The identification and development of probionts for use in marine fish hatcheries

By

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Declaration

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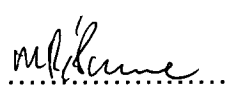

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Abstract

Striped trumpeter (*Latris lineata*) is currently being researched as a new candidate for the Tasmanian aquaculture industry. Larval development is protracted and unusual in the striped trumpeter, and successful mass culture has taken over 15 years. Recent breakthroughs have been made in the successful culture of seedstock, including the use of ozonated water to reduce and control microbial communities. The subject of this thesis was the implementation of research to further improve larval and juvenile rearing success through the use of bacterial probionts.

A knowledge of the microbial bacterial community of the larvae, seawater and live feeds was necessary in order to develop an understanding of what represents a healthy microbial ecology relevant for larval fish rearing success. 16S ribosomal RNA (rRNA) gene-based clone library and terminal restriction fragment length polymorphism (TRFLP) analyses were utilized to examine the microbial community associated with larvae cultured under different "greenwater" conditions. It was discovered that the larvae-associated microbial diversity was restricted but varied considerably between culture conditions. Most bacteria detected on the basis of cloned16S rRNA gene sequence data, belonged to class *Alphaproteobacteria* (predominantly of the *Roseobacter* clade), *Gammaproteobacteria* (genus *Psychrobacter* and *Pseudoalteromonas*) and *Actinobacteria* (genus *Microbacterium*). No association was found between larval survival and microbial community structure. Using TRFLP analysis, similar results were obtained and demonstrated that the bacterial composition was in agreement with the clone

library data. It was found that the microbial community in the larvae was distinct from the bacterial community present in the surrounding water.

Potential probiotic candidates were identified using antimicrobial *in vitro* plate testing against known pathogenic *Vibrio* species, with six out of 25 isolates tested selected for further testing. The 25 test bacteria were obtained in a previous experiment and were determined to be a representation of the bacterial community present in 15 days post-hatch (dph) striped trumpeter larvae. In both the rotifer and *Artemia* challenge trials, it was determined that *Pseudoalteromonas agarivorans* ST18 and *Aliivibrio fischeri* ST7 had the least negative effect on rotifer and *Artemia* survival numbers. To further assess the probiotic capability of strains ST18 and ST7 rotifer and *Artemia* cultures were challenged with pathogenic strain *Vibrio proteolyticus* V760 mixed with strains ST18 or ST7. Strain ST18 was found to have a probiotic effect in that cultures containing both V760 and ST18 were not significantly different from the controls but produced significantly better survival compared to the pathogen-only treatments.

To further investigate ST18 and ST7 probiotic capability, they were added to striped trumpeter larvae cultures either directly through addition to water or through bioencapsulation in live fed rotifers. TRFLP was used to monitor the change in bacterial community and to track individual probionts by detection of unique terminal restriction fragments (TRF). The addition of strain ST7 alone was found to be disadvantageous to the culturing of striped trumpeter yolk sac larvae, while the addition of strain ST18 and a combination of strains ST18 with ST7 showed no significant reduction in survival. Tracking

of strain ST18 using TRFLP was successful with the strain specifically detected in treatments where it was added by both bioencapsulation and direct addition.

When ST18 was directly added to both the water and through rotifers simultaneously it resulted in decreased larval survival due to the high number of bacteria present, and possibly resulting in a reduction in dissolved oxygen levels. However, in first-feeding larvae it was seen that the addition of the probiont via enriched rotifers was a promising mode of addition, as it was not significantly different from the control. Thus the introduction of strain ST18 to the larvae bioencapsulated in rotifers may have resulted in the introduction of comparatively smaller numbers of bacteria that did not compromise the growth of the developing larvae due to excessive biological oxygen demand. This resulted in this treatment being not significantly different to the control in terms of larval survival.

With the knowledge gained from this study we have learnt that during the early larval stages of striped trumpeter, the bacterial flora is low in diversity and opportunistic. The methods developed during this study have shown how it is possible to use bacterial isolates as probionts. It also shows that by using TRFLP it is possible to track a species of bacteria through an experimental aquaculture system.

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Chapter 1: General Introduction

1.1 Introduction

Bacteria are unicellular microorganisms. Typically, they are a few micrometres in diameter, have a wide range of shapes, ranging from spheres (cocci) to rods and spirals. Bacteria are ubiquitous in every habitat on earth, growing in soil, acidic hot springs, radioactive waste, the deepest seawater, and deep in the earth's crust (Madigan et al., 2000).There are typically 40 million bacterial cells in a gram of soil and a million bacterial cells in a millilitre of sea water. Bacteria are vital in recycling nutrients, and many important steps in nutrient cycles depend on bacteria, such as the fixation of nitrogen from the atmosphere (Madigan et al., 2000). In industry, bacteria are important in processes such as wastewater treatment, the production of cheese and yoghurt, and the manufacture of antibiotics and other chemicals (Pianka, 1970). They also play an essential role in aquaculture, none more so than in the seed production of marine fish where the control of the bacterial community in larval rearing tanks is of paramount importance (Verschure et al., 2000).

1.2 Microbial flora of larval fish culture and their control

Many problems associated with the rearing of delicate marine fish relate to excessive bacterial populations. Bacterial blooms that occur in the culture systems may lead to disease, reduced growth rates, or even to a complete crash and loss of stock (Verschuere et al., 2000; Schulze et al., 2006). The reason for this is that these animals are cultured under optimal conditions, e.g. pH, water quality, temperature, and nutrients.

However, this also creates optimal conditions for various species of microorganisms, including pathogenic bacteria, to proliferate. This is particularly true when water treatment reduces or alters the natural microbiota, thus providing space for the colonisation and invasion of different species (Verschuere et al., 1997). The resulting environment is extremely well-suited for the growth of opportunistic bacteria that are classified as *r*-strategists, as they grow quickly and dominate under conditions that are eutrophic (nutrient rich) (Verschuere et al., 1997). In contrast, *k*-strategists are classified as bacteria that can effectively exploit resources under oligotrophic (nutrient-limited) mixed culture conditions, and tend to grow slowly (Verschuere et al., 1997). In general, *r*-strategists are characteristic of unstable environments, while *k*-strategists predominate in stable, nutrient-limited environments and are more likely to be physiologically specialised (Pianka, 1970). Therefore, when we add nutrient to the cultures, be it in the form of live feeds or artificial feeds, it results in a disturbance of the natural relationship between the fish and the bacteria (Verschuere et al., 1997). By understanding bacterial interactions within a culture unit, it may be possible to manipulate the bacterial communities with non-pathogenic bacteria (probionts), which may then in turn inhibit the establishment of potentially pathogenic *r*-strategists (Hansen and Olafsen, 1999).

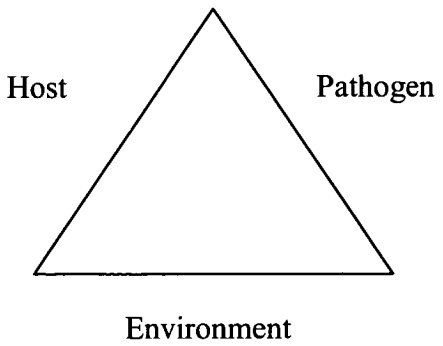

Figure 1.1*:* Disease triangle concept showing the interaction between the host, pathogen and environment and how they are all interconnected.

As with all live animals, larval fish have their own bacterial communities, which they acquire from their surroundings. This can be seen graphically in the disease triangle concept, described in the 1960s by George McNew, a scientist at the Boyce Thompson Institute for Plant Research (Fig. 1.1) (Scholthof, 2007). In larval rearing, the environment is the water, the host is the larvae, and the pathogens, in the case of the systems being explored in this thesis, are typically bacterial species.

With this in mind, hatchery producers of larvae must be informed about the bacteria associated with each stage of production. As the larvae obtain their microbiota from their surroundings, management of the number and types of bacteria present is highly desirable. Bacteria are present throughout the process, from egg collection, egg incubation, and through to the culture tanks.

Fish also contain a specific intestinal microbiota that becomes established at the juvenile stage or soon after metamorphosis (Hansen

and Olafsen, 1999; Verschuere et al., 2000). During the establishment of the gut microbiota it is likely a series of transitional changes occur throughout the stages of the fish's life (Hansen and Olafsen, 1999). This is due in part to the varying conditions to which the larvae are exposed during larval culture. In the Atlantic halibut (*Hippoglossus hippoglossus* L.), gut-associated bacteria are detected towards the beginning of the non-feeding yolk sac stage (Verner-Jeffreys et al., 2003). These bacterial isolates are predominantly non-fermentative, gram-negative rods, (e.g. *Pseudoalteromonas* spp.), and they increase in number once active feeding by the larvae commences. *Vibrio* spp. also become associated with the gut during this stage of growth (Verschuere et al., 2000; Verner-Jeffreys et al., 2003). Other species which have been studied in detail included turbot larvae (*Scophthalmus maximus*). Blach et al, (1997) found that oxidative gram negative rods dominated during the early stages of the turbot larvae while this shifted to *Vibrio sp* during the final stages. It was also observed that high mortality coincided with high heterogeneity of *Vibrio* spp. In Atlantic cod (*Gadus morhua*) larvae this pattern has also been seen (Hansen and Olafsen, 1989).

To control the microbial communities of larval systems, many different approaches have been taken. With the understanding of the above information in regards to the types of bacterial present and the ways by which they interact with the larvae, it is possible to explore ways of managing bacterial populations to avoid pathogenicity. Skjermo et al.

(1997) has suggested a strategy for microbial control that uses a modified disease triangle concept, as shown in Fig 1.2.

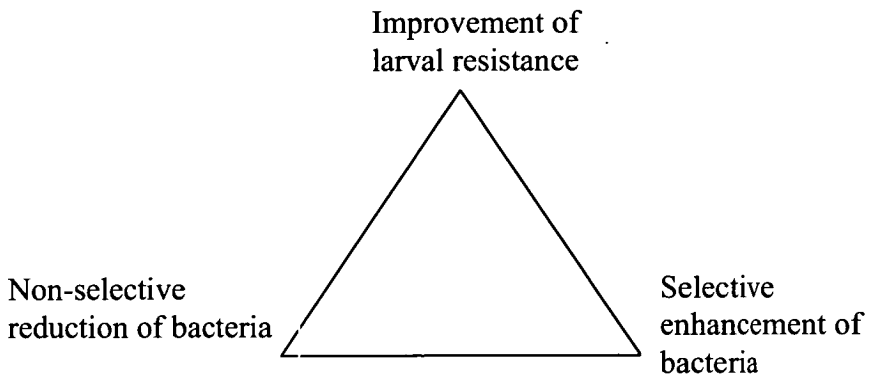

Figure 1.2: Outline of the three elements in the strategy to obtain microbial control in the rearing of marine fish (taken from Skjermo et al., 1997)

Two of the elements involve environmental factors, with the third being the fish itself (Fig. 1.2). Non-selective reduction of bacteria relates to the reduction in microbial load through disinfection of eggs and tanks, through water treatment including filtration, ozonation and UV, and through treatment of live feeds.

Since microbial communities in marine fish culture are influenced by the water treatment systems in place, which can be seen in Fig. 1.2, a common approach is to use greenwater cultivation. This is a term to describe a culturing method in which microalgae are added to the rearing environment of larval fish (Palmer et al., 2007). This may be carried out in tanks or in ponds and this type of greenwater environment affords better larval survival (Palmer et al., 2007). This is generally attributed to: i) better

direct and indirect nutrition of larvae; ii) reduced stress levels; iii) enhanced environmental conditions for feeding from increased turbidity, light scattering and attenuation, and visual contrast enhancement; iv) improved water quality due to stripping of nitrogenous substances and increased oxygenation rates; and v) the possibility of antibacterial properties of the microalgae (Palmer et al., 2007).

The implementation of filtration systems may also control the microbial communities as can the use of ultra violet (UV) and/or ozonation of water. In UV treatment the water passes by a UV light were the UV irradiation inactivates and kills bacteria. Similarly, ozonation of water reduces the microbial numbers, through oxygen free radicals which induce severe oxidative stress in bacterial cells and result in inactivation, reduced growth and death.

