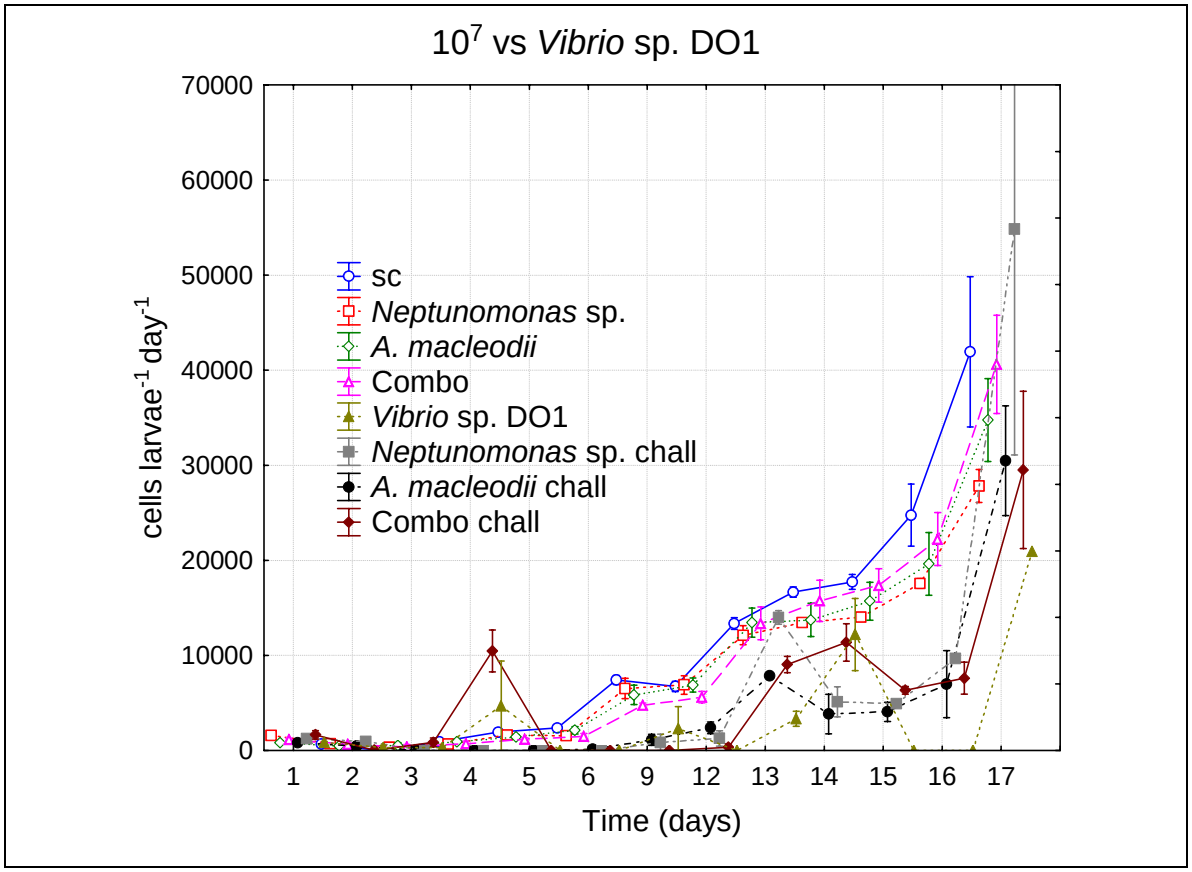
Note: sc = survival control, combo = probiotic combination, chall = challenge experiment.

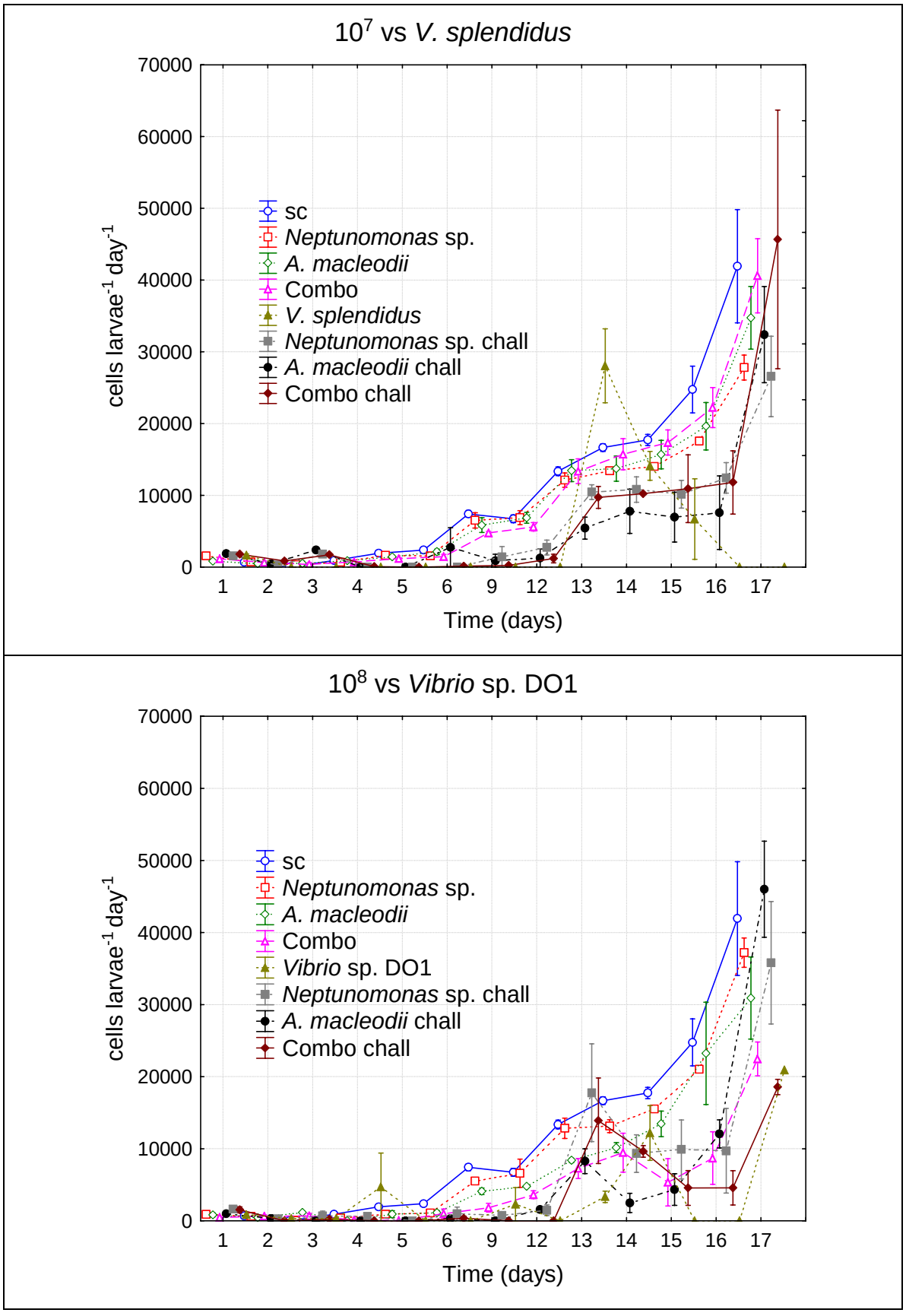
8.3.4 Larval feed consumption

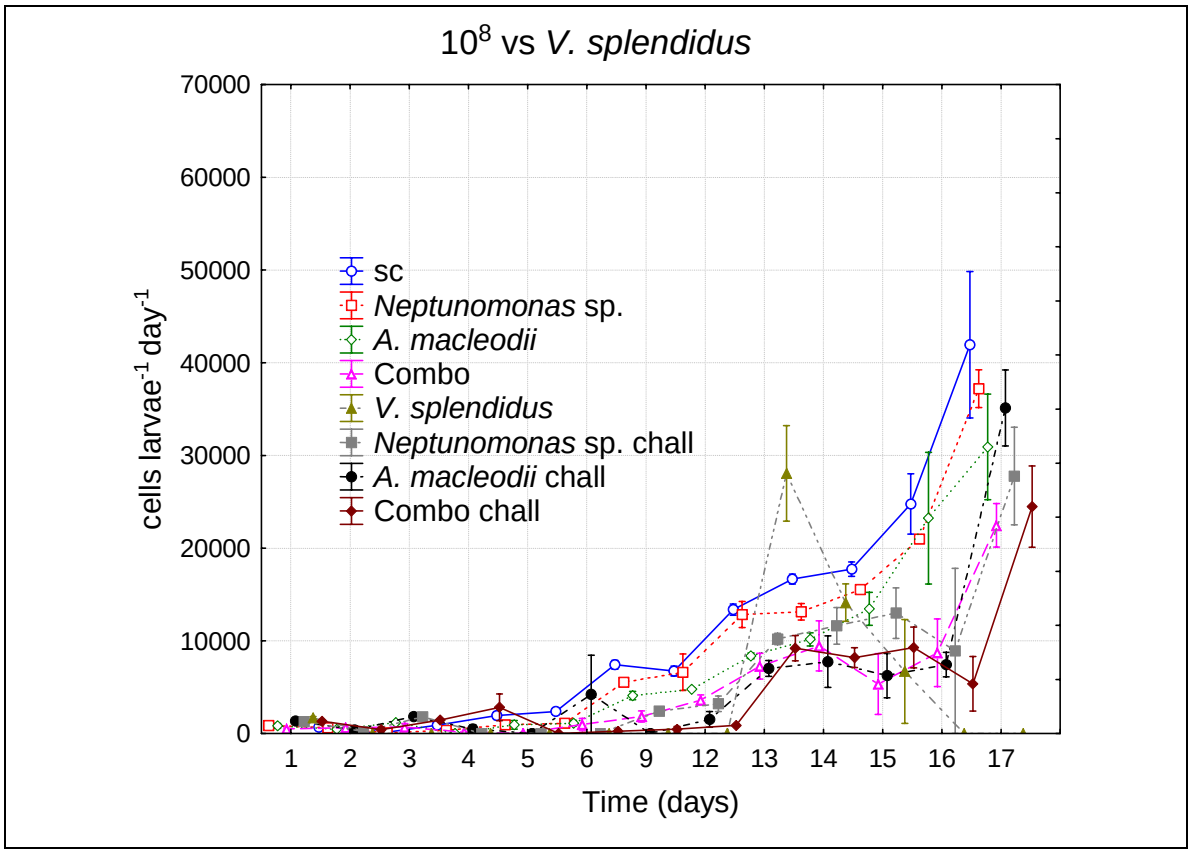
Although there were differences between the treatments in the feed consumption of the larvae throughout the experiment, these were not statistically significant (Fig. 8.4). Unchallenged larvae consumed more microalgae than challenged larvae (Fig. 8.4). Non-

inoculated control larvae and those in combination probiotic controls, at 10^7 CFU ml^{-1}, consistently consumed the most microalgae. Combination probiotics at 10^8 CFU ml^{-1} suppressed feeding activity markedly; 24,760, 22,239 and 8,713 cells $larva^{-1}$ day^{-1} respectively on the 16th day; 41,937, 40608 and 22461 cells $larva^{-1}$ day^{-1} respectively on the 17th day for non-inoculated control larvae, 10^7 CFU ml^{-1} combination probiotics and 10^8 CFU ml^{-1} combination probiotics. This equates to one-day retardation in feeding when compared with other control treatments.

Figure 8.4 Microalgae consumption (cells $larva^{-1}$ day^{-1}) of GSM larvae in different treatments during a probiotic/pathogen challenge experiment utilising probiotics in single-strain administration or in dual combination. Each graph represents either a different pathogen or a different test concentration of the probiotics. Values represent mean consumption (± standard error).




10^7 vs *V. splendidus*
cells larvae^{-1} day^{-1}
70000
60000
50000
40000
30000
20000
10000
0
sc
Neptunomonas sp.
A. macleodii
Combo
V. splendidus
Neptunomonas sp. chall
A. macleodii chall
Combo chall
1 2 3 4 5 6 9 12 13 14 15 16 17
Time (days)
10^8 vs *Vibrio* sp. DO1
cells larvae^{-1} day^{-1}
70000
60000
50000
40000
30000
20000
10000
0
sc
Neptunomonas sp.
A. macleodii
Combo
Vibrio sp. DO1
Neptunomonas sp. chall
A. macleodii chall
Combo chall
1 2 3 4 5 6 9 12 13 14 15 16 17
Time (days)



Note: sc = survival control, combo = probiotic combination, chall = challenge experiment.

8.3.5 Bacterial examination of challenged larvae and tank water

Probiotics were detected in the larvae and water of the treatments to which they had been added (Table 8.3). Despite being detected in the larvae from day 1, *Neptunomonas* sp. 0536 was undetectable in the water until the fourth day. Pathogens were detected from the day following their inoculation, except in the combination 10^8 CFU ml^{-1} treatment against *V. splendidus* challenge, where they were undetected in the water and larvae on the third day. Similarly, on the third day, *V. splendidus* was not detected in the water of tanks where *Neptunomonas* sp. 0536 had been used in single-strain administration, but the pathogen was recovered from the larvae.

Table 8.3 Detection of test isolates from treatments during probiotic combination experiments.

Treatment		Day 0		Day 1		Day 2		Day 3		Day 4		Day 5		Day 6	
		W	L	W	L	W	L	W	L	W	L	W	L	W	L
Non-inoculated Control	Prob	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Path	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Neptunomonas sp. 0536 (low)	Prob	-	-	-	+	-	+	-	+	+	+	+	+	+	+
	Path	-	-												
Neptunomonas sp. 0536 (high)	Prob	-	-	-	+	-	+	-	+	+	+	+	+	+	+
	Path	-	-												
A. macleodii 0444 (low)	Prob	-	-	+	-	+	+	+	+	+	+	+	+	+	+
	Path	-	-												
A. macleodii 0444 (high)	Prob	-	-	+	+	+	+	+	+	+	+	+	+	+	+
	Path	-	-												
V. splendidus	Prob	-	-												
	Path	-	-					+	+	+	+	+	+	+	+
Vibrio sp. DO1	Prob	-	-												
	Path	-	-					+	+	+	+	+	+	-	+
Neptunomonas sp. 0536 (low) vs *V. splendidus*	Prob	-	-	-	+	-	+	-	+	+	+	+	+	+	+
	Path	-	-					-	+	+	+	+	+	+	+
Neptunomonas sp. 0536 (high) vs *V. splendidus*	Prob	-	-	-	+	-	+	-	+	+	+	+	+	+	+
	Path	-	-					-	+	+	+	+	+	+	+
Neptunomonas sp. 0536 (low) vs *Vibrio* sp. DO1	Prob	-	-	-	+	-	+	-	+	+	+	+	+	+	+
	Path	-	-					+	+	+	+	+	+	+	+
Neptunomonas sp. 0536 (high) vs *Vibrio* sp. DO1	Prob	-	-	-	+	-	+	-	+	+	+	+	+	+	+
	Path	-	-					+	+	+	+	+	+	+	+
A. macleodii 0444 (low) vs *V. splendidus*	Prob	-	-	+	+	+	+	+	+	+	+	+	+	+	+
	Path	-	-					+	+	+	+	+	+	+	+
A. macleodii 0444 (high) vs *V. splendidus*	Prob	-	-	+	+	+	+	+	+	+	+	+	+	+	+
	Path	-	-					+	+	-	+	+	+	+	+
A. macleodii 0444 (low) vs *Vibrio* sp. DO1	Prob	-	-	+	+	+	+	+	+	+	+	+	+	+	+
	Path	-	-					+	+	+	+	+	+	+	+
A. macleodii 0444 (high) vs *Vibrio* sp. DO1	Prob	-	-	+	+	+	+	+	+	+	+	+	+	+	+
	Path	-	-					+	+	+	+	+	+	+	+
Combo control (low)	Prob	-	-	+	+	+	+	+	+	+	+	+	+	+	+
	Path	-	-												
Combo control (high)	Prob	-	-	+	+	+	+	+	+	+	+	+	+	+	+
	Path	-	-												
Combo (low) vs *V. splendidus*	Prob	-	-	+	+	+	+	+	+	+	+	+	+	+	+
	Path	-	-					-	+	+	+	+	+	+	+
Combo (high) vs *V. splendidus*	Prob	-	-	+	+	+	+	+	+	+	+	+	+	+	+
	Path	-	-					-	-	+	+	+	+	+	+
Combo (low) vs *Vibrio* sp. DO1	Prob	-	-	+	+			+	+	+	+	+	+	+	+
	Path	-	-					+	+	+	+	+	+	+	+
Combo (high) vs *Vibrio* sp. DO1	Prob	-	-	+	+			+	+	+	+	+	+	+	+
	Path	-	-					+	+	+	+	+	+	+	+

Note: Prob = probiotic, Path = pathogen, W = water, L = larvae.

8.4 Discussion

The results of this study indicated that a combination of the probiotics, *A. macleodii* 0444 and *Neptunomonas* sp. 0536, did not provide additional support against individual pathogen attacks by *Vibrio* sp. DO1 or *V. splendidus* during GSM larval rearing. Despite this, the results did show that a combination administration allowed for the successful

completion of the larval cycle in GSM. This is important because, although not observed in this experiment, it is possible that administration of the two probiotics would provide more comprehensive protection against encounters with opportunistic bacteria during routine larval rearing. Such opportunistic bacteria were not tested in this study. Other studies previously demonstrated enhanced protection with multi-species probiotics (Zoppi *et al*., 2001; Timmerman *et al*., 2007), based on the theory that multiple strain-specific benefits possessed by individual probiotics could broaden the spectrum of probiotic effect. Indeed, in earlier work, probiotics were isolated that were effective only against *V. splendidus* or *Vibrio* sp. DO1, but not both (refer to Chapter 5).

Probiotic application resulted in significantly better survival rates in pathogen-challenged larvae. Survivals were constantly better with *A. macleodii* 0444 usage; however levels were only statistically better than pathogen controls on the 6^{th} and 17^{th} days. Nevertheless, *A. macleodii* 0444 has repeatedly shown protection against vibriosis in GSM larvae (refer to Chapter 6); hence its capacity as a probiotic is well illustrated. Possible reasons for the lower levels of protection in the present study could be genetic differences in the larval batch tested thereby making them more susceptible to pathogens, or environmental parameters at the time, such as natural resident microflora during the experiments. Such variation was observed in the larvae of control treatments during several independent experiments in Chapter 4. Administration of *Neptunomonas* sp. 0536 alone and probiotic combinations afforded significantly better larval survival against attack by *V. splendidus* and *Vibrio* sp. DO1; however probiotic levels of 10^7 CFU ml^{-1} were less effective against *Vibrio* sp. DO1. On most occasions throughout the study,

administration of probiotics at levels of 10^8 CFU ml^{-1}, whether single-strain or in combination, afforded slightly better larval survival against pathogen challenge than did 10^7 CFU ml^{-1}. However, this observation was statistically significant only on a few occasions (Table 8.2).

Despite 10^8 CFU ml^{-1} dosages appearing better in terms of larval survival against pathogen challenge, 10^8 CFU ml^{-1} probiotic control treatments performed worse than 10^7 CFU ml^{-1} control treatments, although this was not significant. However, the survival rate of the 10^8 CFU ml^{-1} combination control was significantly lower than non-inoculated control larvae (42.7% compared with 60.6% survival). Moreover, a dosage of 10^8 CFU ml^{-1} reduced the size of unchallenged larvae and also the proportion of larvae retained upon a 178 μm screen at day 19, when compared with 10^7 CFU ml^{-1} dosages. Percentage of larvae retained on a 178 μm screen incorporates the measures of larval survival and size and is the parameter of most commercial importance. For the most part, feed consumption was higher for larvae in the single-strain 10^7 CFU ml^{-1} probiotic controls compared with 10^8 CFU ml^{-1} controls, although not statistically so. From days 1-6 and 9-14 microalgae consumption was 33% and 17% less, respectively, in 10^8 CFU ml^{-1} probiotic control treatments compared with 10^7 CFU ml^{-1} treatments; consumption was similar during days 15-17. Additionally, those larvae which received a 10^8 CFU ml^{-1} dose of probiotics in combination appeared hampered. This was expressed by a feeding activity which was considerably lower than all other control treatments; 34% lower at day 17, roughly the same as day 16 consumption in the other treatments. It would appear that the health of larvae was better in treatments receiving probiotics at a 10^7 CFU ml^{-1} dose.

This was accentuated when the probiotics were administered in combination, whereby the 10^8 CFU ml^{-1} dose appeared to be detrimental as judged by slowed growth and reduced survival rates.

One potential reason for the better survival rates obtained with the 10^8 CFU ml^{-1} dosages of probiotics might relate to the feed consumption of the larvae. The observation that larvae were feeding less at the higher dosage might translate into less pathogen uptake during pathogen challenge and, therefore, presumably less mortality. Previously, it was documented that GSM larvae administered *A. macleodii* 0444 at 10^8 CFU ml^{-1} consumed approximately 20% less microalgae than non-inoculated control larvae during the first four days of the larval period, suggesting the larvae might be feeding upon the probiotic during the early larval stages (refer to Chapter 7). In the present study, larvae in the 10^7 and 10^8 probiotic control treatments consumed 7% and 38% less microalgae than non-inoculated control larvae during the first six days. This reinforced the notion that larvae administered probiotics consume fewer microalgae than do non-inoculated larvae. It is worth considering whether, in the present study, the higher dose of probiotics was unfavourable to the larvae, leading to a reduced feeding behaviour and lower pathogen consumption and mortality, or whether the probiotic was consumed by the larvae, resulting in reduced microalgae consumption but increased protection against the pathogens. Despite probiotic addition ceasing on the sixth day, the larvae administered 10^8 CFU ml^{-1} levels of probiotic continued to consume fewer microalgae than those provided 10^7 CFU ml^{-1} until the 14^{th} day. This might indicate that, indeed, the larvae were compromised in some way by the higher levels of the probiotic, hence reducing their

ability to feed. Alternatively, it might also be the result of lower microalgae consumption during the early stages, when larvae also consumed bacteria, leading to size differences and, hence, continual consumption differences between larvae in the 10^7 and 10^8 CFU ml^{-1} treatments. If the latter concept were true and, indeed, the larvae were uncompromised by the higher dosages of probiotic, albeit growing slower, then the determination of which was the better concentration of probiotic to administer would need to be reconsidered. Laing & Earl (1998) showed that slow growing Pacific oyster larvae were equally as competent as fast growers. What that means in terms of the present study is that, although larvae might be better protected from pathogen attack with higher dosages, they grow slower and take longer to settle, yet still remain as viable as faster-growing larvae provided the lower dose. Cost analysis models would then need to determine the potential extra safety provided by the higher dosages of the probiotics in relation to the extra running time, considering that a GSM hatchery costs approximately US$600 per day to operate (D. McCall, personal communication). One aspect to consider is that, when administered in combination, larvae treated with a 10^8 CFU ml^{-1} combination of the probiotics had 7% lower survival than 10^7 CFU ml^{-1} combination treated larvae and 18% lower than non-inoculated controls, the latter being statistically significant.

It should be noted also that the combinations applied here are not exhaustive. It would be prudent to examine other combinations of the probiotics, such as different proportions of each in addition to the 1:1 ratio applied in the current study. Douillet (2000b) tested six mixtures of probiotics upon the growth of rotifers, each containing a different composition of the same four isolates. He found that just one of the mixtures performed

better than single-strain administration of *Alteromonas* sp., which was one of the components. Likewise, two *Lactobacillus* strains were administered in combination to larval sea bream at a ratio of 4:1 (Carnevali *et al*., 2004; Picchietti *et al*., 2007). Although further ratios were not tested, a positive effect was seen in the survival of sea bream larvae and fry at the 4:1 ratio. In the present study, based on single strain administration, it would appear that *Neptunomonas* sp. 0536 is more beneficial than *A. macleodii* 0444 to GSM larvae, perhaps indicating that a larger proportion of the probiotic combination consisting of the former would benefit GSM. On the other hand, previous tests showed that larvae administered *A. macleodii* 0444 performed better, in terms of larval survival, than non-inoculated control larvae, highlighting the usefulness of this bacterium (refer to Chapter 6). The effect of each probiotic might be expected to act differently upon separate batches of larvae reared during different times of the year. While probiotics can be expected to be effective, they cannot be relied upon to produce the exact same result every time because of variations in the environment in which they are applied. Multi-strain probiotic administration might be a good way to counter such uncertainties.