Larvae are fed live feeds, which have their own bacterial communities associated with them (see Section 1.3 Role of live feed). Therefore, at different stages, the larvae are exposed to an array of different bacterial communities that often contain pathogens, albeit usually at very low levels. It is standard practice to clean culture tanks on a regular basis to remove detritus and to reduce potential bacterial proliferation.

Antibiotics have been used widely in aquaculture, but they are non-selective, meaning they will act on all bacteria present, both harmful and beneficial. Also the use of antibiotic prophylaxis may have reduced

effects over time, if the target microorganisms become resistant (Vadstein et al., 2004). Antibiotics are further discussed in Section 1.5.

Use of a selective method such as the incorporation of probiotic bacteria may prove advantageous. As here, known bacteria can be added to the community with the knowledge that it will only have an interaction with the target, here being pathogenic bacteria. However, with the modification of any microbial environment, there are always challenges. The major difficulties in the use of probionts are that they can modify the native microbial communities (affecting the microbial balance) and the choice of the correct balance of the probiont (how much and when). During the development of fish larvae, the stomach, foregut, midgut and hind gut become compartmentalized in terms of pH, enzyme content and digestive mechanisms. Consequently, the probionts chosen must be able to cope with these varying environments and be maintained in different areas of the gut (Vine et al., 2006). Probionts are further discussed in Section 1.6.

Finally, improvement of resistance against bacteria can be obtained by stimulation of the immune system of the larvae to better cope with microbial challenges (Fig. 1.2). Fish larvae, in particular striped trumpeter, do not have the ability to develop specific immunity as their immune systems are not yet fully developed (Covello et al. 2009). An immunostimulant can be defined as an agent that stimulates the non-specific immune system mechanisms when given alone, or the specific aspects of the immune system when given together with an adjuvant (e.g.

through vaccination). Immunostimulants include bacterial products, muramyl dipeptides, polysaccharides and synthetic chemicals (Vadstein et al., 1997).

1.3 The role of live feed

Over 85% of cultured marine species are fed live feeds in aquaculture (Hameed and Balasubramanian, 2000). The purpose of a live feed is to simulate the naturally occurring food source upon which the larvae would normally prey. These natural preys are often free-living zooplankton, such as copepods. However, as copepods are difficult to rear intensively (Cheng-Sheng et al., 2005), the industry relies heavily on more easily cultured zooplankton such as rotifers (*Brachionus* spp.) and brine shrimp (*Artemia* spp.) (Douillet, 2000).

Rotifers are often used as an initial feed, as their small size (0.1-0.5 mm length) allows effective feeding by larval fish. They are common in freshwater throughout the world and some saltwater species also exist. They are an easy animal to culture at densities up to 10^3 ml^{-1} and can be cultured to densities exceeding 10^4 ml^{-1} (Douillet, 2000, Vine et al, 2006). They are also an easy animal to nutritionally enrich, thus providing the means to improve their nutrient value, which can then be passed onto the fish larvae. Skjermo and Vadstein (1993) reported that some of the bacteria associated with rotifers may have a detrimental effect on the larval fish to which they are fed. Mass culture of rotifers, which involves high densities, warm temperatures of around 25°C and enriched media,

encourage proliferation of undesirable bacteria. The numbers of *Vibrio* spp. have been found to increase significantly during mass culture and to displace other bacterial groups(Olsen et al., 2000) These bacteria can contribute to the poor reproducibility often seen in replicated systems in terms of survival and growth rates of rotifers (Skjermo and Vadstein, 1993). When the rotifers are fed an enrichment diet, a shift in the bacterial communities also occurs. Opportunistic bacteria bloom at this point and after a few days, the "normal" communities re-establish; these often being members of the phylum *Bacteroidetes* (formerly referred to as the *Cytophaga/Flavobacterium/ Bacteroides* group) (Skjermo and Vadstein, 1993).

Brine shrimp (*Artemia* spp.) are an important live feed in aquaculture due to their ease of production and their suitable biochemical composition (Verschuere et al., 1999). They have been used as a live feed since the 1940s and often follow the initial use of rotifers as a first stage feed (Rollefsen 1940 in Straub et al., 1993). Extensive cultivation-based research has been undertaken to investigate the bacterial communities that are present in *Artemia* populations (Solangi et al., 1979; Austin and Allen, 1982; Straub and Dixon, 1993; Lopez-Torres and Lizarraga-Partida, 2001). Freshly hatched *Artemia* nauplii are deficient in essential fatty acids, particularly docosahexaenoic acid (DHA) (Sargent et al., 1999). To overcome this dietary limitation, *Artemia* are enriched in a similar way to rotifers with lipid emulsions, to optimise lipid nutrition (Sargent et al., 1999). As with rotifers, increasing the nutrient load of

Artemia culture can result in increased bacterial populations. Bacteria isolated from *Artemia* have been shown to belong to genera *Bacillus, Micrococcus, Staphylococcus, Erwinia* and *Vibrio* (Austin and Allen, 1982). However, the problem for fish culturists is that *Vibrio* spp. may become dominant during the hatching process, which is usually conducted at incubation temperatures around 28°C for 24 hours. As the cysts break down, an organic reserve (glycerol) is released into the water and this provides an organic substrate that is used efficiently by the *Vibrio* spp. already present in the surrounding water (Vine et al., 2006). The resultant bloom of *Vibrio* spp., when transferred with the *Artemia* to the larvae, may lead to larval mortalities. It is important to stress that *Vibrio* spp. are not directly associated with *Artemia,* but instead originate from the seawater in which they are hatched (Lopez-Torres and Lizarraga-Partida, 2001).

Factors associated with both nutrition and bacteria have been suggested for the high numbers of mortalities during the first feeding of larval fish (Olsen et al., 2000). As discussed, both rotifers and *Artemia* may carry a large bacterial load that, when transferred to the larval fish, can account for fish mortalities (Hameed and Balasubramanian, 2000). The relationship between the presence and predominance of *r*-strategists and fast growing bacteria in *Artemia* intestinal tracts is a direct result of the systems in which they are cultivated. The food, fishmeal, and fish oil commonly used for the cultivation of *Artemia* are rich substrates suitable for the proliferation of opportunistic bacteria (Olsen et al., 2000). *Artemia*

that are fed microalgae (*Tetraselmis* sp.) have a 75% lower bacterial population load and a more diverse community with fewer *Vibrio* spp. when compared to *Artemia* fed commercial feeds (Olsen et al., 2000). The domination by *Vibrio* spp. can be as high as 58% in *Artemia* fed commercial feeds (Olsen et al., 2000). Verschuere et al. (1997) observed that *r*-strategist bacteria constituted 90% of the bacteria present in three day-old *Artemia* cultures. Verschuere et al. (1999) showed that axenic cultures of *Artemia* grown under suboptimal conditions yielded better survival and/or growth rates when grown in a preemptively colonized culture medium, compared with strains of *Artemia* fed in the standard way. This suggests that the bacterial flora present is important for the growth and survival of *Artemia.*

1.4 Methods used to reduce the bacterial populations of rotifers and *Artemia*

With the knowledge of bacterial associations within live feed, culture methods have been developed to reduce the bacterial loads. Rotifers are now commonly cultured in recirculation systems rather than in batch cultures, to stabilise bacterial loads (Doulillet, 2000). Commercial enrichment products are also better designed to reduce bacteria, either through the inclusion of disinfectants or by reducing the enrichment time required. The emphasis on lipid nutrition is also now better understood. A method widely used in the industry is called decapsulation, which is the process of removing *Artemia* cysts with sodium hypochlorite. Gomez-Gil (1994) reported on the use of disinfectants for *Artemia* nauplii and showed that exposures to sodium hypochlorite (5 mg l^{-1} for 15 min),

formaldehyde (70 mg l^{-1} for 5 min) or hydrogen peroxide (60ml l^{-1} of a 6% solution for 5 min) provided the greatest reduction in bacterial loads. The best results were found for sodium hypochlorite or formaldehyde, both of which resulted in a 90% reduction in the heterotrophic bacteria. When sodium hypochlorite was used, it also resulted in the complete elimination of *Vibrio* spp.

More traditional enrichment procedures can also decontaminate *Artemia.* Using algae such as *Chaetoceros muelleri* and ozone (4 ppm for 5 min), final bacteria loads can be reduced by 99.5% (Tolomei et al., 2004). The use of enrichment products to decontaminate *Artemia* is feasible because they have a relatively short gut passage time, as quick as 10 minutes (Smith et al., 2002).

1.5 Antibiotics

Reducing antibiotic usage helps prevent the development of antibiotic resistant bacteria, which have become a serious problem in the prawn industry, where a *Vibrio* spp. has become resistant to multiple antibiotics, leaving farms in South America unable to treat disease outbreaks (Moriarty, 1998). In this case, the solution has been to use a probiotic strain of the genus *Bacillus.* The probiont acted prophylactically against *Vibrio* spp. when added to the water and feed (Moriarty, 1998).

Antibiotics have been used widely in the aquaculture industry to reduce and control disease outbreaks; however, it has been shown that

indiscriminate use can lead to an increase in antibiotic resistance and problems with tissue residues (Vine et al., 2006). Antibiotics still play an important role in the control of new fish diseases or as a tool for identifying bacterial disease as a cause of fish mortalities (Battaglene et al., 2006; Kersarcodi-Watson et al., 2008). Oxytetracycline (OTC) has been studied as an antibiotic addition to modify the bacterial flora of striped trumpeter (*Latris lineata*) larvae (Battaglene et al., 2006). The bacteriology of this study provided a snapshot of Vibrionaceae the primary flora colonizing the gut of larval fish (Munro et al., 1994) and was based on culture techniques. The bacterial load was found to be reduced by the OTC treatments, and the larvae were significantly larger than the ones not treated, and had a five-fold increase in survival. The OTC treated larvae also had less "grey gut", a measure of intestinal dysfunction and the results indicated that bacterial infection was a major source of mortality in the larvae and also reduced growth. The study, indicated that microbial control had a greater influence than lipid nutrition on the survival and growth of the larvae during rotifer feeding stages (Battaglene et al., 2006).

Increased political and environmental pressure has led to a decrease in use of antibiotics, particularly the latest generation of antibiotics reserved for human health treatment (Vine et al., 2006; Kersarcodi-Watson et al., 2008). Other therapeutic chemicals have also been developed in agriculture and aquaculture, particularly in developed countries (Vine et al., 2006; Ziaei-Nejad et al., 2006). Importantly, there has been a push for

the development and use of alternative strategies to manage and control bacterial contamination and disease. These activities have opened up the field of probiotics as a viable and effective management approach in aquaculture.

1.6 Probiotics

The word probiotic originates from the Greek word for 'for life' and is currently used to describe bacteria that have beneficial effects in both humans and animals (Farzanfar, 2006). They have been used in terrestrial farming since the 1970s, with the chicken, cow and pig industries being the biggest users. Recent probiotic research (Verschuere et al., 2000) has centered on two major strategies: bioremediation (modification of an established biota to one that is supportive of the host) and biocontrol (use of a specific microorganism to minimize the impact of a specific pathogen). The former introduces benign bacteria that either displace or deny access to more aggressive opportunistic bacteria (Skjermo et al., 1999; Gomez-Gil et al., 2000; Huys et al., 2001). In contrast, biocontrol focuses on the use of host-benign species of bacteria to control specific pathogens that are associated with disease outbreaks (Skjermo et al., 1999; Gomez-Gil et al., 2000; Huys et al., 2001). In terms of fish culture, one strategy is to first establish a process that can be used for assessing the type of bacterial interactions associated with fish larvae and to devise a means of evaluating the properties of potential probionts. These processes can then be extended to develop standard methods that can be used for evaluating the effectiveness of probionts for

bioremediation or biocontrol in hatcheries. Isolation and evaluation of probionts involves a mixture of *in vivo* and *in vitro* experimentation. The use of larval animals reared under hatchery conditions is essential for the development of a bioremediation strategy, since the bacterial ecology of larval fish is complex (Vine et al., 2006).

A number of criteria have been determined for bacteria to be considered as candidates/probiotics to improve the health of their host (Gatesoupe 1999; Vine et al., 2006). Put simply, a probiont must:

1) Be antagonistic to pathogens *in vitro.*
2) Have a colonization potential that ensures that it does actually colonize the target's gut.
3) Pass challenge tests that confirm that it does increase the host's resistance to disease.