The results presented herein did not provide unequivocal support with regard to the best probiotic administration. Despite the lack of increased protection against pathogen attack following combination probiotic treatment, the advantages of such treatments to protect against multiple infections have been described by others. For prophylactic purposes, it would seem advantageous to administer multi-species probiotic treatments. The findings of this study indicated that the combination of *A. macleodii* 0444 and *Neptunomonas* sp. 0536 at the 10^8 CFU ml^{-1} level might be detrimental to GSM larvae. However,

combination levels of 10^7 CFU ml^{-1} performed no different when compared with non-inoculated control larvae. Importantly, these levels provided protection against pathogen attack with *V. splendidus* and *Vibrio* sp. DO1. However, the levels were not statistically significant against *Vibrio* sp. DO1 (a pathogenic event which, in the present study, was fast and severe). Additionally, in terms of the proportion of GSM larvae reaching settlement competency, 10^7 CFU ml^{-1} levels of probiotic performed better. In the situation where *A. macleodii* 0444 and *Neptunomonas* sp. 0536 are administered in combination, a level of 10^7 CFU ml^{-1} of each probiotic would be recommended. However, further work into the potential detriment of 10^8 CFU ml^{-1} concentrations, along with cost analysis on the implications of a slower growth rate versus increased pathogen safety, might determine 10^8 CFU ml^{-1} to be preferred in the long term.

Chapter 9

Synthesis

9.1 Synthesis

The work contained within this thesis was designed to address an industrial problem, namely the health management of GSM larvae. Regular antibiotic usage in GSM larviculture was not sustainable and alternatives in health management were needed. The success of this research was underlined by the discovery and demonstration of two novel probiotics, *A. macleodii* 0444 and *Neptunomonas* sp. 0536; both worthy additions to the literature on aquatic probiotics. Despite probiotic properties having been documented previously for *Alteromonas* spp., it is the first time *A. macleodii* has been named as a probiotic. Furthermore, this is the first time a member of the *Neptunomonas* genus has been demonstrated as a probiotic. Both probiotics displayed beneficial effects to GSM larvae under pathogen attack and are in the process of patent submission. Importantly, both were non-detrimental to GSM larval development and possessed an ability to persist in the larvae in a flow-through rearing system. The ability to maintain presence in the hatchery environment suggests these bacteria attached to GSM larvae. It was suggested that the probiotics out-competed the pathogens for attachment sites in the GSM gut; this would potentially protect the larvae from a pathology which was concentrated around the GSM digestive system. However, modes of probiotic action were not in the scope of this research program and future work will elucidate the mechanisms by which the probiotics are effective.

Demonstration of the benefit of both probiotics during routine hatchery rearing of GSM was yet another large milestone of the work presented. It progressed the work to a level at which it can easily be accepted by industry. Application of these probiotics is planned in

the near future and will provide future assurance to the industry. During regular use, the probiotic would be administered in a flow-through system. Therefore, it was opportune that a two-hour period of static water, with probiotics, was capable of facilitating a protective effect against pathogens and allowed for the establishment of the probiotic in the larvae; this was made evident by the ongoing detection of the probiotic after cessation of its administration. Additionally, it is quite possible that these probiotics would be beneficial for other aquaculture species. This notion is supported by the suppression of *Vibrio* spp. that was observed when *Neptunomonas* sp. 0536 was administered. This ability is advantageous in a culture environment where *Vibrio* spp. are the main disease agents. Although not in the scope of this research, work on other aquaculture animals, to determine the host range for the probiotics, is anticipated.

Another substantial contribution of this thesis was the development of a direct, small and fast screening technique for probiotic bacteria. Most importantly, the technique theoretically allowed all modes of probiotic action to be expressed. As demonstrated by BLIS testing, production of diffusible inhibitory substances was not occurring at noticeable levels for the majority of shortlisted test isolates, indicating that the TCD bioassay was a more sensitive method for conducting first stage screens. The technique offers a new alternative to aquatic probiotic screenings and can offer a high hit-rate for identifying potential probiotics, as was witnessed with a 58% success rate in this research. This success rate is high and strongly demonstrates the advantages of the bioassay technique in identifying potential probiotics. Given that the target animals could

withstand the conditions of the TCD, having shown no ill effects for the duration of the experiment, the bioassay could be a valuable tool for future probiotic research.

In the course of this work it was necessary to identify bacteria pathogenic to GSM larvae. Establishing *Vibrio* sp. DO1 and *V. splendidus* as two pathogens of GSM larvae was the first time pathogenic bacteria of marine mussel larvae have been demonstrated. Moreover, it extended the knowledge on the pathogenic spectrum of bacteria previously shown as pathogens of other aquaculture species. GSM larval mortality problems have occurred for the past eight years at GACL, hence, knowledge concerning the pathogenic bacterial agents was much needed. The identification of GSM pathogens will help in ongoing health management of GSM larvae. For the purposes of the research contained in this thesis, elucidation of these pathogens was essential and the starting point for all future work. The development of experimental infection models in the hatchery was the first to report these for mollusc larvae in a flow-through rearing process. Although the models are specific to GSM larvae challenged with *Vibrio* sp. DO1 and *V. splendidus*, they have contributed information and a point-of-reference to others wishing to conduct similar research. For the purpose of my own research, importantly, it allowed the testing of potential probiotics under routine hatchery conditions when exposed to a controlled experimental infection.

Testing of *A. macleodii* 0444 and *Neptunomonas* sp. 0536 under non-challenged conditions demonstrated that GSM larvae were able to develop through metamorphosis and settle similar to non-treated larvae. This apparently benign activity underpinned their

appropriateness as probiotics for GSM larvae and suggested that the probiotics were prophylactic measures for disease prevention rather than growth aids. Nevertheless, GSM larvae, administered the probiotics, consumed less food during the early life stages, indicating that they might be consuming the bacteria. This could be important in ongoing GSM larviculture because suitable food items are limited by their size and the processing ability of early GSM larvae. Replacing part of the diet with probiotic bacteria could provide a way of reducing the food requirements while improving the health protection of larvae. The observation that larval growth was not compromised during the early stages, despite probiotic-administered larvae consuming less microalgae, might be a result of lipid stores aiding in the growth of early larvae, or that the probiotics hold nutritional value for early GSM larvae.

A noteworthy observation was the non-detection of *A. macleodii* 0444 in on-grown GSM four months after being placed in the field for cultivation to market size. Although this bacterium has not been reported as a health concern for humans, it should not be forgotten that the mussels are intended for human consumption and, hence, non-presence of the bacterium would encourage acceptance of its use in GSM production.

Despite a lack of additive protection in GSM larvae when the probiotics were applied in combination, they did allow completion of the larval period. It should be remembered also that the probiotic combination tests were applied only against two specific bacterial pathogen challenges. In the routine rearing of GSM larvae, unknown bacterial threats exist and the extra protection which is provided, in theory, during multi-species

administration is desirable. At this stage, a combination administration of 10^7 CFU ml^{-1} would be recommended, since 10^8 CFU ml^{-1} levels, in combination, might be detrimental to the larvae. The aspect of optimising the administration of the two probiotics warrants further investigation.

9.2 Conclusion

The work presented in this thesis was concerned with identifying probiotic alternatives for GSM larval production. Because of a lack of previous work, the research had to commence with other background studies such as bacterial pathogenesis. Revisiting the initial aims of this thesis, all have been successfully achieved:

1. allow identification of probiotics exhibiting any mode of beneficial action,
2. isolate probiotics that allow for at least 20% improved GSM larval survival when faced with a pathogen attack,
3. identify probiotics that allow successful production of GSM larvae in the absence of antibiotics,
4. assess the benefit of the probiotics to larvae at a functional hatchery.

To achieve these goals, new information was presented on mussel pathogenesis, an effective bioassay was developed and two novel probiotic strains were demonstrated. These aspects contribute not only to the use of probiotics in GSM larval rearing, but also to literature in the field of aquaculture. Routine use of *A. macleodii* 0444 and *Neptunomonas* sp. 0536 during GSM larval rearing is intended in the near future.

9.3 Future directions

Various follow-up studies of *A. macleodii* 0444 and *Neptunomonas* sp. 0536 can be suggested. Initial work might investigate the potential they hold in the disease prevention of other aquaculture species and in other parts of the world. Additionally, the method of administration should be looked at. It is desirable to have a lyophilised product which offers ease of storage, transportability and later use. Work needs to determine whether the probiotic effect can be maintained after lyophilisation. Moreover, with a view towards optimising a commercial product, it is worth investigating strain improvements and bio-reactor culture conditions.

The mechanisms by which the probiotics are effective should be investigated further. This would provide an understanding of the interactions between the probiotics and pathogens which, in turn, would aid probiotic success. It was shown that the probiotics did not produce noticeable amounts of inhibitory substances, but that they might provide benefit through attachment competition. The effect of applying both probiotics in combination at 10^8 CFU ml^{-1} has scope for further research; was this level actually detrimental to the larvae, or did it provide better protection for the larvae? Furthermore, do different ratios of the two probiotics provide more effective protection?

A. macleodii 0444 and *Neptunomonas* sp. 0536 were just two of 11 shortlisted probiotic isolates. The remaining nine isolates would be worthy of further investigation. If beneficial, these isolates could be used to create a "probiotic cocktail" affording increased protection to the larvae. These aspects are all intended for detailed research in the future.

Appendices

Appendix 1

Pilot study in the development of a TCD bioassay

Aim:

To determine the survival of GSM larvae over time when the larvae were contained in TCDs without aeration, water exchange or food.

Materials and methods:

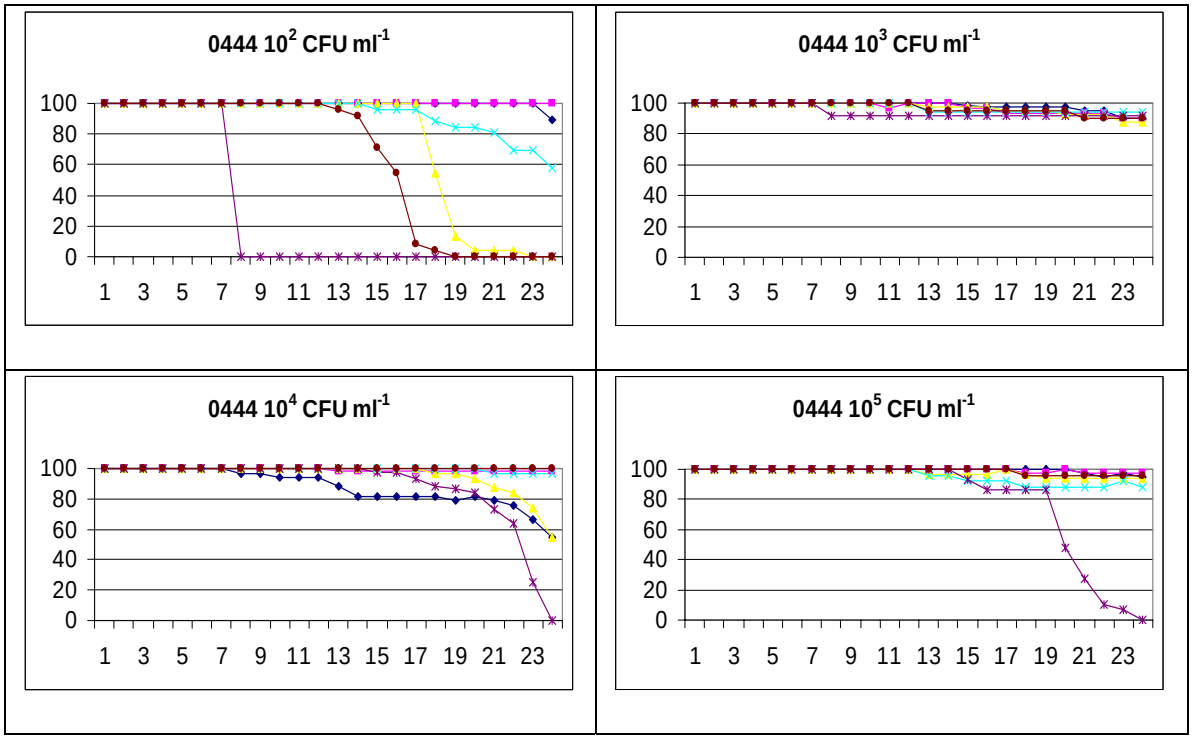
GSM larvae were obtained from GACL as previously described (Chapter 2). Larvae were placed at a concentration of 10 larvae ml^{-1} into TCD wells containing sterile seawater. No food, water exchange or aeration was provided to the TCDs during the experiment. A separate TCD was dedicated to each treatment. Treatments included TCDs inoculated with a bacterium, strain 0444, isolated from the GACL, at concentrations of 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9 CFU ml^{-1}. Two control treatments included TCDs with non-inoculated larvae. Each treatment had six replicates, contained within the same TCD. Larval survival was measured daily for 24 days. TCDs were maintained at 19°C throughout the experiment.

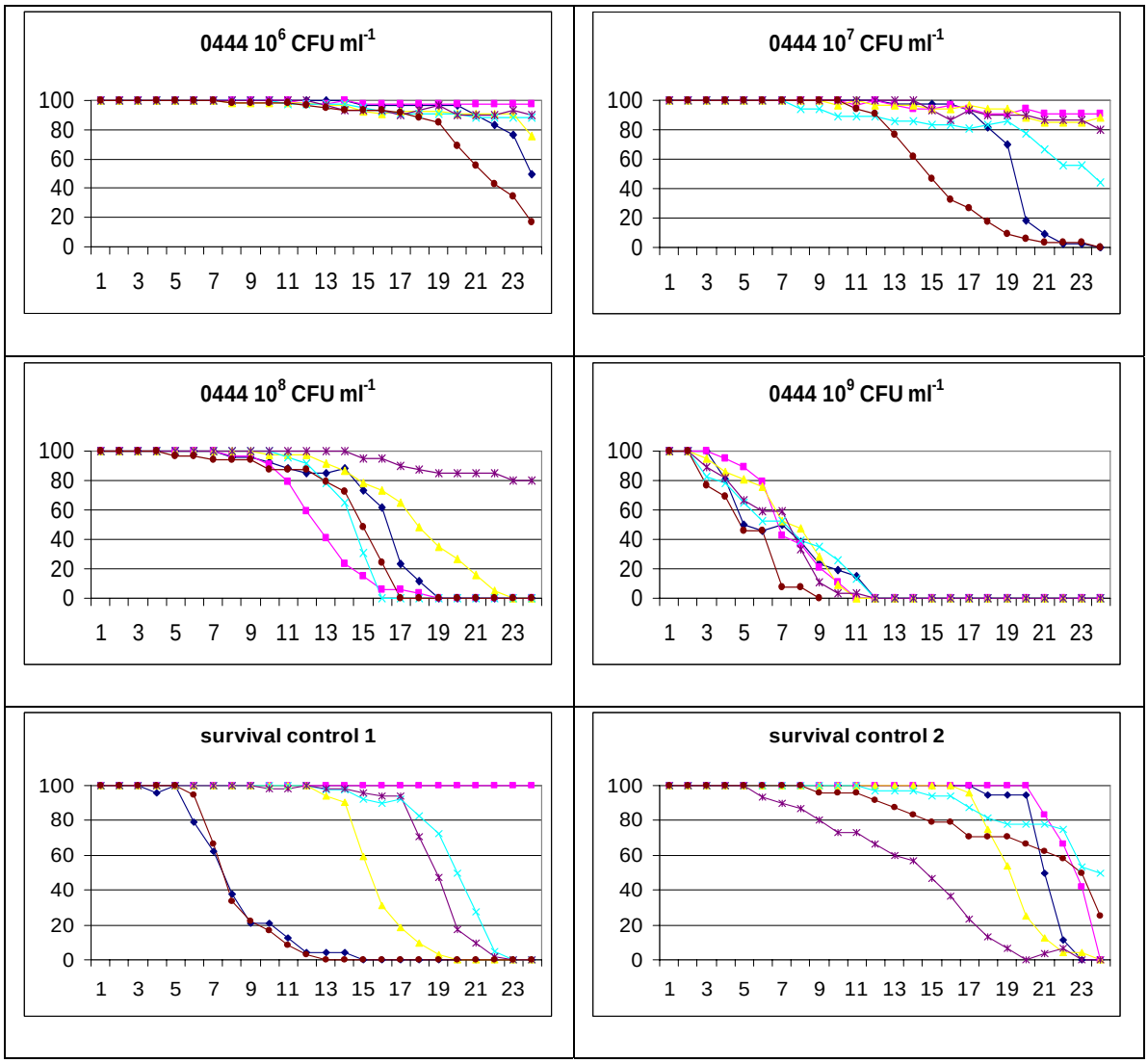
Results:

The survival figures show that, under the tested conditions, GSM larvae were able to survive for the duration of the observation period, 24 days (Fig. A1). However, mortality did become more apparent in certain replicates of the two control treatments after about 15 days.

In the inoculated treatments, larvae began dying on the third day at the highest concentration, 10^9 CFU ml^{-1}. This may have been due to lack of oxygen in the non-aerated environment of the TCD plus the high bacterial load. Despite this, complete mortality took 10-11 days to occur at this high inoculum. At inoculum sizes of 10^7 CFU ml^{-1} and below, large mortality was not witnessed in the larvae, except for certain replicates. When 0444 was administered at 10^8 CFU ml^{-1}, mortality did not occur until about day 14.

Figure A1 Survival rates of GSM larvae over 24 days when inoculated with different concentrations of 0444, and when non-inoculated. The x-axis represents time (days) and the y-axis represents larvae survival (%). Each line displays an individual replicate of each treatment.





Conclusions:

Larvae have the ability to survive at least 24 days without aeration, food or water exchange when placed into a sterile TCD dish with sterile seawater. Mortality did become more noticeable after 15 days in certain treatments, including the controls. After seven days, minimal mortality occurred in any treatment apart from the highest inoculum treatment of 0444 at 10^9 CFU ml^{-1}.

Slight variation was witnessed between some replicates of the same treatment. This was possibly due to extraneous larvae-associated bacteria entering a replicate at the beginning of the experiment, since larvae were not submitted to antibiotic treatment prior to testing.

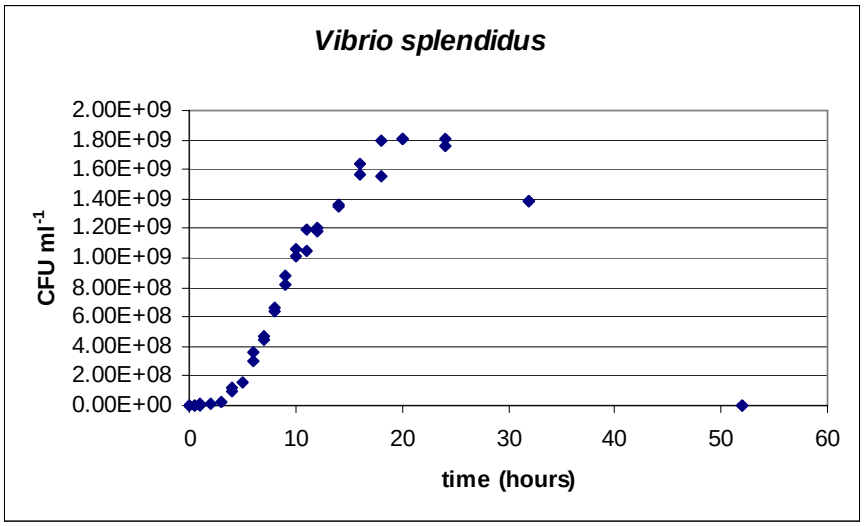
Appendix 2

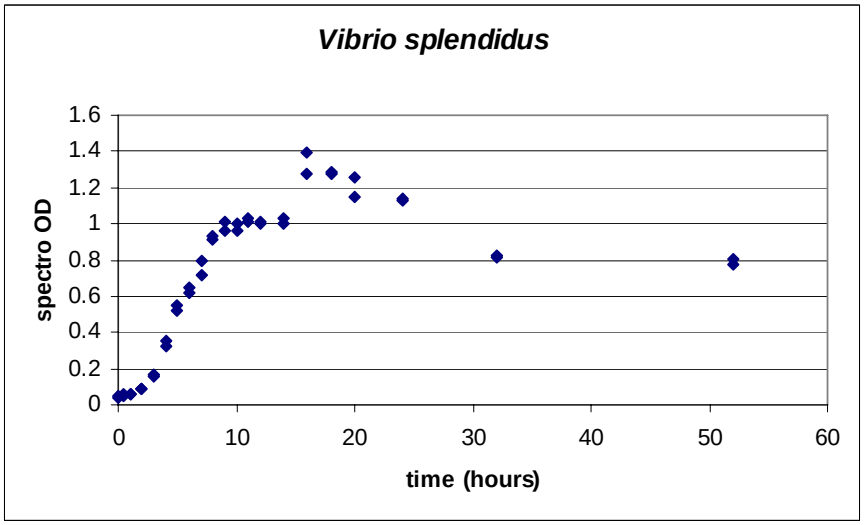
Bacterial growth curves and determination of bacterial concentrations by spectrophotometry

Growth curves were obtained by the following method:

Isolates were sub-cultured into 10 ml volumes of Marine Broth (MB, Difco) and incubated at 25°C degrees for 10 hours. 0.1 ml of each culture was further sub-cultured into another 10 ml MB and further incubated for 10 hours at 25°C. The final 10 ml volume of cultures were aseptically transferred into 500 ml MB and incubated at 24°C-27°C on a New Brunswick G10 Gyrotory Shaker (N.J., USA) at 150 rpm. Growth measurements were taken from 500 ml flasks at hourly intervals. Flasks were run in duplicate for each isolate. Bacterial concentration was enumerated by triplicate plating of serial dilutions on TSA-2%Sea. Spectrophotometer readings were determined at 600nm using a PharmaSpec UV-1700 spectrophotometer (Shimadzu).

Figure A2.1 Growth curves of *Vibrio splendidus* (isolate 0529)





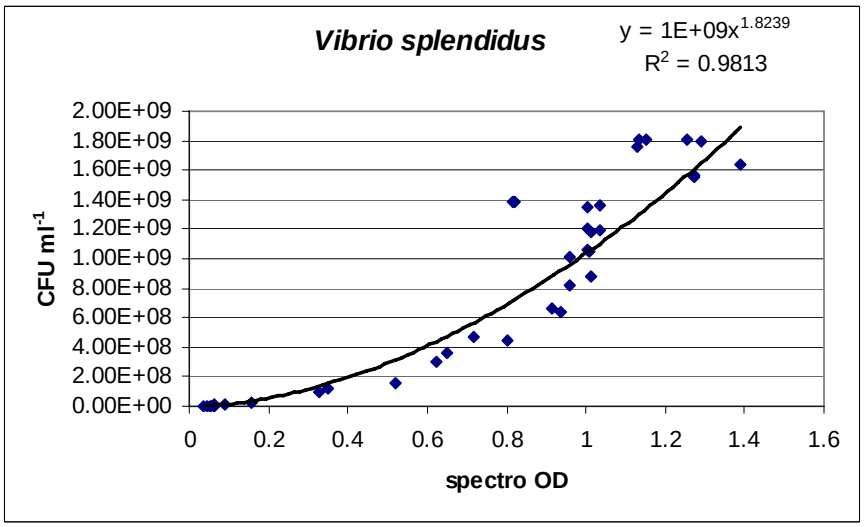
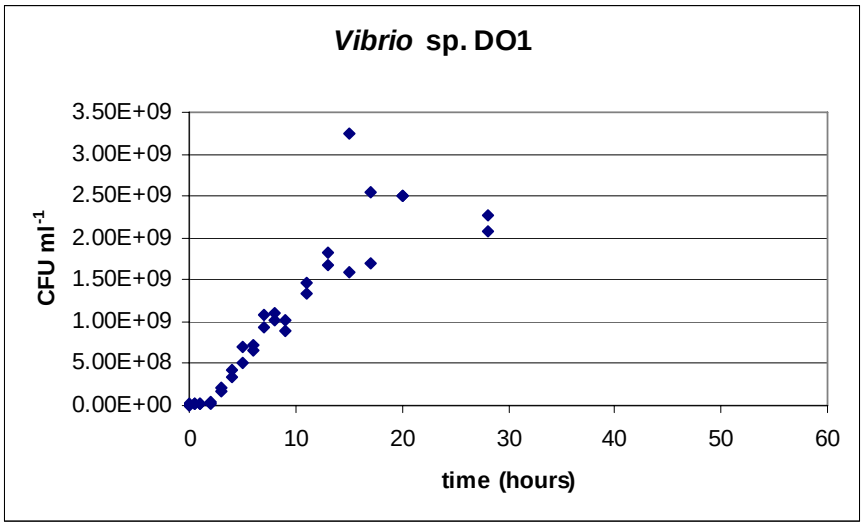
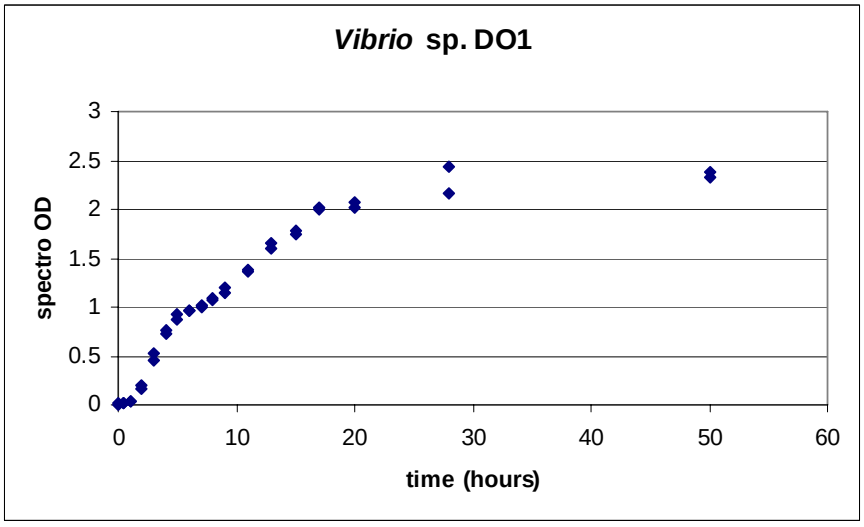