Previous research has looked into methods of manipulating the bacterial communities of culture environments (Gatesoupe, 1991; 1997; 1999; Bruce et al., 2003). Bacteria have been added directly to the water andto the artificial feed while live feeds have also been enriched with probionts. There are a number of reviews written that describe the various probionts used in aquaculture; for example Vine et al. (2006) and Kesarcodi-Watson et al. (2008) and Tinh et al. (2008). It is important to note here that even *Vibrio* spp. can be probionts, although they are thought to be indicators of poor conditions (Garriques and Arevalo, 1995; Gatesoupe, 1997; Ringo and Vadstein, 1998; Ottesen and Olafsen, 2000; Huys et al.,

2001; Makridis et al., 2001). Inclusion of probiotic bacteria may alter the indigenous microbiota of the target species. Tinh et al. (2008) suggests this alteration may come about by (1) the stimulation of the humoral and/or cellular immune responses, (2) the modification of the metabolism of bacterial pathogens by changing their enzyme levels, and/or (3) competitive exclusion either through production of inhibitory compounds that are antagonistic towards pathogens, or by competing for nutrients, attachment sites, or oxygen.

Previous studies have shown that when probiotic bacteria were added to the ambient water, larval fish (Turbot, *Psetta maxima*) and prawns (*Penaeus monodon*), had an increased survival rate, which was considered to be due to *Bacillus* spp. being antagonistic to the pathogenic bacteria in the culture water (Skjermo and Vadstein, 1999; Verschuere et al., 2000; Devaraja et al., 2002; Bruce et al., 2003). Addition of a probiont can be incorporated at the yolk stage, just prior to first feeding. Fish begin to osmoregulate and drink water at this time (Verschuere et al., 2000), thereby introducing bacteria into the intestinal tract. As well as having a potentially positive effect on larval survival, probiotics also have the added benefit of reducing the need for antibiotics. The need to add probiotics to artificial feed has received attention because some probiotics are not native to the microbiota of the animal or are not in high enough numbers to be of use via the water. One example is the use of lactic acid bacteria in turbot *Lactobacillus* spp., which are native to the turbot gut but only occur there in low numbers (Ringo and Gatesoupe,

1998). In this case, *Lactobacillus* spp. needed to be administered through the feed at all times, as it could not build up an effective population due to other bacteria out-competing it. To overcome this problem, research has looked at other bacteria that are present in the gut of fish and that adhere to the intestinal wall (Ringo and Gatesoupe, 1998; Gatesoupe, 1999). As previously mentioned, members of the genera *Vibrio, Pseudomonas, Achromobacter, Micrococcus,* and members of the *Actinobacteria* and *Flavobacteria,* can be readily cultured from the gut of fish (Hansen and Olafsen, 1999). Bacteria that have probiotic properties and the ability to adhere, will also persist within the fish intestinal environmental to avoid fish culturists having to repeatedly re-administer probiotics (Ringo and Gatesoupe, 1998).

1.7 Development and delivery of selected probionts

Recent research has focused on encapsulation of probiotic bacteria into live feeds, a process which is called 'bioencapsulation' and which enables transmission of probiotic bacteria to larval fish.

Studies have investigated the benefits of encapsulating bacteria (Verschuere et al., 1999) and drugs (Dixon et al., 1995) into *Artemia*, which then acts as a live feed for larval fish. These studies have concluded that *Artemia* are a useful vector to transmit these products to the larval fish (Dixon et al., 1995; Gomez-Gil et al., 1998; Verschuere et al., 1999). Drugs such as the antimicrobials romet-30, sulphonamides, erythromycin, and sarafloxacin, can also be bioencapsulated by rotifers

and *Artemia* in quantities that could be therapeutic (Benavente and Gatesoupe, 1988; Verpaet et al., 1992; Dixon et al., 1995; Majack et al., 2000). However, it should be noted that each of these studies used different life stages of *Artemia,* which makes it difficult to draw general conclusions on the exact usefulness of bioencapsulation. In addition, the active feeding rate of *Artemia* changes with time and each drug has a different particle size, making the concentration different for each.

Probionts may also be used as a benefit to raising live feed species. Adding *Pseudomonas* spp. delayed mortality in *Artemia* when no other food was available and was a major source of amino acids and other proteins (Verschuere et al., 1999). Villamil et al. (2003) have reported that the use of *Lactobacillus brevis* and *L. casei* significantly reduced the amount of *Vibrio alginolyticus* load in *Artemia* cultures. It has also been hypothesized that dissolved nutrients that are normally unavailable to *Artemia* may be converted to bacterial biomass when probionts are added, which are in turn grazed upon by the *Artemia* (Verschuere et al., 1999).

It is also possible to encapsulate introduced bacteria into live feed, such as rotifers. Lactic acid bacteria had been fed to rotifers which in turn were fed to turbot (Ringo and Gatesoupe, 1998). Rotifers acted as a vector to introduce the probiotic bacteria into the intestinal tract of the larvae. Douillet, (2000) demonstrated that the growth of rotifers was affected by the presence or absence of bacterial communities and showed that best

growth rates were achieved with the cultures that had been inoculated with an *Alteromonas* strain or a mixed bacterial culture. The lowest growth rates were observed in the controls that were inoculated with bacteria freshly collected from seawater samples. Martinez-Diaz et al. (2003) found that encapsulation of probiotic bacteria was promoted by reducing the initial bacteria load of the culture system.

Bioencapsulation success and derived benefits strongly depends on the type and strain of bacteria used, time of exposure and whether the bacteria are dead or alive (Gomez-Gil et al., 1998). Lysed cells or extracellular activity may be an important factor as they can deliver enzymes that remain active in the gut (i.e. acquired bacterial enzymes), and may therefore result in an increased ability for the host to digest food . If the bacteria are dead, then only the concentration is an important factor in the uptake of bacteria by *Artemia* nauplii, but if the bacteria are alive, the species or strain is also a significant factor . It has been demonstrated that in most cases, the bioencapsulation of bacteria occurs during the first 30 minutes, in both rotifers and *Artemia* (Gomez-Gil et al., 1998; Makridis et al., 2001). This is a direct result of the live food having relatively short gut passage times.

It is possible, after short term incubation, to replace opportunistic bacteria present in live food cultures with other bacteria, which persist for a relatively long period of time (4-24h) as a dominant part of the bacterial community of live food

(Majack et al., 2000). The composition of the bacterial community changes, as the bioencapsulated strains comprise up to 100% of the colony forming units (CFU). Some *Vibrio* strains are grazed on more effectively than other strains and thus may persist longer (Gomez-Gil et al., 1998).

1.8 Experimental approach

1.8.1 Molecular analysis of microbial communities

With the addition of a probiont to the culture system, it is difficult to monitor changes that may occur in the microbial community. A valid approach is to track populations using microbiological and molecular methods.

Traditionally, microbiology methods are culture based. These methods have the advantage of providing information about the phenotypic characteristics (e.g. morphology, growth rate and metabolic capabilities) of a culture. However, this is very time consuming, and often takes many days to culture and process. The identification of fish microbiota, in the past, has typically relied on phenotypic and biochemical key characteristics (Cahill, 1990). Molecular analysis now reveals a vast diversity of bacteria, but only a relatively small proportion of bacterial species have been so far cultured (Hugenholtz et al., 1998). This is mainly due to the lack of knowledge of growth requirements for many

bacteria, the often limited nature of diversity surveys, and the inherent bias towards the analysis and study of fast-growing species.

1.8.1.1 Clone libraries

The highest resolution assessment of microbial diversity of an environmental sample is obtained by constructing a 16S rRNA gene clone library. Here, after the nucleic acids have been extracted from the sample, a clone library can be constructed in three different ways: shot gun cloning of DNA; cloning of rRNA genes after reverse-transcriptase-polymerase chain reaction (RT-PCR); and direct cloning of PCR-amplified 16S rRNA genes. The most popular and widespread method is the latter approach, since the PCR products can be easily cloned into several commercially available sequence ready vectors (Theron and Cloete, 2000). These clones can then be sequenced directly and the sequences compared to nucleotide databases (e.g. Genbank). For complex communities, it is necessary to sequence a large numbers of clones (in the 1000s) in order to gain an accurate insight into the community diversity (Kemp and Aller, 2004). While more recent studies have shown that the use of 16S rRNA microarray analysis can offer great insights into community structure (DeSantis et al., 2007), exhaustive analysis of diversity is very expensive to complete and rather time-consuming. Instead a valid goal that can be attained cost- and time-effectively is to simply establish the major players in systems i.e. the most numerically dominant species. Though it is possible to deeply sequence or analyse diversity, many basic questions remain about the role or significance of

low abundance members of the community. Clone libraries therefore still offer the highest resolution in assessing community structure, however, practicality may also demand that a more focused approach be undertaken.

1.8.1.2 Denaturing gradient gel electrophoresis (DGGE)

DGGE was first used by (Muyzer et al., 1993) to analyse natural microbial communities. DGGE is based on the concept that DNA PCR fragments of the same length but with varying sequences have different melting properties (Heuer and Smalla, 1997). With this approach, PCR-amplified DNA is electrophoresed through a linearly increasing gradient of chemical denaturants, usually urea and formamide, which partially melts the DNA strand, causing the DNA to become branched and leading to sharply decreased mobility of the DNA through the gel. A GC rich clamp on one end of the primer set prevents the complete melting of the DNA fragment. As the melting temperature of a fragment is determined by its sequence, the fragments remain double stranded until a point is reached that causes melting of domains within the DNA duplex. DGGE has been in favour for many years in the analysis of environmental samples, as it allows the rapid generation of snapshots of complex microbial communities (Muyzer and Ramsing, 1995). However, the method is technically demanding, can lack resolution when complex samples are investigated and is subject to PCR and gel bias (Powell et al., 2003).

1.8.1.3 Automated ribosomal intergenic spacer analysis (ARISA)

To overcome the limitations of cultivation, molecular techniques that adapt the well established (PCR) technique for amplification of target genes from organisms have been used along with automated ribosomal intergenic spacer analysis (ARISA). ARISA involves the use of PCR to obtain fluorescently labeled amplicons comprising the Internal Transcribed Spacer (ITS) region between the 16S and 23S rRNA genes. These amplicons are then resolved using capillary electrophoresis to obtain a profile of the bacterial community, which appears as a series of peaks of specific sizes. The ITS region has highly variable lengths between different bacterial taxa and has proven very useful for tracking bacteria in natural samples, e.g., seawater (Brown et al., 2005). By correlating the length of each fragment corresponding to a peak in the electrophoretogram obtained, it is also possible to identify the source micro-organism.

1.8.1.4 Terminal restriction fragment length polymorphisms (TRFLP)

Another relatively new molecular approach that allows the assessment of the diversity of complex bacterial communities is terminal restriction fragment length polymorphism (TRFLP) analysis. This technique has been used for rapid assessment of ecosystem diversity and the structure of complex bacterial communities in various environments. TRFLP is a community profiling method, usually based on the 16S rRNA gene, and can be used with universal primers down to species level primers, depending on the resolution required. This method does require

amplification of the 16S rRNA gene with specific primers and is thus more susceptible to biases and skewing of the native community. The technique itself depends on the amplification of the DNA with a primer set, one with a fluorescently end labeled primer and restriction of the resulting product with frequently (4-base) cutting restriction enzymes. Due to sequence variations, the terminal restriction site for each species in the community should be different. The output is digital and provides information on the size of the product in base pairs (i.e. species) and the intensity of fluorescence or relative abundance of the various community members. Only the fluorescently labeled terminal restriction fragments are detected. Their sizes are determined by comparison to those of a known internal standard consisting of DNA of known length (Hewson et al., 2003; Smith et al., 2007). The approach also has a relatively high signal-to-noise ratio requiring the application of both technical and biological replicates. A source of signal noise is due to the formation of pseudo terminal restriction fragments (TRF), as explained in reviews by Egert and Freidrich, (2003), Nocker et al. (2007) and Orcutt et al. (2009). It was found that like PCR pseudo-TRFs can be formed with the left over primer bases and thus give false peak readings. It is therefore advised to perform virtual digestion of corresponding sequence data such as clone library-derived sequences. It is also important to design the primers being used correctly, to optimise primer levels in the TRFLP PCR thermocyling process, and to take account of PCR run-to-run variations (Schutte et al., 2008) thus minimising pseudo-TRF artifacts. If this is not done, there is a risk of missing important TRFs and thus data for later analysis. Primers

10F and 907R were used in this study. This primer set was used for the formation of the clone libraries and TRFLP, constancy through the study with the primers was believed to be the best approach.