Figure A2.2 Growth curves of *Vibrio* sp. DO1 (isolate DO1)





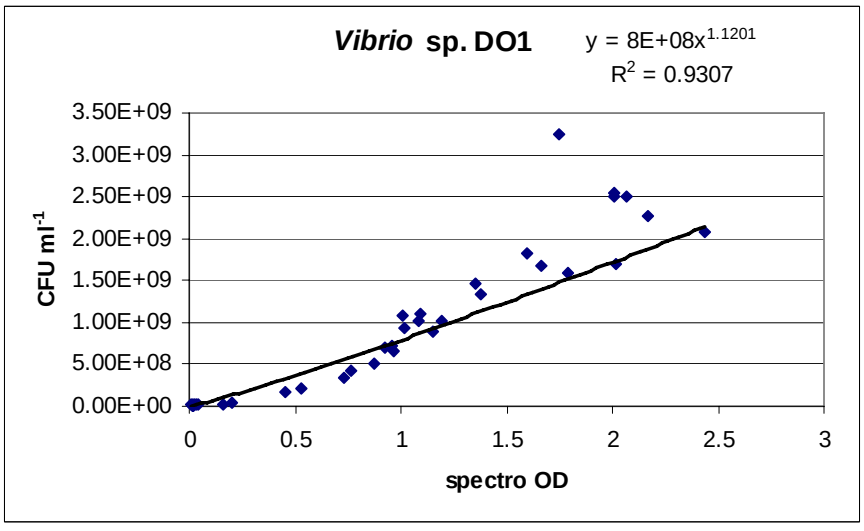


Figure A2.3 Growth curves of *Alteromonas macleodii* (isolate 0444)

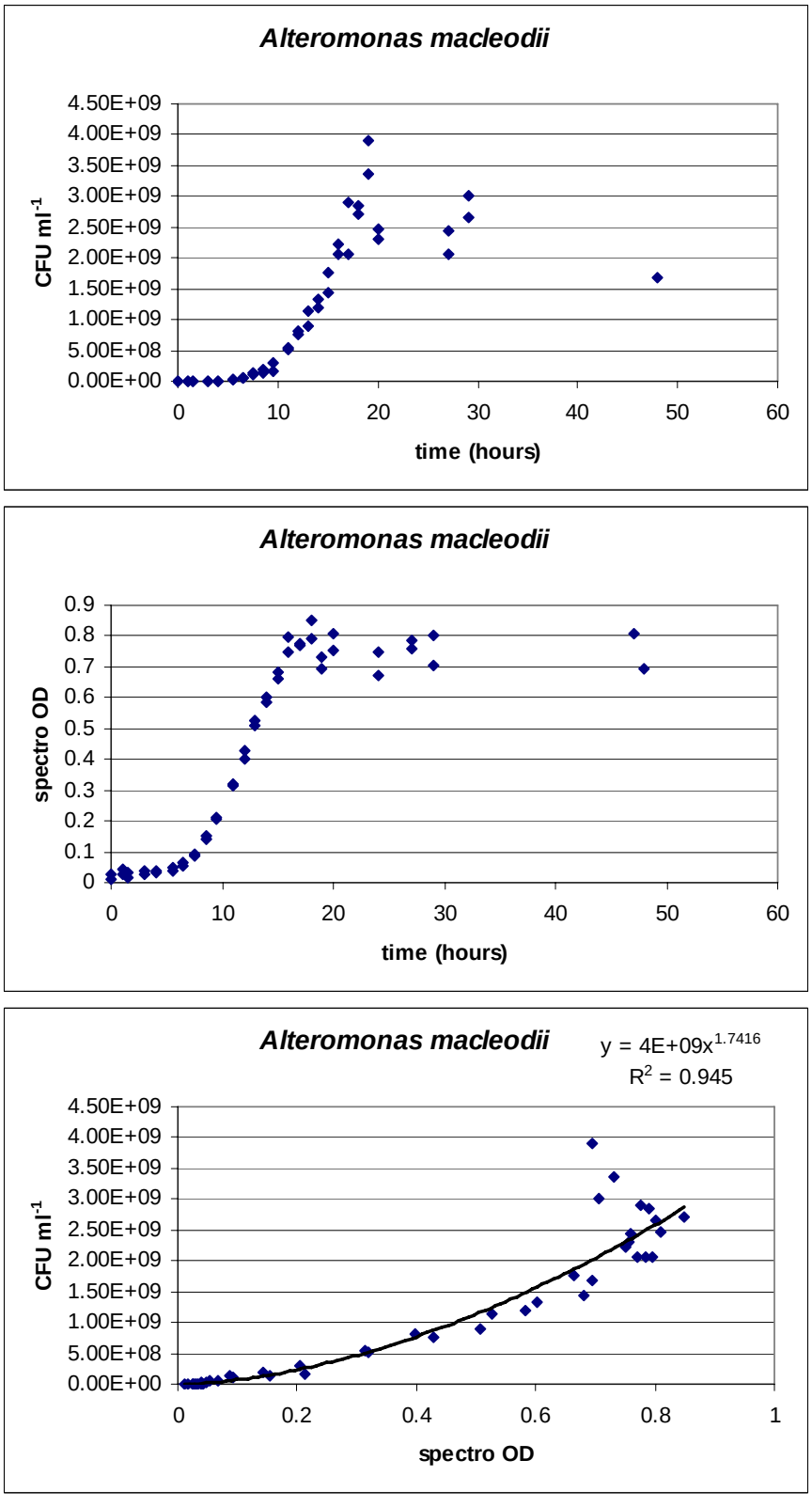
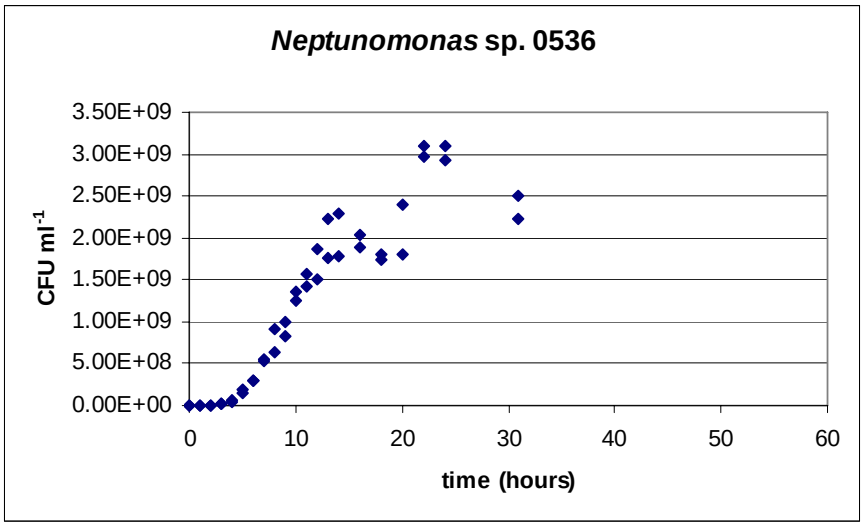
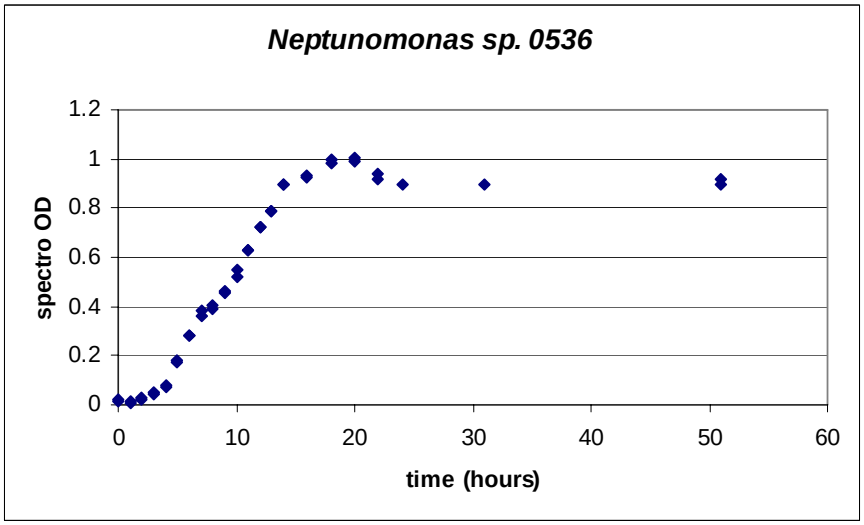
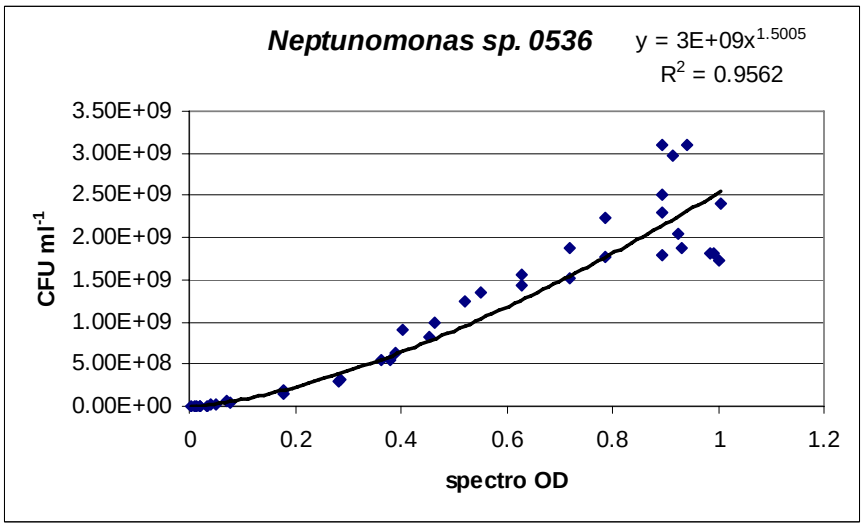


Figure A2.4 Growth curves of *Neptunomonas* sp. 0536 (isolate 0536)







Appendix 3

Screening 11 potential probiotics for production of BLIS using agar diffusion tests

Aims:

To determine whether production of bacteriocin-like inhibitory substances (BLIS) occurs in a shortlist of potential probiotics of GSM larvae.

Materials and methods:

Bacteriocin-like inhibitory substance (BLIS) testing

Eleven potential probiotics were used in this study: 04287, 0444, 0448, 04143, 0532, 0536, 0548, 0593, 0594, 0598, and 0599. Production of BLIS was tested against two pathogens of GSM larvae, *V. splendidus* and *Vibrio* sp. DO1. Two methods were used: the diametric streak method (Gibson *et al*., 1998) and a modified stab method (Smith & Davey, 1993). *Aeromonas media* (ATCC 33907) was used as a positive control. Cultures of *A. media* were prepared for experimentation using TSB (diametric streak method) or TSA (stab method). Preparations of test isolates were cultured with 2% seawater supplement. All tests were performed on TSA-2%Sea.

Diametric streak method

Overnight cultures of each probiotic were grown in 10 ml TSB-2%Sea, or TSB for *A. media*. Cultures were streaked across agar plates (TSA-2%Sea, including *A. media*),

approximately 5 mm wide, and incubated for 48 hours at 30°C. Bacterial growth was removed using the end of a glass slide containing sticky tape. Plates were exposed to chloroform vapour for 25 min to kill remaining viable cells. Each plate was then streaked, at right angles to the original streak, with overnight culture of test pathogens, *V. splendidus* and *Vibrio* sp. DO1 (TSB-2%Sea, 25°C for *V. splendidus*, 30°C for *Vibrio sp*. DO1). Plates were incubated for 24 hours at 25°C, *V. splendidus,* and 30°C *Vibrio sp*. DO1, and zones of inhibition recorded.