1.8.1.5 Real time PCR

Through the use of specific primers targeting the probionts ITS region, RT-PCR can be correlated to other experimental data, such as survival rates and effects of nutrients. By allowing the binding of a DNA-intercalating dye, Sybr-Green II, the amount of amplified product being produced during the PCR thermocycling process is automatically determined. By determining the minimum time for the fluorescence to exceed a certain threshold, the copy number of the target gene (16S or 23S rRNA genes) is calculated by comparison to a standard curve calculated from measurements of known target gene copy numbers. From this, a relative abundance of the probiont population can be established. In addition, the same approach can be used to rapidly monitor bacterial pathogen populations, which are also detected by using a specific primer set (Theron and Cloete, 2000).

1.9 Fish model used in study

The model fish used in this study was striped trumpeter *Latris lineata*. This species was identified as a new candidate for aquaculture in the late 1980s and is being investigated as an alternative cold temperate species for sea cage culture of salmonids in Tasmania (Battaglene and Cobcroft, 2007). The fish was chosen for its docile nature, fast growth and

tolerance to high holding densities. It is a highly prized fish in restaurants. The species is widely distributed in the temperate latitudes of southern Australia, around Tasmania and New Zealand, and in isolated island groups in the Indian and Atlantic Oceans (Tracey et al., 2006). Striped trumpeter was once plentiful in Tasmania but the Tasmanian fishery has declined to a catch of less than 30 metric tonnes per annum. Work started on striped trumpeter during the late 1980s with a production method based on that of Atlantic salmon. It was quickly seen that striped trumpeter were not easy to culture and had a complex life cycle, including a nine month neustonic 'paperfish' stage. Due to the extended larval stages, it has been difficult to rear large numbers of larvae for a variety of reasons, including key development issues such as poor swim bladder inflation, jaw malformations, inadequate larval nutrition and bacterial problems (Battaglene and Cobcroft, 2007).

Striped trumpeter are now cultured under "greenwater" conditions and fed enriched live feeds, which has resulted in increased production. Measures are taken to reduce the bacterial loads including egg disinfection, washing of the live feeds, the use of ozone treated seawater and use of OCT (Battaglene, 2006, Battaglene and Morehead 2006; Battaglene and Cobcroft, (2007).

Pilot sea cage trials are underway with cultured striped trumpeter but production of high quality juveniles is still a bottleneck in commercial development of this new candidate for aquaculture. Research is

underway to improve larviculture techniques, through the use of formulated diets, improved tank design, and microbial control (Battaglene and Brown 2006; Battaglene and Cobcroft, 2007).

1.10 Thesis aims

The aim of the thesis was to establish a process that could be used for assessing the type of bacterial interactions associated with fish larvae, using striped trumpeter larvae as a model and developing the means for evaluating the properties of potential probionts. The goal was to develop standard methods that can be used to identify possible probionts for bioremediation or biocontrol in hatcheries and also to develop methods of identifying/ tracking the probionts once added. Isolation and evaluation of probionts involved a mixture of *in vivo* and *in vitro* experimentation. The use of larval animals reared under hatchery conditions is essential for the development of a bioremediation strategy, since the bacterial ecology of larval fish is complex. To develop a robust process that could identify and culture probionts in larval fish cultures, the following goals were developed:

1) Identification of the bacterial communities in striped trumpeter larval cultures through the use of a 16S rRNA bacterial gene clone library and TRFLP analysis.

2) Determination of the bacteria that possess probiotic activity against *Vibrio* spp., by using *in vitro* antimicrobial plate tests and assessment of possible candidates on live feeds.

3) Development of the use of TRFLP to enable tracking of the probionts in a mixed environment.

4) Exploring the best mode of action to introduce the probiont to the larvae and at which stage. The tracking of the probionts in an experimental striped trumpeter larvae system was to be achieved using TRFLP to match the probionts' unique TRF.

1.10.1 Experimental constraints

It must be noted that this thesis has certain experimental constraints associated with it. Replication in Section 2.3.2.3 is lacking due to samples coming from a previous experiment. During the study the use of clone libraries and TRFLP required considerable optimisation. These processes took a substantial amount of time and had an associated cost-burden and thus led to the number of replicates used for TRFLP analysis being suboptimal (typically n=3) and clone libaries (typically n=50).

1.10.2 Thesis structure

The thesis consists of Five Chapters:

Chapter One includes a general introduction into the microbiology of the larvae of marine fish and the methods to be used in this study. Chapter Two involves an investigation of the bacterial communities, using 16S rRNA clone libraries and TRFLP analysis, associated with 15 dph striped trumpeter larvae grown under different conditions. It includes an assessment of whether microbial communities could be linked with larval survival performance. Chapter Three includes the results of experiments in which potential probionts were identified. This research also included utilisation of the TRFLP approach to track the isolates within experimental

systems. The chapter also explores the efficacy of probionts against a pathogenic *Vibrio proteolyticus* strain in experiments involving live feed species, rotifers and *Artemia*. Chapter Four utilizes findings from the previous chapters and assessed different ways to introduce the probionts to larval fish, be it by directly adding them to the tank water, or via bioencapsulation within live feed species. Within this chapter there was also an assessment of the probionts performance to determine whether they had a detrimental, neutral, or positive benefit to larval survival and growth during the yolk sac and first feeding phases. Chapter Five summarizes the thesis. It also discusses further directions and areas of importance to be researched that could further increase our understanding of the microbiota communities of striped trumpeter.

Chapter 2: Microbial communities of post hatch striped trumpeter (*Latris lineata*) larvae held under different rearing conditions, determined using cultivation-independent approaches

2.0 Abstract

A knowledge of the microbial bacterial community of the larvae, seawater and live feeds was believed to be necessary in order to develop an understanding of what represents a healthy microbial ecology relevant for larval fish rearing success. 16S rRNA bacterial clone library and terminal restriction fragment length polymorphism (TRFLP) analysis was utilized to examine the microbial community associated with striped trumpeter larvae (*Latris lineata*) cultured under different "green water" conditions. It was discovered that the larvae-associated microbial diversity was restricted but varied considerably between culture conditions. Most bacteria detected belonged to class *Alphaproteobacteria* (predominantly the *Roseobacter* clade), *Gammaproteobacteria* (genus *Psychrobacter* and *Pseudoalteromonas*) and *Actinobacteria* (primarily genus *Microbacterium*). No association was found between larval survival and microbial community structure. Similar results were obtained using TRFLP analysis, though it was found that the larval microbial community was distinct from the bacterial community present in the surrounding water.

2.1 Introduction

Intensive cultivation of most marine larval fish is potentially subject to bacteria-associated problems that result in poor growth and mass mortalities (Skjermo and Vadstein, 1999; Vadstein et al., 2004). An understanding of the microbial bacterial community of the larvae, seawater and live feeds is necessary in order to develop an understanding of what represents a healthy microbial ecology relevant for larval fish rearing (Nicolas et al., 1989; Hansen and Olafsen, 1999; Verschuere et al., 1999). As with all live animals, larval fish have their own bacterial communities that they acquire from their surroundings. It is possible that, as in mammals, fish contain a specific suite of intestinal microbiota that establish during juvenile developmental stages (Hansen and Olafsen, 1999; Verschuere et al., 2000).

New techniques are required to better understand these bacterial communities and their interactions. Traditionally, culture-dependent studies of hatchery fish have been conducted on identifying the major families of bacteria associated with marine fish at different stages of life. Hansen and Olafsen, (1989) found that larvae and juvenile cod (*Gadus morhua*), were made up of predominantly *Vibrio, Lactobacillus* and *Bacillus*, while this changed to *Vibrio, Photobacterium, Pseudomonas*, and *Alteromonads* during the adult stages. Studies on the Atlantic halibut (*Hippoglossus hippoglossus*) have also shown a shift during the yolk sac stage through to larval stages (Verschuere et al., 1999). During the yolk

sac stage, it was observed that the dominant genera present were *Cytophaga, Flexibacter, Flavobacterium and Pseudomonas*, while during the feeding larval stages, *Vibrio and Aeromonas* were detected (Bergh et al., 1992). While using a culture-dependent approach does give an insight into the bacterial communities, often a high proportion of bacteria are overlooked, either due to inadequate surveying or because they cannot be cultured on standard agar media. For this reason, more recent studies have applied molecular-based approaches (Jensen et al., 2004; Romero and Navarrete, 2006; McIntosh et al., 2008; Zhong et al., 2008). These methods have allowed the identification of bacteria without isolation and allow the phylogenetic affiliation of the community present to be accurately determined. Overall, this information has greatly increased our knowledge of the microbiology of fish (Romero and Navarrete, 2006).

Methods based on the amplification of the 16S rRNA gene, have emerged as powerful tools (Holben et al., 2002). Examples of these are DGGE, TRFLP, clone libraries and real time PCR, which have been discussed in Section 1.8. These techniques are subject to PCR biases and resolution limits, meaning that even when precautions, such as multiple PCR reactions are made, PCR can skew community composition data. This may also result in species with a smaller amount of DNA present to remain undetected. Using these methods, it has been found that the microbial diversity of larval fish is relatively similar on a broad level with most taxa belonging to the phyla *Proteobacteria, Actinobacteria,* and *Bacteriodetes*. On a species level, the differences are

much greater. It has also been reported that these differences can occur within different parts of the fish, e.g. foregut or hind gut (Verner-Jeffreys et al., 2003) and that skin mucus and the surrounding water also have different microbial populations (Smith et al., 2007). The age of the fish, as well as whether it is either wild or captive, can also influence microbial communities (Romero and Navarrete, 2006). Therefore, by utilizing molecular biology techniques, it is possible to obtain insights into the microbiota and thus better understand how the microbial ecology of the fish and its environmental influences health and growth.

While the microbial community of the striped trumpeter larvae has been previously investigated following culture (Battaglene et al., 2006) to gain a greater understanding of the microbial community of striped trumpeter larvae, a molecular approach was applied. This study uses 16S rRNA and TRFLP molecular techniques described in Sections 1.8.1.1 and 1.8.1.4 respectivly. Samples were obtained from 15 day-old striped trumpeter larvae that were grown under three different green water conditions. The main objectives of this study were to:

1. Determine the bacterial communities associated with 15 day-old striped trumpeter larvae reared under different conditions, by using 16S rRNA gene clone library analysis.
2. Identify and track the dominant bacterial species on fish tissue samples using TRFLP analysis.

3. Determine how the microbial communities are influenced by different greenwater rearing approaches and to determine whether microbial communities affect growth and survival of larvae.

2.2 Methods

2.2.1 Background

The study formed part of a larger experiment investigating the effects of three water treatments on the survival and growth of striped trumpeter larvae (Cobcroft et al., 2010).

2.2.1.1 Larval rearing

Eggs were collected from a female striped trumpeter broodstock by strip-spawning, and fertilized with the milt of four males. Fertilized eggs were incubated and hatched as previously described (Bransden et al., 2004). Larvae (4500 per tank) were stocked into twenty-four, 300 l black hemispherical fiberglass tanks at 1 dph. Larvae were held under static, clear water conditions from 1 to 5 dph. A photoperiod of 18 hours light: 6 hours dark was used throughout the experiment and was produced by a computerized halogen light source (~11 µmol s^{-1} m^{-2} at the water surface) using a gradual fade in and fade out process. During 6 dph internal screens (390 µm mesh size) were placed into the centre of each tank to allow outflow and removal of rotifers, algae and incoming seawater overnight (see below).

There were three treatments each with eight replicated tanks. The first treatment, designated "Fresh Algae" (FA), consisted of rearing larvae in a

'greenwater' environment that included live *Nannochloropsis oculata* at a turbidity level of 3 Nephelometric Turbidity Units (NTU) (HACH 2100 portable turbidity meter). The second treatment, designated "Instant Algae" (IA) was an algal paste of concentrated *N. oculata* (Reed Mariculture Inc., California, USA) that was also used to produce a green water environment by re-suspension of the concentrate in seawater to a turbidity level of 3 NTU. The final treatment, designated "Clear Water" (CW) consisted of rearing larvae without addition of algae (<0.01 NTU). The algal additions were added to each tank at a rate of 8.8 l min^{-1} (total 100 l) from the reservoirs accompanying each individual tank. The algal suspensions or seawater had been acclimated to tank temperature in the reservoirs overnight and provided with aeration. Tanks remained static during the light phase with gentle aeration (200 ml min^{-1}). A central screen (mesh size 390 µm) was placed in the tank and incoming seawater (112.5 l hr^{-1}) that had been passed through primary filtration was supplied. The screen prevents the loss of larvae but allows the passage of algal cells and live food (see below). All tanks were supplied with live food rotifers (*Brachionus plicatilis*) enriched on Algamac 2000 (Aquafauna Biomarine, USA) as described by Bransden et al. (2004). Enriched rotifers were supplied at 10ml^{-1} every morning. Surface skimmers were used from 8-15 dph to remove surface oil and promote swim bladder inflation (Trotter et al., 2003). Water quality was measured daily with a range of temperatures of 15.5–16.5°C, salinity of 34.1-34.7ppt, pH of 8.1 and dissolved oxygen of greater than 90% saturation. Mortalities were spot siphoned and counted daily, up to the conclusion of

the experiment (15 dph) when all live larvae were removed and counted to determine final survival.

2.2.2 Larval sampling

Larvae were sampled from each tank on 15 dph. Fifty larvae were removed from each tank and following anesthesia; 30 were used to determine morphormetric indices and dry matter content and 20 were used for the assessment of the microbial community. At the same time 20 ml of water was removed via a sterile pipettor from each tank and placed into sterile containers. All sampling took place prior to feeding.

The larvae and water samples selected from the six treatment tanks for the study were chosen from tanks that performed at different levels for each of the three treatments, designated "good performance" and "poor performance" i.e., the best and worst performing tanks for each treatment. Further information on performance of tanks not chosen for analysis can be found in Cobcroft et al. (2010). In addition water samples relating to each of these six larvae samples were also obtained for TRFLP analysis. No replication was possible because of the constraints described in Section 1.10.1.

2.2.3 DNA extraction

Extraction of bacterial DNA from the homogenized striped trumpeter larvae was performed utilizing the DNeasy Tissue Kit (Qiagen Pty. Ltd., Australia). An initial pretreatment step employing lysozyme was

incorporated to lyse Gram-positive bacteria. Additional wash steps were employed in addition to the manual protocol which resulted better yields of DNA as it removed salts and other inhibitors that were present in the samples. This was done by repeating the initial wash step as per manual. Extraction of bacterial DNA was also performed on water samples, utilizing the MoBio water extraction kit (MoBio Laboratories, Inc) following the manufacturer's instructions. The amount of DNA present in larval and water extracts was measured by spectrophotometry using a SmartSpec 3000 (BioRad).

2.2.4 Clone library polymerase chain reaction (PCR)

Clone libraries were generated using DNA extracted from the six samples types. PCR amplification of the 16S rRNA gene, used the primers 10f (GAG TTT GAT CCT GGC TCA G) and 907r (CCG TCA ATT CCT TTG AGT TT). Each reaction was a 50µl reaction mix with 5µl of 10X buffer, 1µl of Advantage Taq (1.1 µg/µl of TITANIUM taq DNA polymerase and TaqStart Antibody, Clontech), 4µl of a deoxynucleotide mix solution, 4µl the forward primer, 4µl of the reverse primer, and approximately 10ng DNA template. A final volume of 50 µl was obtained using milliQ water. The following thermal cycling program was used: initial denaturation at 94°C for 3 minutes, 30 cycles of denaturation for 1 minute, annealing at 50°C for 1 minute, extension for 3 minutes: final extension at 72°C for 10 minutes. The reactions were purified using the Qiagen PCR cleanup kit

2.2.5 Clone library construction

PCR-amplified 16S rRNA gene samples to be cloned were ligated into the pGem-T vector (Promega) according to the manufacturer's instructions and transformed into Epicurian coli XL ultracompetent cells (Stratagene). Transformants were then screened using blue-white selection on Luria agar containing XGal/IPTG and 100 mg l^{-1} ampicillin. Approximately 50 white colonies from each library were then transferred to fresh plates and re-incubated overnight. Plasmids were extracted using the Ultraclean miniplasmid extraction kit (MoBio). Positive clones were sequenced in both directions using primers M13 forward and M13 reverse using the Ready Reaction Dideoxy Cycle Sequencing kit (Beckman-Coulter). Sequencing was performed using a Beckman-Coulter CEQ2000XL automated capillary sequencing system.

2.2.6 Sequence analysis

Sequences were aligned against reference sequences obtained from GenBank (http:/www.ncbi.nlm.nih.gov/Bast (Altschul et al., 1997) using BioEdit (version 7.0.9) (Hall, 1999). Automated alignment using Clustal X was performed and aligned data exported to the CLC free workbench (v4) where similarity trees were produced, using the Neighbour-joining algorithm. The 16S rRNA gene sequences from *Thermotoga maritima* and *Coprothermobacter platensis* were used as outgroup references on the *Alphaproteobacteria*, *Gammaproteobacteria* and *Actinobacteria* sequence based-trees, while the plastid 16S rRNA gene sequence from *Fucus versiculosus* was used for the plastid sequence-based tree. Clones

with a sequence similarity of 98% were considered to be the same phylotype (Keswani and Whitman, 2001) for the purposes of assessing diversity. Clone library comparisons method utilized the LIBSHUFF v. 1.22 computer program (http://libshuff.mib.uga.edu/) (Singleton et al., 2001) to generate homologous and heterologous coverage curves from clone libraries, which were then compared statistically. The DNADIST program of PHYLIP using the Jukes-Cantor model for nucleotide substitution was used to construct the distance matrix submitted to LIBSHUFF.

2.2.7 TRFLP sample preparation

TRFLP was used to determine the changes in bacterial communities between the six larvae samples held under different conditions and to assess whether there were differences in the bacterial community that could have resulted in higher or lower survival rates of the larvae. Fluorescently labeled primers were labeled with Beckman Coulter WellREDtm fluorescent dyes. Primer10f (GAG TTT GAT CCT GGC TCA G) was labeled with the D4 (Blue) dye while primer 907r (CCG TCA ATT CCT TTG AGT TT) was labeled with the D3 (Green) dye. Each PCR reaction comprised of 12.5 µl HotstartTaq MasterMix (Qiagen, Australia), 1.0 µl of each primer and approximately 10ng DNA template, with a final volume of 25 µl obtained with milliQ water. The following thermal cycling program was used: initial denaturation at 94°C for 15 minutes, 34 cycles of 94°C for 1 minute, 50°C for 1 minute, 72°C for 1 minute with a final extension at 72°C for 10 minutes. Four identical PCRs per sample as

outlined above were run separately and the resulting products were pooled during the PCR purification process. The reactions were purified using the Qiagen PCR cleanup kit.

Purified PCR products were digested with 20U of enzymes *HaeIII, HhaI* and *HinfI* (New England BioLabs) 10µl reaction were prepared using 2 µl of DNA template, 20U of enzyme 1µl of bovine serum albumin solution, 1µl of NEL Buffer and milliQ water was used to obtain a volume of 10 µl. Each digest was incubated at 37°C for 3 h. At the end of the incubation the digestion was stopped using heating in a thermocycler with the *Hha*I digestion stopped by incubating at 65°C for 20 min while *Hinf*I and *Hae*III digestion were stopped by incubation at 80°C for 20 min. Each digest was done separately in duplicate. Digests were then desalted and purified using an ethanol precipitation method. To each well, 1µl of 3M sodium acetate and 0.5 µl of glycogen was added and mixed; 30 µl of ice-cold absolute ethanol was added and the sample vortexed. Plates were covered with an aluminum sealing mat and incubated at -20°C for 20 minutes. Plates were then centrifuged at 4°C for 30 minutes at 4300rpm (Sorvall Super T21 Thermo Electron Corp. Waltham, USA). Removal of ethanol was achieved by inverting the plate onto size cut absorbent paper, followed by centrifugation for 30 s at 300 rpm (Quantum Scientific, Murarrie, Australia). This was followed by a wash with 200 µl 70% (vol./vol.) ethanol, which was removed as previously described. Plates were left to air dry in a laminar flow cabinet, until there was no more remaining ethanol residue. Each digest was performed in duplicate.

The dried pellets were re-suspended in 30 µl Sample Loading Solution (Beckman Coulter) and 0.3 µl of the 600bp DNA size standard (Beckman Coulter) was added to each well. Samples were then analyzed on a Beckman Coulter CEQ800 Genetic Analysis System, using the Frag-4 program, injection 2.0 kV/30sec, run at capillary temperature 50 °C /4.8kV for 60 minutes.

2.2.8 TRFLP data analysis

The TRFLP analysis procedures employed by Dann et al. (2009) were used to ensure TRFLP profiles were reproducible and also to minimize baseline noise. In summary this was done by producing *in silico* digests using workbench 4.0. Clone sequences were submitted and digested within the program generating the corresponding base position size for each TRF. Duplicate samples were analysed using T-ALIGN (Smith et al., 2005), with a confidence interval of 0.5 bp in order to generate a consensus TRFLP profile in which TRFs present in both replicates were represented. Multi-dimensional scaling (MDS) plots were obtained using Primer v. 6 (Primer-E Ltd., Plymouth UK) to determine the similarity of the microbial community between treatment replicates and between separate treatments. Stress values obtained with each MDS plot provide an indication of the "goodness of fit" with the data set with the lower the value indicating a more representative analysis of the dataset. The similarity of treatments was determined using ANOSIM analysis using Primer v. 6. This analysis produces R statistic values that provide an

indication of the level of similarity between sample sets within a spatial scale, where 1 indicates completely different sets of samples while a value of 0 indicates sample sets that completely overlap. Permutation analysis was also performed to obtain a significance value. Percent similarity within sample sets and the average percent contributions of individual TRFs to the total peak area TRFLP profile were determined using SIMPER analysis within the Primer 6 software package.

In relation to the MDS plots the actual location of each data point in space is arbitrary, and the axes can be rotated freely. It is the relationship of the data points to each other that is of importance, with two near points representing more similarity to each other than to another point located at a distance. The stress of the plot, generated as part of the MDS analysis in Primer 6, is a measure of how much distortion was introduced to allow the representation of the data in the specified dimensions, i.e. a "goodness of fit". A stress of 0 gives a perfect representation while a measure of stress <0.2 indicates the plot is a good represnatation of the data set and can be used for interpretation. A measure of >0.3 indicates that the level of distortion that was required to display the data on the map is too high for any reliable inferences to be made from the configuration.

2.3 Results

2.3.1 Clone library data and comparisons between treatments

Larval survival, 15 days post-hatch, was most consistent in the clear water treatment tanks (38 ± 5% survival) (Table 2.1) and also yielded larvae of the greatest size. The use of fresh algae resulted in similar outcomes, but was more variable (36 ± 9% survival). The use of "instant algae" preparations resulted in poor outcomes (7 ± 4% survival) (Table 2.1). Overall, the microbial communities directly associated with the larvae were low in diversity (Table 2.2) with the microbial community dominated by members of the class *Alphaproteobacteria* (Fig. 2.1 to 2.4). Members of the *Actinobacteria* (Fig. 2.6) and *Gammaproteobacteria* (Fig. 2.7) were also detected in certain samples. 16S rRNA-like gene sequences derived from the plastids of *Nannochloropsis* species (Fig. 2.5) were detected in all samples (Fig. 2.1 to 2.3).

2.3.1.1 Clear Water tanks

Both clear water-based tank samples were dominated by the seawater species *Sulfitobacter pontiacus*, *Ruegeria mobilis* and *Microbacterium oxydans/M. marinotypicum* (Table 2.2). Collectively, these species made up 95% and 48% of the bacterial clones, respectively in the two samples analysed. The species *Sulfitobacter dubius* and *Sphingomonas paucimobolis* were also detected in the clear water treatment sample with the poorest survival outcome (CWP). In the case of the sample with the best survival outcome (CWG) 38% of bacterial clones were most closely

related to the species *Psychrobacter nivimaris* (Fig. 2.2). In the same sample additional clones were found to belong to a clade of uncultivated alphaproteobacteria found in various marine ecosystems based on unpublished cloned sequence data on GenBank; these are most closely related to the genera *Thalassobius* and *Thalassobacter*.