Stab method

Overnight cultures of *V. splendidus* and *Vibrio sp*. DO1 (10 ml TSB-2%Sea, 25°C *V. splendidus,* 30°C *Vibrio sp*. DO1) were used to create 0.5 MacFarland standards in 10 ml sterile 2% seawater. A lawn culture of each pathogen was seeded onto agar plates (TSA-2%Sea) by spreading with a cotton-wool swab. Using sterile toothpicks and agar-derived cultures (test isolates: TSA-2%Sea, 30°C, 24 hours; *A. media*: TSA, 30°C, 24 hours), each probiotic was stabbed to the base of the freshly lawn-seeded agar plates. Plates were incubated for 24 hours at 25°C for *V. splendidus* and 30°C for *Vibrio sp*. DO1. After incubation, a clear zone surrounding a probiotic strain stab culture (due to inhibited growth within the pathogen lawn culture) indicated the production of a diffusible inhibitor by that probiotic.

Results:

No test isolates produced clearly delineated zones of inhibition (Table A3). A few isolates did cause minor reduction of pathogen growth (Fig. A3), but this could have been

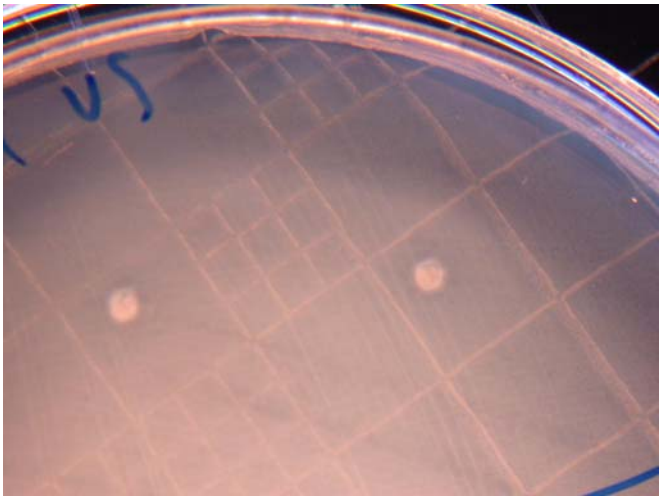
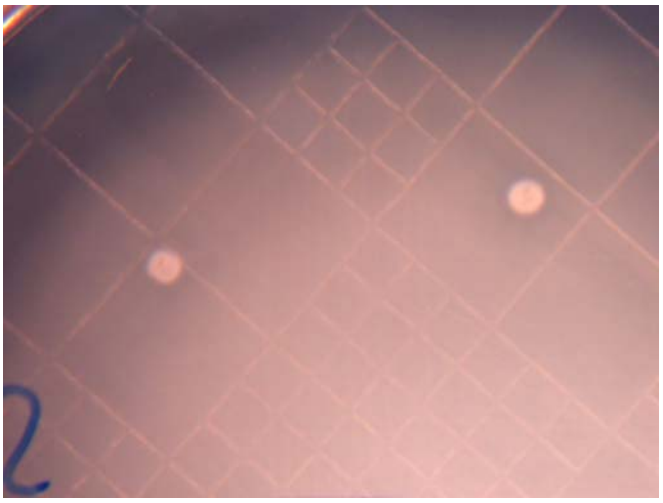
caused as a result of nutrient depletion by the producer strain. Hence, these isolates have been noted as "possible BLIS producers" and marked with a question mark in Table A3. Isolate 0598 had the most consistent and clear pattern of inhibition, and 0599 also exhibited possible inhibition in two assays. However, the other possible producers did not cause inhibition in more than one assay.

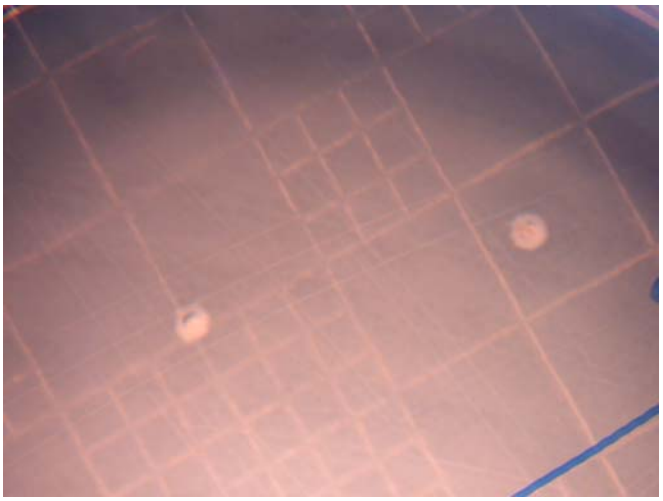
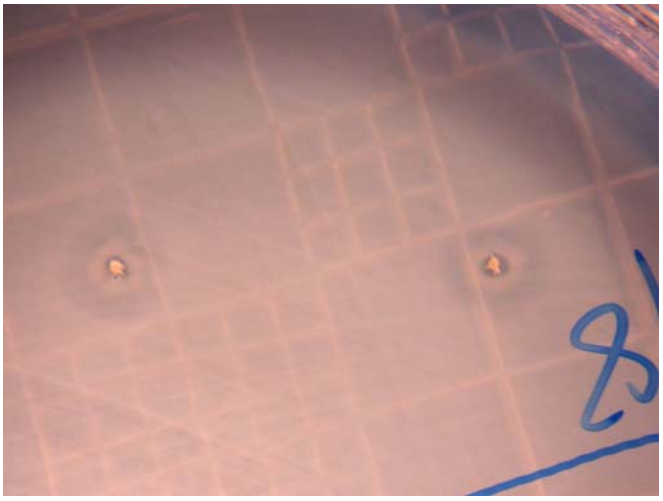
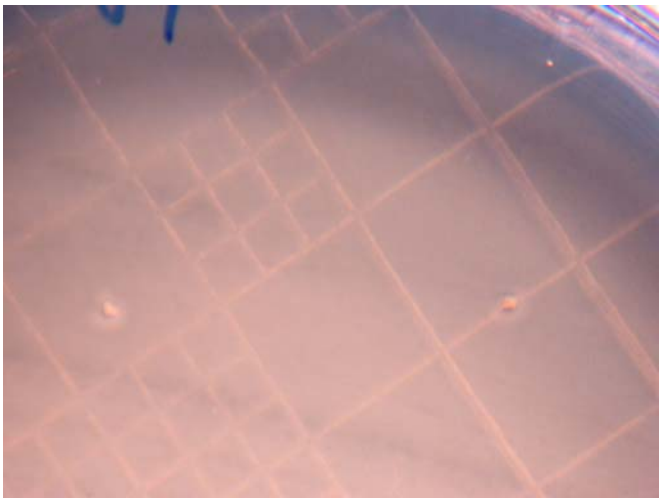
Table A3 Results of BLIS production tests. Putative probiotics were tested for inhibitory activity against GSM larval pathogens *V. splendidus* and *Vibrio sp*. DO1.

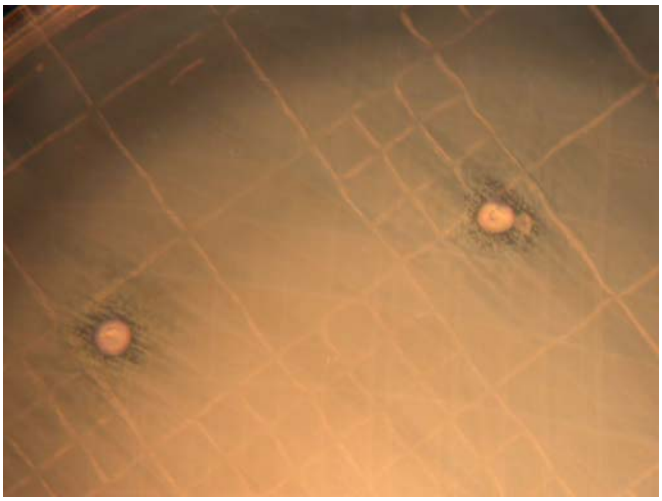
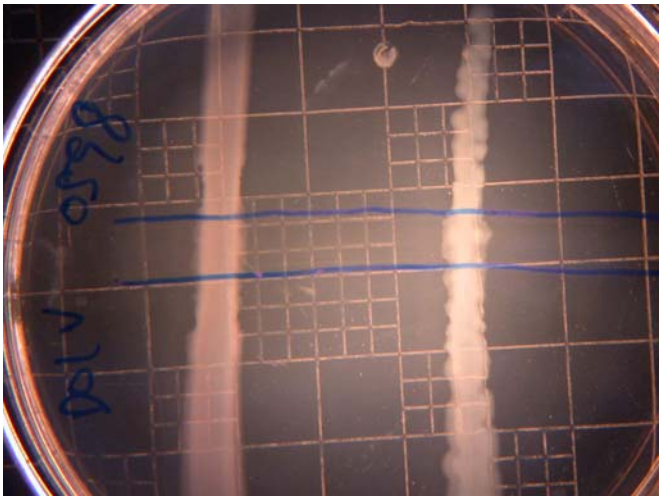
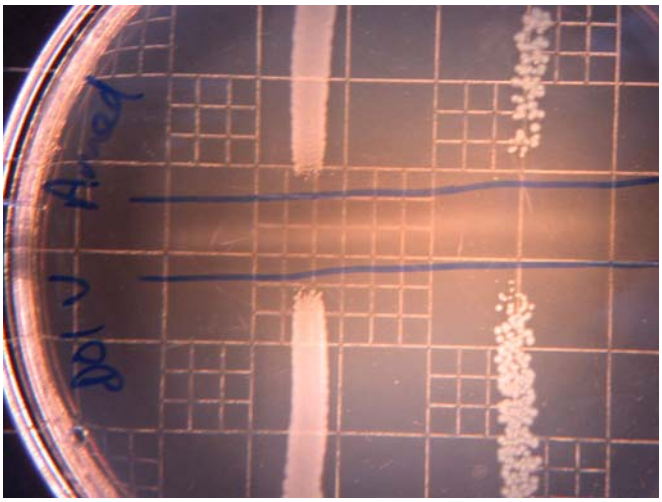
Putative Probiotic	Stab vs *Vibrio sp*. DO1	Diametric streak vs *Vibrio sp*. DO1	Stab vs *V. splendidus*	Diametric streak vs *V. splendidus*
04287	-	-	-	-
0444	-	-	-	-
0448	?	-	-	-
04143	-	-	-	-
0532	?	-	-	-
0536	?	-	-	-
0548	-	-	-	?
0593	-	-	-	-
0594	-	-	-	-
0598	?	?	-	?
0599	?	-	-	?
A. media	+	+	+	+

Note: ? = possible inhibition, - = no inhibition, + = inhibition

Figure A3 Apparent inhibition of *Vibrio* sp. DO1 and *V. splendidus* by potential probiotics using agar diffusion tests (stab method and diametric streak method).