2.3.1.2 Instant Algae

Only *Sulfitobacter pontiacus* was found to occur in both good and poor performinginstant algae treatment samples. Clones related to *Psychrobacter nivimaris* and *Microbacterium oxydans* were detected in the sample with the poorest survival (designated IAP). In comparison the alphaproteobacterial species *Phaeobacter gallaeciensis* and *Nautella italica* were only detected in a second sample (designated IAG) that demonstrated better but still comparatively poor survival (Table 2.1).

Table 2.1: Striped trumpeter larval survival and size for three different water treatments: fresh algae, instant algae and clear water. Survival of fish in single tanks chosen for anaylsis from the best and worst tanks in the treatment ("good" and "poor" n=1), along with overall treatment means for survival and Total Length (TL) (n=8 see Cobcroft et al., 2010)

	Fresh Algae	Instant algae	Clear water
		% survival:	
Good Performance	50	13.4	45.6
Poor Performance	22.5	1.1	30.1
Overall % survival (mean, SD)	35(± 9)	7± 4	38± 5
Overall larval size TL (mm ,SD)	7.1±0.3	6.6±0.3	6.6±0.3

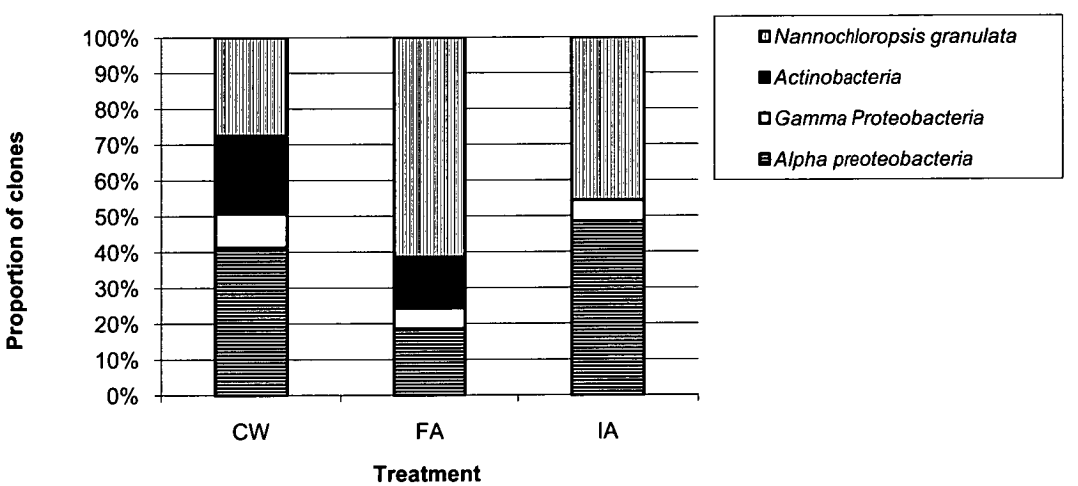

Figure 2.1: Percent proportion of major taxonomic groups occurring between the samples divided between the three different green water treatments. Abbreviations: clear water, CW; fresh algae, FA; and instant algae, IA.

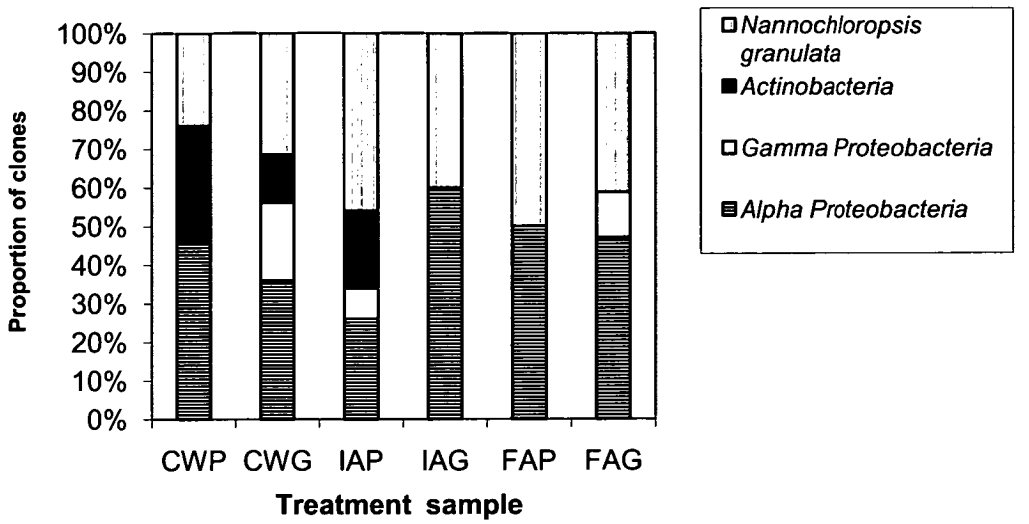

Figure 2.2: Percent proportion of major bacterial taxonomic groups occurring between the different treatment samples analysed.

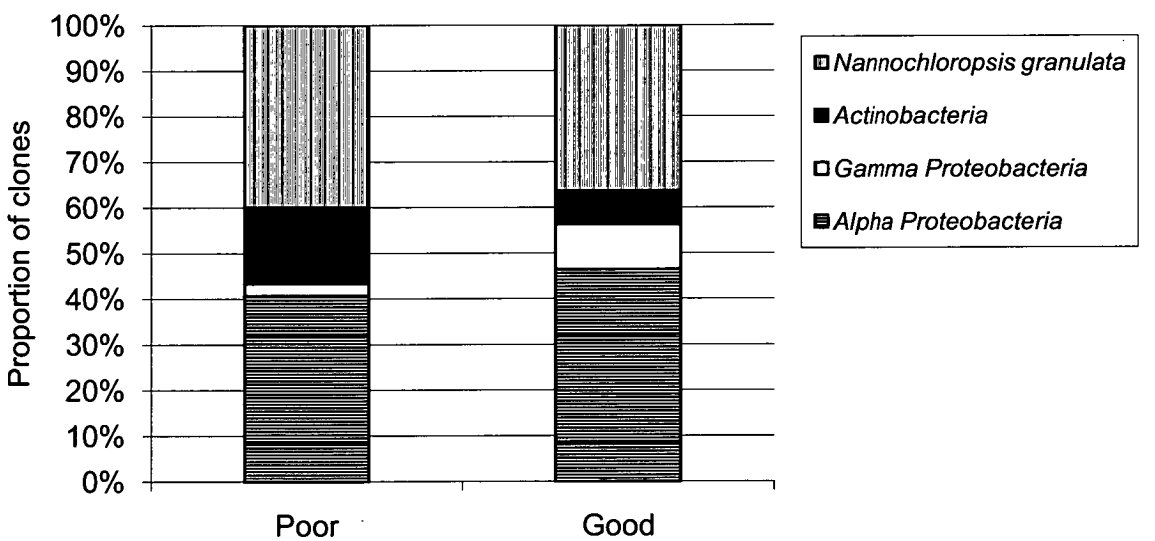

Figure 2.3: Percent proportion of major bacterial taxonomic groups occurring in samples pooled on the basis of poor and good larval survival performance as indicated in Table 2.1.

2.3.1.3 Fresh Algae

Overall diversity was found to be greatest in the fresh algae supplied tanks and the communities differed almost completely between the samples analysed. The only phylotypes in common were related to *Ruegeria mobilis* and to deep-branching *alphaproteobacteria* distantly related to the family *Rickettsiales*, very similar to clones derived from the gut microbiota of zebrafish (Rawls et al., 2004). The sample with the best survival outcomes (FAG, 50% survival, Table 2.1), also possessed phylotypes related to uncultured marine *alphaproteobacteria*, *Psychrobacter* and *Pseudoalteromonas*. The comparatively poorer performing tank (FAP), though still several times better than both IA samples, had phylotypes related to *Phaeobacter gallaeciensis* as well as *Methylobacterium aquaticum* and *Sphingomonas paucimobilis*.

2.3.1.4 Comparison of rearing performance based on clone library data comparisons

In order to find whether microbial communities influenced larval rearing, samples were defined in terms of performance 'pools' (Table 2.1), designated simply as either "good" and "poor" performers. From the clone library data (Table 2.2) most species detected were found in tanks with different performance outcomes and thus could not be expected to be influencing larvae survival. The species *Methylobacterium aquaticum*, *Sphingomonas paucimobilis* and *Sulfitobacter dubius* were only found in poorly performing tanks based on fresh algae and clear water treatments.

Table 2.2: Distribution of cloned bacterial 16S rRNA sequences detected in striped trumpeter larvae grown under different green water conditions.

Species:	Similarity (%)	Clear water		Instant algae		Fresh algae	
		Sample name and relative larvae survival (performance):					
		CWP	CWG	IAP	IAG	FAP	FAG
		Proportion of clones sequenced (%):*					
Class *Alphaproteobacteria:*							
Sulfitobacter pontiacus	>99	36	27	50	36		35
Sulfitobacter dubius	>99	2					
Uncultivated species (closest species *Thalassobius mediterraneus*)	96		10				
Phaeobacter gallaeciensis	>99				43	16	
Nautella italica					21		
Ruegeria mobilis	>99	6	13			16	23
Uncultivated species (closest species *Bartonella bacilliformis*)	90						3
Methylobacterium aquaticum						16	
Sphingomonas paucimobilis	>99	2				8	
Uncultivated species (most similar to *Rickettsia* & relatives)	<80					41	19
Class *Gammaproteobacteria*:							
Psychrobacter nivimaris/P. glacincola	98-99	16	38	15			9
Pseudoalteromonas elyakovii	>99						9
Phylum *Actinobacteria*:							
Marinobacterium oxydans; *M. maritypicum*	>99	16	12	35			

However, as these species were not detected in the corresponding instant algal treatments it cannot be suggested they are linked to poor survival outcomes. Furthermore, the species were encountered only sporadically and at lower proportions than other species detected. Similarly, no one species can be indicated as being responsible for improving outcomes based purely on the clone library data.

The null hypotheses that the three tank treatment libraries derived from 15 day post hatch larvae would not be significantly different were tested by using a statistical approach useful for comparing clone library data - the LIBSHUFF method (Singleton et al., 2001). It was found that all sample clone libraries were significantly different from one another ($p<0.01$) (Table 2.3) due to the low diversity but distinct speciation was encountered. Under-sampling may thus contribute to this finding as LIBSHUFF analysis sensitivity is considerably affected by the sampling effort (Singleton et al., 2001). When the performance data is pooled, coverage values derived from LIBSHUFF analysis are relatively small, suggesting that performance variations are not linked to microbial community composition within the resolution limits of the available data.

Table 2.3: LIBSHUFF comparisons of the heterologous and homologous coverages (delta-C) of bacterial clone library data on the basis of larval sample green water treatments and larval survival outcomes.

Comparison Number	Homologous Data	Heterologous Data	*P*[b]	delta-C
1	FAP[a]	FAG	0.003	4.198
	FAG	FAP	0.003	5.232
2	IAP	IAG	0.003	4.364
	IAG	IAP	0.009	0.616
3	CWP	CWG	0.012	0.173
	CWG	CWP	0.003	1.766
4	IAP	CWP	0.003	1.96
	CWP	IAP	0.006	0.634
5	IAP	CWG	0.003	0.899
	CWG	IAP	0.009	0.615
6	IAP	FAG	0.003	1.232
	FAG	IAP	0.006	1.107
7	IAP	FAP	0.006	1.087
	FAP	IAP	0.003	2.205
8	FAG	IAG	0.003	2.276
	IAG	FAG	0.003	3.01
9	FAG	CWG	0.003	1.394
	CWG	FAG	0.003	1.893
10	FAG	CWP	0.003	2.95
	CWP	FAG	0.003	2.303
11	FAP	CWP	0.003	4.198
	CWP	FAP	0.003	5.232
12	FAP	IAG	0.003	3.93
	IAG	FAP	0.003	3.11
13	CWP	IAG	0.003	2.692
	IAG	CWP	0.003	0.844
14	FAP	CWG	0.003	1.812
	CWG	FAP	0.003	5.246
15	IAG	CWG	0.003	3.093
	CWG	IAG	0.003	4.474
16	Poor	Good	0.003	0.287
	Good	Poor	0.003	0.457

[a]Abbreviations for treatments are provided in the Methods section.

[b]*P*-values were Bonferroni-corrected to account for the possibility of false positive significance values.