Test	Photo of inhibition
0448 stab vs *Vibrio* sp. DO1	
0532 stab vs *Vibrio* sp. DO1	

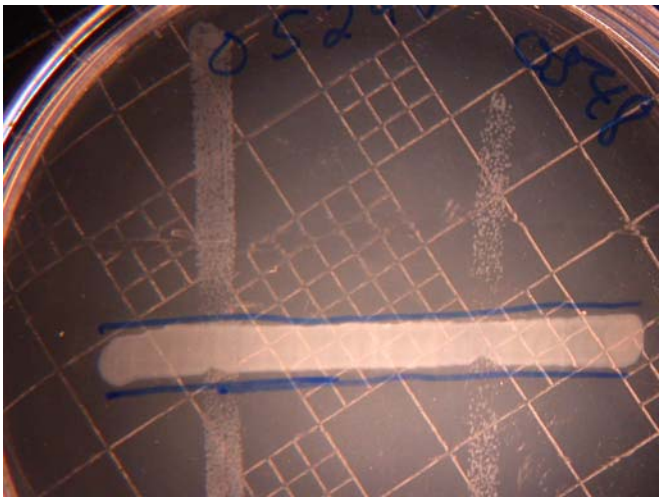
0536 stab vs *Vibrio* sp. DO1	
0598 stab vs *Vibrio* sp. DO1	
0599 stab vs *Vibrio* sp. DO1	

A. media stab vs *Vibrio* sp. DO1	
0598 diametric streak vs *Vibrio* sp. DO1 (note- blue lines represent guidelines for the outer edges of where the producer strain was initially grown)	
A. media diametric streak vs *Vibrio* sp. DO1	

0548 diametric streak vs

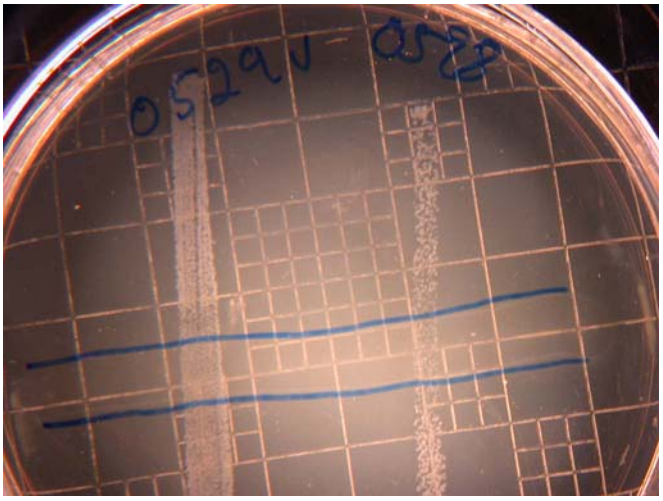
V. splendidus

(note- 0548 was tightly attached to agar and could not be removed totally prior to chloroform stage)



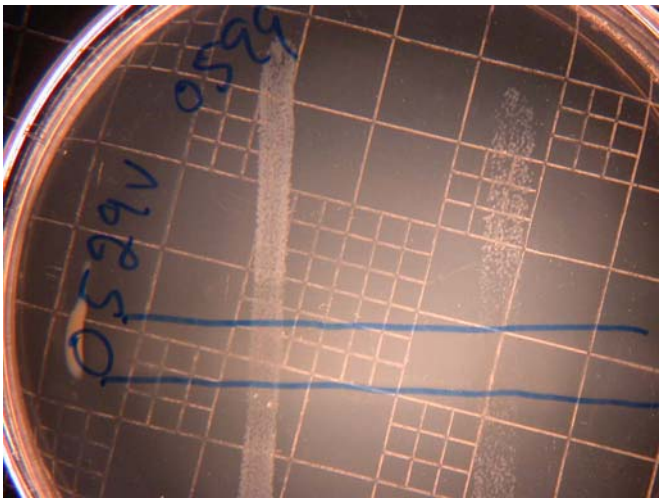
0598 diametric streak vs

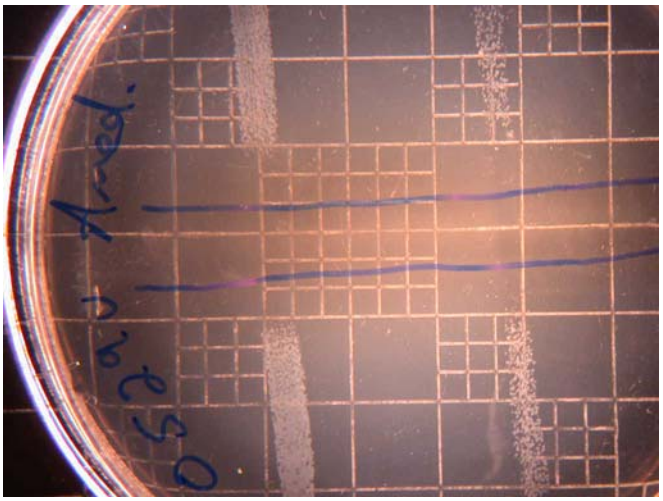
V. splendidus



0599 diametric streak vs

V. splendidus



A. media diametric streak vs *V. splendidus*	

Conclusions:

Only six out of 11 putative probiotics screened for production of BLIS appeared to be producing an inhibitory activity, albeit at very low levels. It is possible that inhibition of pathogen growth was not caused by a BLIS substance, but rather could have been caused by nutrient depletion by the producer strain. It is quite likely that not all six would have been pursued for further testing due to their questionable pathogen inhibition. The lack of clear benefit by these 11 strains (previously shown to be beneficial in TCD bioassays) reinforces the notion that many candidate probiotic strains get overlooked in screening trials employing agar diffusion tests. During this experiment, preparation cultures of *Aeromonas media* were grown in TSA/TSB to ensure healthy cultures of the freshwater isolate at the start of testing. However, since the indicator strains (pathogens) grew best at 2% salinity, tests incorporating *A. media* were conducted on TSA-2%Sea. This was unavoidable, but not a significant concern since *A. media* displayed growth under conditions of 2% salinity. *A. media* caused inhibition on the BLIS tests, but not in earlier

TCD bioassays (refer to Chapter 5). The inability of *A. media* to be protective in TCDs might stem from the fact that it is a freshwater isolate being tested at 35 ppt salinity, whereas BLIS tests were performed at 20 ppt salinity.

Appendix 4

Antibiotic resistance tests

Aims:

To determine the antibiotic resistance patterns of two probiotic bacteria with a view towards development of selective media for the purpose of re-isolation from environmental sources

Materials and methods:

Testing followed the disc diffusion method (Woods & Washington, 1995). Tests were conducted on TSA-2%Sea and incubated at 26°C. Zones of inhibition were measured at 24 and 48 hours. The recommended control strains of this method were not marine bacteria and, therefore, comparison was not made against controls with regard to definite resistant or susceptible status. Rather, published susceptibility values (Woods & Washington, 1995) were used as a guide for potential antibiotic resistance for further testing using Etests (Etest®; AB BIODISK, Sweden).

Results:

The slow growth of 0444 meant that no readings could be made until 48 hours incubation (Table A4). Strains 0444 and 0536 were both resistant to the Gram-positive targeted antibiotics bacitracin, oxacillin and novobiocin. Both strains appeared resistant to the Gram-negative targeted antibiotic aztreonam. Strain 0536 appeared resistant to the bacteriostatic antibiotics erythromycin and trimethoprim.

Table A4 Antibiotic resistance profiles, for probiotic strains 0444 and 0536, using the disc diffusion method. Values represent diameter zone of clearing (mm).

	0536			0444		
Antibiotic	24 hrs	48 hrs	Decision	24 hrs	48 hrs	Decision
Amoxycillin clavulanic (augmentin) 30µg	30	35	S	Reinc	35	S
Ampicillin 10µg	24	26	S	Reinc	20	S
Aztreonam 30µg	10	10	S	Reinc	6	R
Bacitracin 0.04 iue	6	6	R	Reinc	6	R
Cefaclor 30µg	30	38	S	Reinc	32	S
Ceftazidime 30µg	28	28	S	Reinc	32	S
Chloramphenicol 30µg	28	30	S	Reinc	40	S
Erythromycin 15µg	11	15	R	Reinc	30	S
Gentamicin 10µg	15	18	S	Reinc	19	S
Nalidixic 30µg	28	29	S	Reinc	31	S
Nitrofurantoin 300µg	30	32	S	Reinc	36	S
Norfloxacin 10µg	24	28	S	Reinc	30	S
Novobiocin 5µg	13	10	R	Reinc	10	R
Oxacillin 1µg	6	6	R	Reinc	6	R
Piperacillin/tazobactam (100/10µg)	20	24	S	Reinc	32	S
Sulfamethoxazole/ trimethoprim (23.75/1.25µg)	21	19	S	Reinc	28	S
Tetracycline 30µg	32	30	S	Reinc	25	S
Tobramycin 10µg	20	21	S	Reinc	20	S
Trimethoprim 5µg	13	14	R	Reinc	22	S

Note: As a guide, based on recommended susceptibility limits, S = susceptible, R = resistant. Reinc = indicates plates lacking bacterial growth for assessment and which were re-incubated.

Conclusions:

Because strains 0444 and 0536 are both Gram-negative bacteria, resistance to antibiotics that are effective against Gram-positive bacteria was of little surprise and confirmed that the tests were successful using the modified technique adopting TSA-2%Sea agar. Resistance against aztreonam is worthy of further testing to determine minimum inhibitory concentrations (MICs) for strains 0444 and 0536. Likewise, MIC

determination of erythromycin and trimethoprim is warranted for strain 0536. MIC values will allow the development of selective conditions in order to re-isolate these strains in mixed community samples.

Appendix 5

Continued rearing of GSM larvae until settlement, after pathogen challenge

Aims:

To determine whether probiotic treated larvae, challenged with a pathogen infection, continued their development successfully until settlement.

Materials and methods:

The experiment was set up as described in Chapter 6. This trial involved testing *Alteromonas macleodii* 0444 against *Vibrio* sp. DO1. Following the routine experimental duration of four days following pathogen exposure, larvae, subjected to select treatments, were cultivated until settlement: the various treatments were control larvae, those treated with the probiotic alone and those treated with the probiotic followed by challenge with the pathogen. Those larvae exposed to the pathogen alone, had undergone severe mortality and were discarded from further rearing.

With regard to those larvae where growth and development was continued, treatments were further divided into two sub-treatments: (1) probiotic cessation on the third day following pathogen exposure (six days post-larval hatching), (2) probiotic treatment continuing until settlement, being provided every second day from day 6 post-hatching

onwards (i.e. days 8, 10, 12, 14). Treatments were run in triplicate. The experimental design resulted in the following treatments:

1. non-inoculated control larvae
2. *A. macleodii* 0444 treated larvae (probiotic stopped on the third day following pathogen exposure)
3. *A. macleodii* 0444 treated larvae (probiotic continued)
4. *A. macleodii* 0444 treated larvae challenged with 10^6 CFU ml^{-1} *Vibrio* sp. DO1 (probiotic stopped on the third day following pathogen exposure)
5. *A. macleodii* 0444 treated larvae challenged with 10^6 CFU ml^{-1} *Vibrio* sp. DO1 (probiotic continued)
6. *A. macleodii* 0444 treated larvae challenged with 10^7 CFU ml^{-1} *Vibrio* sp. DO1 (probiotic stopped on the third day following pathogen exposure)
7. *A. macleodii* 0444 treated larvae challenged with 10^7 CFU ml^{-1} *Vibrio* sp. DO1 (probiotic continued)

Larval survivals were measured on the eighth and 16^{th} day post-larval hatching. Presence of the probiotic and pathogen were determined throughout the larval period, until settlement, as described in Chapter 6.

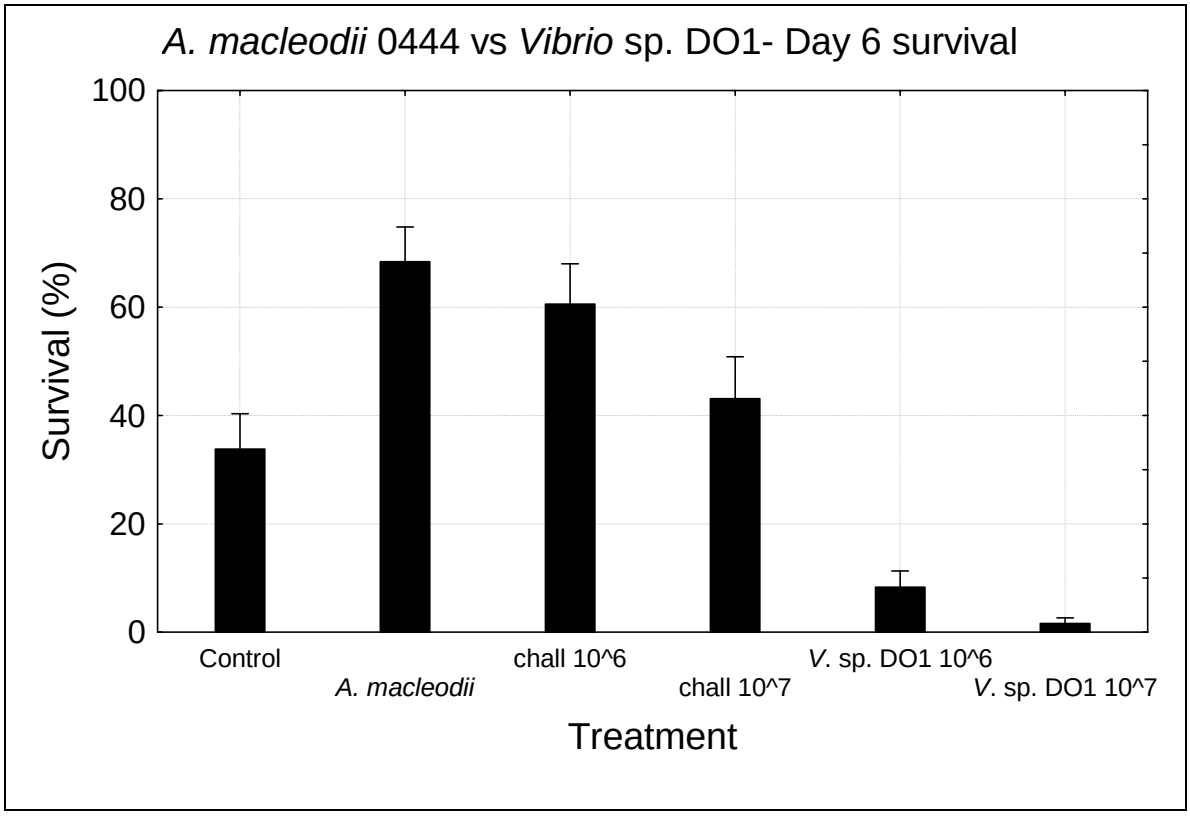
Results:

On the sixth day, at the end of the normal experimental timeframe, those larvae subjected to pathogen alone had severe mortality significantly greater than the other treatments (Fig. A5). Larvae in the probiotic and challenge 10^6 treatment had statistically greater

survival than those of the control and challenge 10^7 treatments. The two latter situations were not statistically different from each other.