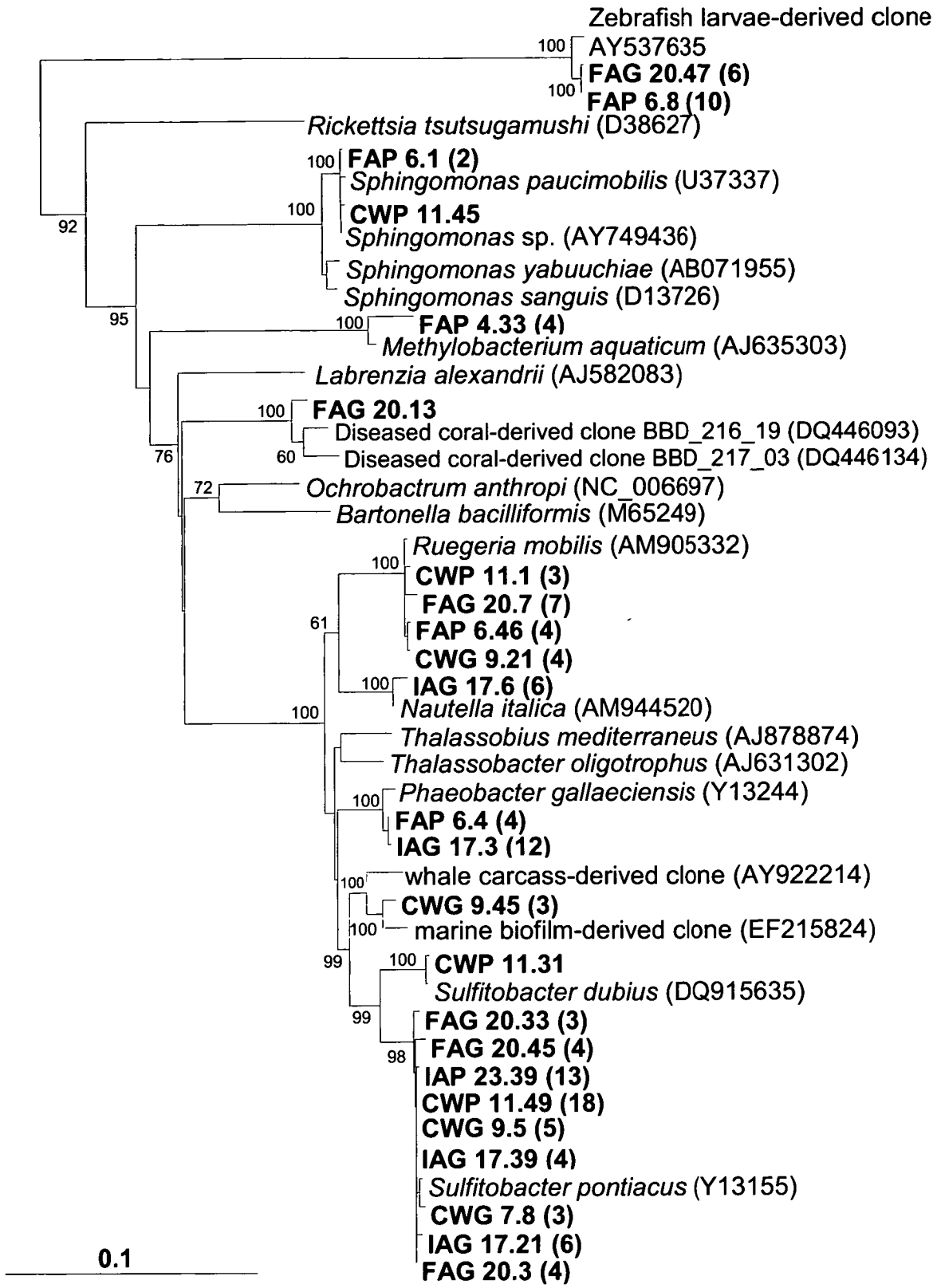

Figure 2.4: 16S rRNA gene-based phylogenetic tree showing position of phylotypes in comparison to other members of the class *Alpha-proteobacteria*. Values in parentheses indicate the number of clones found for each phylotype. Numbers at each branch point are bootstrap values. See methods section for abbreviations.

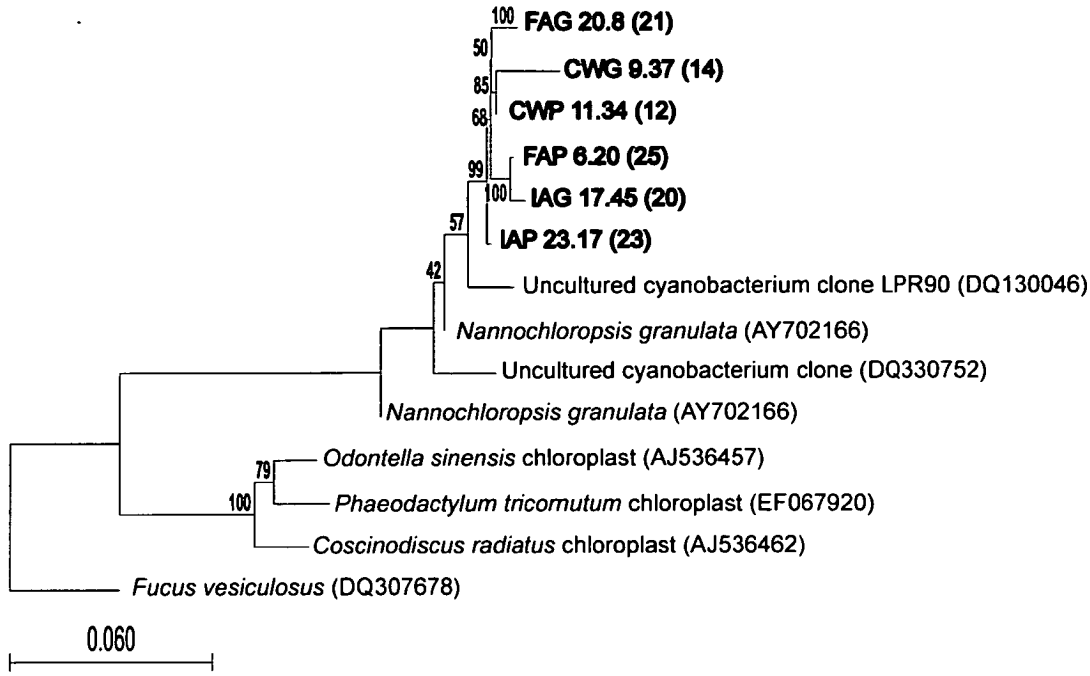

Figure 2.5: Phylogenetic tree showing position of clones in comparison to algal plastid-derived 16S rRNA gene sequences. Clones labelled as "cyanobacteria" are erroneously labelled in the GenBank database. Values in parentheses indicate the number of clones found for each phylotypes. Numbers at each branch point are bootstrap values. See methods section for abbreviations.

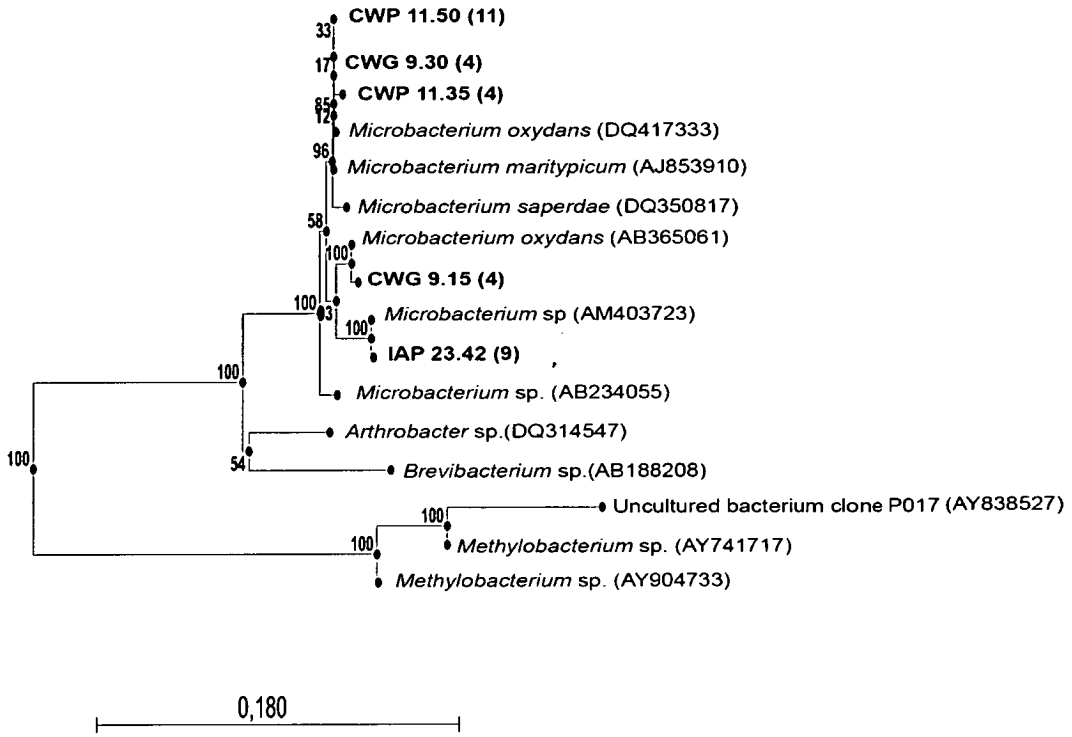

Figure 2.6: 16S rRNA gene-based phylogenetic tree showing position of clones in comparison to closely related members of the phylum *Actinobacteria*, in particular genus *Microbacterium*. Values in parentheses indicate the number of clones found for each phylotype. Numbers at each branch point are bootstrap values. See methods section for abbreviations.

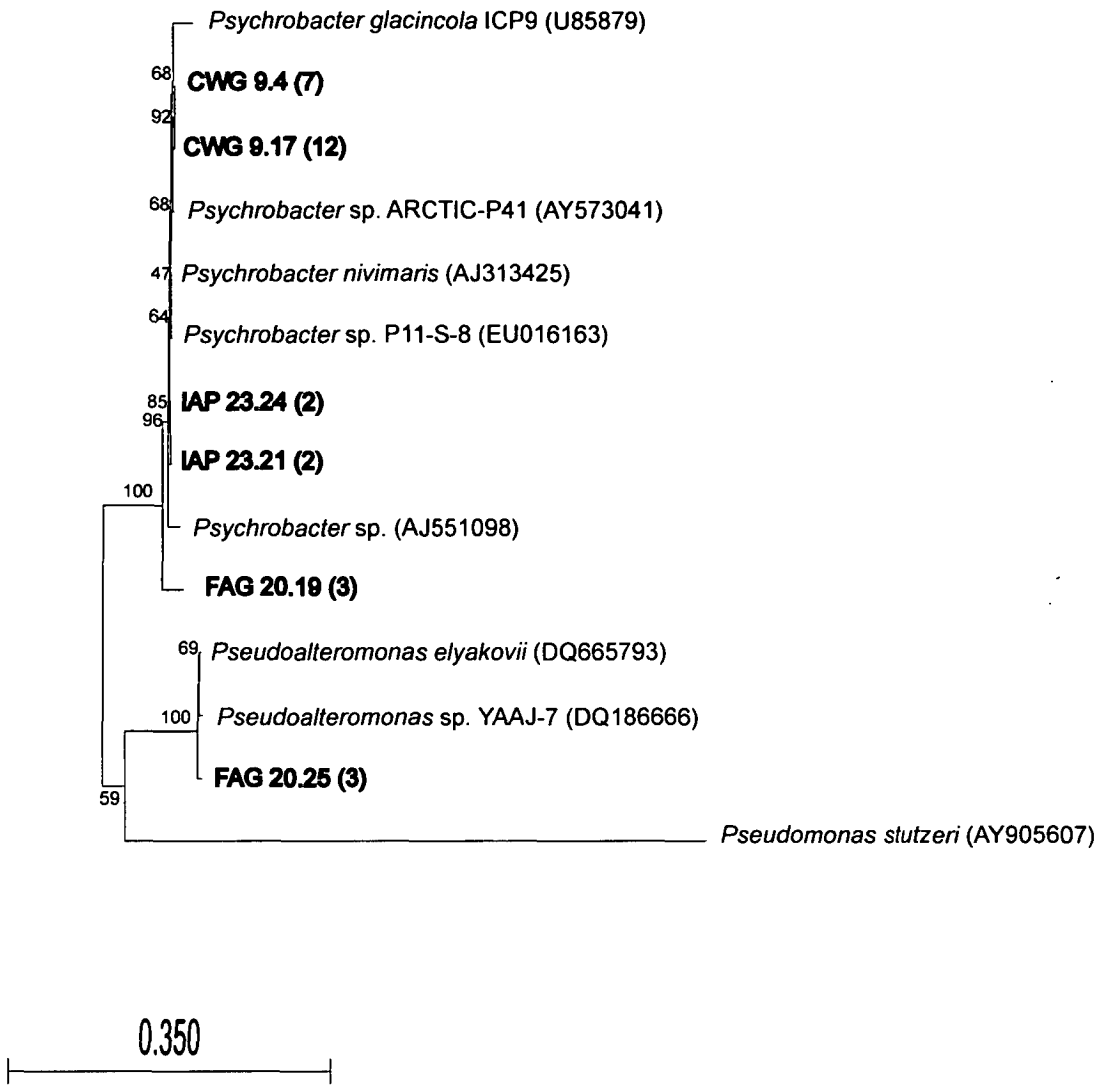

Figure 2.7: 16S rRNA gene-based phylogenetic tree showing position of clones in comparison to other members of the class *Gammaproteobacteria*. Values in parentheses indicate the number of clones found for each phylotype. Numbers at each branch point are bootstrap values. See methods section for abbreviations.