At settlement, on day 16, larvae that had been challenged with the higher dose of pathogen (10^7 CFU ml^{-1}) had statistically lower survival than the other treatments (Table A5.1). Probiotic treated larvae, challenged with the pathogen at a concentration of 10^6 CFU ml^{-1}, had survival no different to that of the probiotic controls or the non-inoculated controls. Continuation of probiotic treatment did not improve larval survival; however, probiotic was still detected in the larvae upon settlement in treatments where probiotic use had stopped 11 days previously (Table A5.2).

Figure A5 GSM larval survival on days 6, 8 and 16 in a challenge experiment involving probiotic *Alteromonas macleodii* 0444 and *Vibrio* sp. DO1.



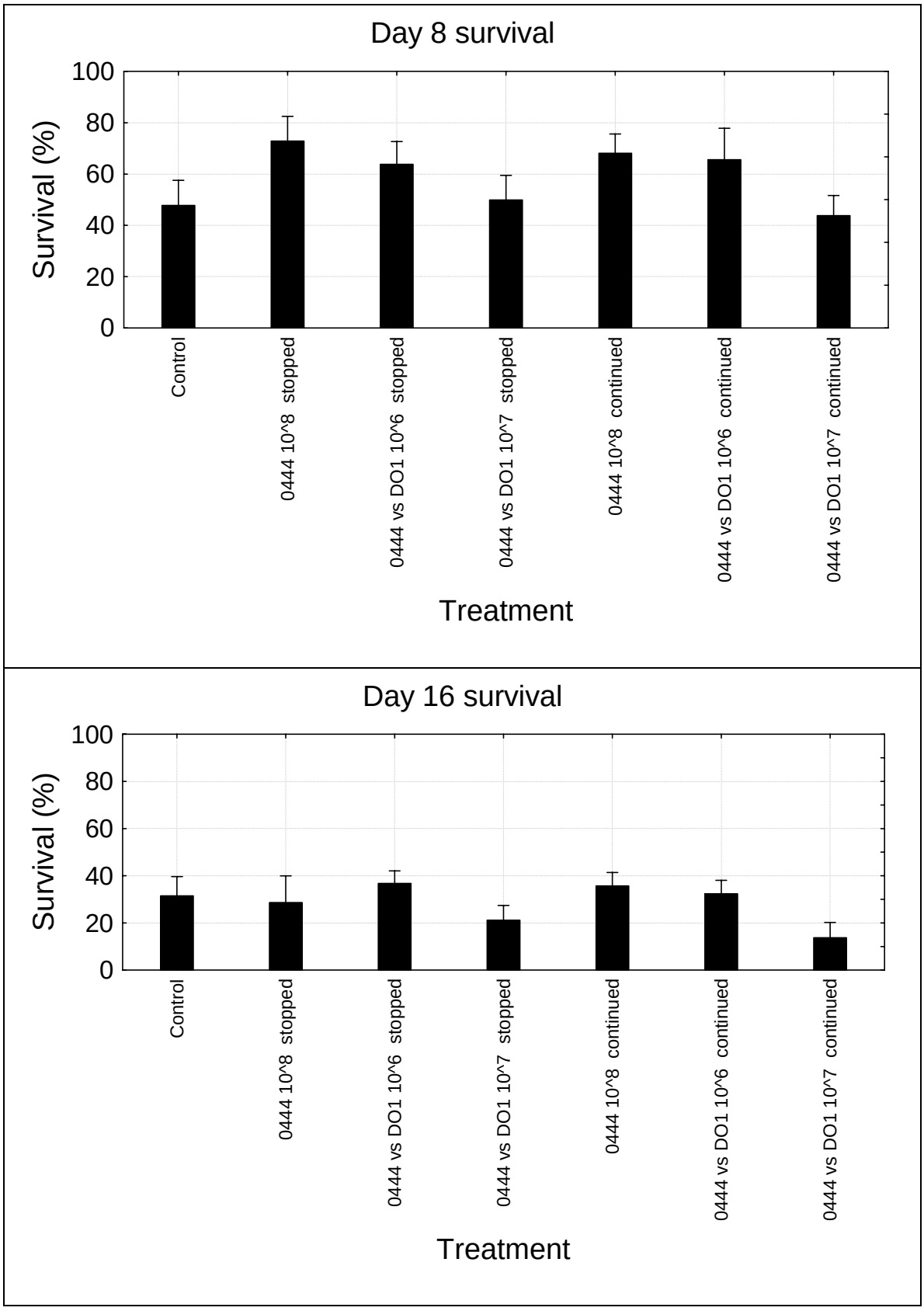

Day 8 survival
Survival (%)
100
80
60
40
20
0
Control
0444 10^8 stopped
0444 vs DO1 10^6 stopped
0444 vs DO1 10^7 stopped
0444 10^8 continued
0444 vs DO1 10^6 continued
0444 vs DO1 10^7 continued
Treatment
Day 16 survival
Survival (%)
100
80
60
40
20
0
Control
0444 10^8 stopped
0444 vs DO1 10^6 stopped
0444 vs DO1 10^7 stopped
0444 10^8 continued
0444 vs DO1 10^6 continued
0444 vs DO1 10^7 continued
Treatment

Table A5.1 GSM larval survival on days 8 and 16 in a challenge experiment involving probiotic *Alteromonas macleodii* 0444 and *Vibrio* sp. DO1. Values represent mean survival ± 95% confidence intervals. Values in a column not sharing the same superscript are statistically different ($p < 0.05$).

Treatment	Day 8	Day 16
Survival control	47.8 ± 9.8^{cd}	31.4 ± 8.2^{ab}
Probiotic stopped	72.9 ± 9.7^{b}	28.6 ± 11.4^{ab}
Challenge 10^6 stopped	63.8 ± 8.8^{abd}	36.8 ± 5.3^{a}
Challenge 10^7 stopped	49.9 ± 9.6^{acd}	21.1 ± 6.3^{bc}
Probiotic continued	68.1 ± 7.5^{ab}	35.7 ± 5.7^{a}
Challenge 10^6 continued	65.6 ± 12.3^{ab}	32.3 ± 5.8^{ab}
Challenge 10^7 continued	43.8 ± 7.8^{c}	13.7 ± 6.5^{c}

Table A5.2 Detection of test isolates from treatments during continued cultivation of GSM larvae until settlement following a pathogen challenge. Days are the stage of the larval period, post-hatching; note: pathogen was inoculated on the third day post-hatching.

Treatment		Day 8		Day 11		Day 12		Day 14		Day 16	
		W	L	W	L	W	L	W	L	W	L
Non-inoculated control	Prob	-	-	-	-	-	-	-	-	-	-
	Path	-	-	-	-	-	-	-	-	-	-
0444 stopped	Prob	+	+	-	+	-	+	-	+	+	+
	Path	+	+	-	-	-	-	-	-	-	-
0444 continued	Prob	+	+	+	+	+	+	+	+	+	+
	Path	+	+	-	-	-	-	-	-	-	-
0444 vs 10^6 DO1 stopped	Prob	+	+	+	+	-	+	+	+	-	+
	Path	+	+	-	-	-	-	-	-	-	-
0444 vs 10^6 DO1 continued	Prob	+	+	+	+	+	+	+	+	+	+
	Path	+	+	-	-	-	-	-	-	-	-
0444 vs 10^7 DO1 stopped	Prob	+	+	+	+	+	+	-	+	-	+
	Path	+	+	-	-	-	-	-	-	-	-
0444 vs 10^7 DO1 continued	Prob	+	+	+	+	+	+	+	+	+	+
	Path	+	+	-	-	-	-	-	-	-	-

Note: Prob = probiotic, Path = pathogen, W = water, L = larvae.

Conclusions:

GSM larvae treated with probiotic, *Alteromonas macleodii* 0444, and challenged with a pathogen attack by *Vibrio* sp. DO1, during their early larval stages, reached settlement similar to healthy, non-inoculated control larvae. The continuation of probiotic treatment

throughout the larval period did not further improve the larval survival achieved following pathogen attack on the third larval day. However, in routine GSM larval production, where timing of pathogen influence cannot be known, continued administration of probiotic would be advised. In treatments where the probiotic was stopped on the fifth day, it was still detected in larvae 11 days afterwards, suggesting a colonization capacity of the bacterium on the larvae.

Publications originating from this thesis

Publications in refereed journals:

Kesarcodi-Watson, A., Kaspar, H., Lategan, M.-J. & Gibson, L. 2008. Probiotics in aquaculture: the need, principles and mechanisms of action and screening processes. Aquaculture, 272:1-14.

Kesarcodi-Watson, A., Kaspar, H., Lategan, M.-J. & Gibson L (2009). Two pathogens of Greenshell™ mussel larvae, *Perna canaliculus*: *Vibrio splendidus* and a *V. coralliilyticus/neptunius*-like isolate. Journal of Fish Diseases, 32:499-507.

Kesarcodi-Watson, A., Kaspar, H., Lategan, M.-J. & Gibson, L. (in press). Challenge of New Zealand Greenshell™ mussel larvae, *Perna canaliculus,* using two *Vibrio* pathogens: a hatchery study. Diseases of Aquatic Organisms (in press).

Submitted manuscripts:

Kesarcodi-Watson, A., Kaspar, H., Lategan, M.-J. & Gibson, L. Screening for probiotics of Greenshell™ mussel larvae, *Perna canaliculus*, using a larval challenge bioassay (manuscript submitted, Aquaculture).

Manuscripts awaiting patent prior to submission:

Kesarcodi-Watson, A., Kaspar, H., Lategan, M.-J. & Gibson L. *Alteromonas macleodii* and *Neptunomonas* sp. 0536, two novel probiotics for Greenshell™ mussel larvae, *Perna canaliculus*: protection in a hatchery facility during pathogen-challenge with *Vibrio splendidus* and a *Vibrio coralliilyticus*-like isolate.

Kesarcodi-Watson, A., Kaspar, H., Lategan, M.-J. & Gibson, L. Performance of two probiotic strains, administered in combination, during hatchery production of Greenshell mussel™ larvae, *Perna canaliculus*, and exposed to pathogen challenges.

Presentations at conferences:

Kesarcodi-Watson, A., Gibson, L. & Kaspar, H. A highly reproducible *in vivo* screening assay for direct recognition of probiotic bacteria in Greenshell™ mussel larvae. New Zealand Microbiology Society 2005, Dunedin, New Zealand.

Kesarcodi-Watson, A., Kaspar, H., Lategan, M.-J. & Gibson, L. Probiotic bacteria for Greenshell™ mussel larviculture in New Zealand. Australian Society for Microbiology 2007, Adelaide, Australia.

Kesarcodi-Watson, A., Kaspar, H., Lategan, M.-J. & Gibson, L. Probiotic bacteria for hatchery production of Greenshell™ mussel. New Zealand Marine Sciences Society 2007, Hamilton, New Zealand.

Kesarcodi-Watson, A., Kaspar, H., Lategan, M.-J. & Gibson, L. Hatchery performance of two novel probiotic bacteria on Greenshell™ mussel, *Perna canaliculus*, larvae. World Aquaculture Society 2008, Busan, South Korea. *Winner of Best Student Abstract Award.*

Kesarcodi-Watson, A., Kaspar, H., Lategan, M.-J. & Gibson, L. Trials of two novel probiotics in a Greenshell™ mussel hatchery. Australian Society for Microbiology 2008, Melbourne, Australia.

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