2.3.2 TRFLP results

The relationship between the composition of total bacterial communities of the six larval samples, including treatment effects and performance outcomes, with the surrounding tank water was investigated using TRFLP analysis. In order to further understand microbial communities associated with 15 day post-hatch larvae TRFs were compared to clone library sequence data that was digested *in silico* (Table 2.4).

2.3.2.1 Changes in the composition of the larval bacterial communities within Good versus Poor performing tanks.

On the basis of ANOSIM no differences in the structure of the total bacterial community of the tanks defined on the basis of performance (Table 2.1) were detected (Table 2.1, Fig. 2.8). To further evaluate the data, SIMPER analysis was performed to specifically delineate TRFs that may differ on the basis of performance (Table 2.5). It was found that for poor and good performance pooled samples, there was on average 61.7% dissimilarity. Two TRFs were found that were significant contributors to the microbial communities in both tanks while four TRFs were identified that contributed the greatest to differences on the basis of performance (Table 2.5). The source species of several of these TRFs could not be confidently identified on the basis of the clone library data digests (Table 2.4). In the poor performance sample pool TRF 568 *HinfI* (r) was likely derived from a *Nannochloropsis* plastid; TRF 257 *HaeIII* (f), observed as a significant contributor to the better performing tanks, is

also likely derived from the same source. The tanks contained a number of slightly different plastid phylotypes (Fig. 2.7), suggesting that different source *Nannochloropsis* strains may be present across the different greenwater tank treatments with TRFLP analysis results suggesting that they are not present equally between tanks.

Similarity based SIMPER analysis of the poor performance tanks revealed only a low average similarity between replicates of the CWP, IAP and FAP samples (31.4% similarity). TRF 66 *HhaI* (r) was the largest contributor in the dataset, possibly deriving from members of the *Roseobacter* clade. The next highest was TRF 570 *HinfI* (r), likely deriving from chloroplasts of ingested *Nannochloropsis* (Table 2.5). SIMPER analysis indicated slightly higher congruence between the CWG, IAG and FAG samples (42.5% similarity) with TRFs 66 *HhaI* (r), 66 *HinfI* (f) and 257 *HaeIII* (f) contributing approximately equally in these sample (10-14%; Table 2.5). As previously indicated, some of these TRFs could not be identified based on clone library data. This suggests cloned 16S rRNA genes were not retrieved from the microorganisms that are the source of these TRFs during the clone library analysis.

Table 2.4: Restriction enzyme TRFs of microorganisms detected in larval samples determined by in silico digestion[a].

Clone identification:	*HinfI* (f)	*HinfI* (r)	*HhaI* (f)	*HhaI* (r)	*HaeIII* (f)	*HaeIII* (r)
Sulfitobacter pontiacus	296	538	60	66	~	568
Sulfitobacter dubius	296	538	60	66	~	568
Uncultivated species (closest species *Thalassobius mediterraneus*)	282	538	60	66	~	483
Phaeobacter gallaeciensis	296	538	60	66	~	483
Nautella italica	296	538	60	66	~	483
Ruegeria mobilis	296	538	60	66	~	483
Uncultivated species (closest species *Bartonella bacilliformis*)	296	563	~	~	192	483
Methylobacterium aquaticum	81, 298	367	515	68	62	292
Sphingomonas paucimobilis	102	272	81	68	70	~
Uncultivated species (most similar to family *Rickettsiales*)	~	~	~	~	197	71
Microbacterium oxydans/M. marinotypicum	123, 129	79	138, 144	228	223, 229	~
Psycrobacter nivimaris/P. glacincola	118	473	~	~	254	~
Pseudoalteromonas elyakovii	324	588	366	545	~	~
Nannochloropsis plastids	199	567-569, 575	~	~	258, 260	~(489)

[a]TRFs <60 bp are not included.

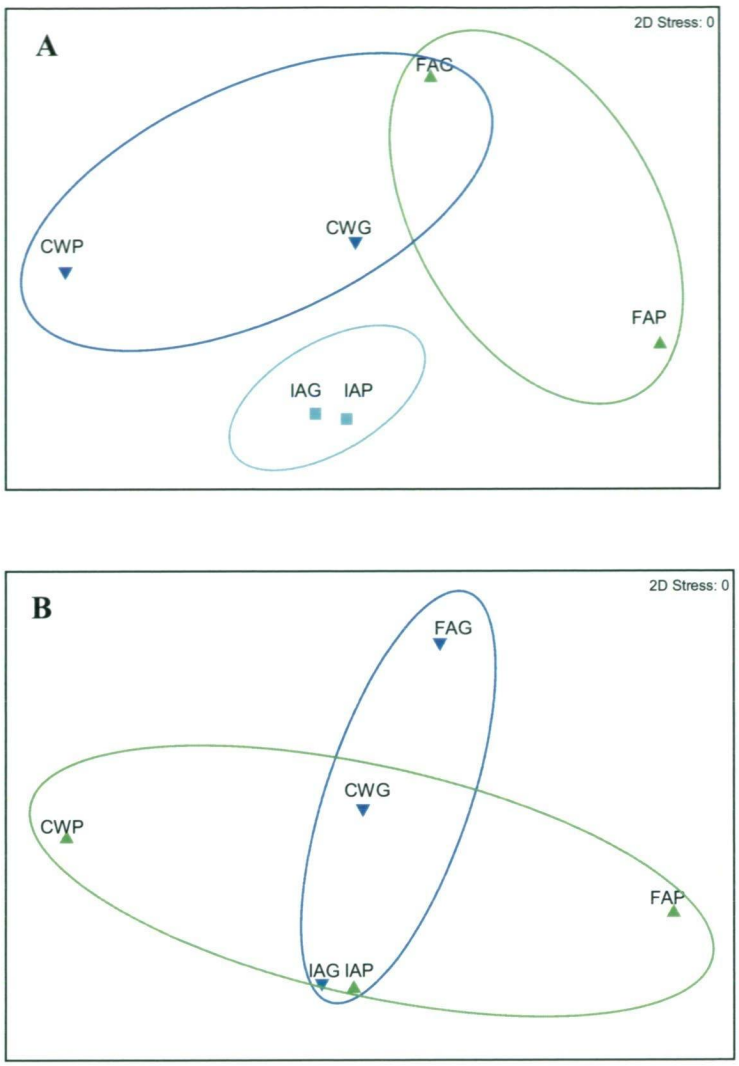

Figure 2.8: MDS plot of pairwise comparisons between 16S rRNA gene-based TRFLP profiles from larvae reared using - (A) instant algae (IA), clear water (CW) and (FA) fresh algae green water treatments. (B) Data compared on the basis of survival performance (P, poor; G, good performance).

Table 2.5: SIMPER analysis results for TRFLP profile data obtained from larvae-associated bacteria derived from tanks samples pooled on the basis of survival performance and treatment.

TRF[a]	Predicted species:[b]	Green water treatment			Larval survival Performance	
		Fresh Algae	Clear Water	Instant Algae	Poor	Good
		% contribution to % similarity:				
66 *HhaI* (r)	*Sulfitobacter/ Roseobacter* clade	23.95	33.58	35.78	28.12	34.08
66 *HaeIII* (f)	-	-	15.67	15.45	8.37	12.37
66 *HinfI* (f)	-	28.67	-	32.92	12.26	3.42
67 *HhaI* (r)	*Methylobacterium/Sphingomonas*	-	11.10	-	9.38	4.59
69 *HaeIII* (f)	*Sphingomonas*	25.49	-	-	7.59	-
76 *HaeIII* (r)	-	-	-	-	-	2.62
90 *HhaI* (f)	-	7.03	-	-	4.35	-
197 *HinfI* (f)	*Nannochloropsis* plastids	10.74	-	7.49	10.80	8.79
202 *HaeIII* (r)	-	-	-	-	-	4.06
257 *HaeIII* (f)	*Nannochloropsis* plastids	16.48	12.87	-	-	17.24
296 *HinfI* (f)	*Sulfitobacter/ Roseobacter* clade	-	-	-	-	3.73
326 *HinfI* (f)	*Pseudoalteromonas*	-	-	2.79	-	2.11
484 *HaeIII* (r)	*Sulfitobacter/ Roseobacter* clade	-	5.42	-	-	-
568 *HaeIII* (r)	*Sulfitobacter pontiacus*	-	-	6.09	6.91	2.65
570 *HinfI* (r)	*Nannochloropsis* plastids	17.29	10.68	12.27	16.33	10.49
Similarity%:		32.2	44.0	42.3	31.4	42.5

[a]TRFs indicated are the main contributing TRFs matching between replicate samples.

[b]TRF peak values can vary by ±1-2 bp due to inaccuracies in the size estimation during electrophoresis. The species or genera indicated are the closest matches based on the in silico digests indicated in Table 2.4. However, a significant caveat should be noted that the identification is at best a prediction.

2.3.2.2 Changes in the composition of the larval bacterial communities between fresh algae, instant algae and clear water treatments.

TRFLP analyses indicated that CW and FA treatments had higher similarity to each other (R = 0.25) compared to that of the IA supplied tanks (Figure 2.9, Table 2.6, R = 0.5 & 0). Similarity for the FA samples was only 32.2% while the other treatment similarities were 42-44% (Table 2.6). However variation in the microbial communities between tanks of the same treatments was high, indicating that specific relationships between microbial community differences are difficult to determine. This is suggested by the fact that only one TRF was common between all treatments – 66 *Hhal* (r). The comparatively higher similarity between the CW and FA treatments despite high inter-tank variation is suggested by three TRFs contributing 47-55% of the peak area (Table 2.6).

2.3.2.3 TRFLP analysis of tank water samples comparing treatment and performance

Water samples from the corresponding tanks were also analysed using TRFLP. Data mining of the possible TRF originators again relied on the clone library data derived from larvae samples as it was assumed that microorganisms colonising the larvae mainly derived from surrounding tank water. As previously described water samples were collected from two tanks for each treatment including one in which survival was the best and a second tank in which survival was the least.

TRFLP profile data indicated water samples for the IA treatment were very similar (63.8% similarity) but the other treatments had less similar

communities particulary the FA treatments (23.3% similarity) (Table 2.6). No differences in community structure were present for samples pooled on the basis of larval survival (R=-0.07, p=0.8). ANOSIM data indicates the FA and CW water samples based TRFLP profiles were similar overall (R=0) but that IA and CW water samples were completely different (R=1). Some overlap was observed for the FA and IA samples (R=0.25). MDS analysis (Fig. 2.9) suggests a polarisation occurs between TRFLP profiles when based purely on the basis of performance *independent* of treatment. IAP, IAG and FAP (survival 1-22.5%, Table 2.1) water sample profiles were completely separate to the profiles from water from the better performing tanks (FAG, CWP, CWG, survival >30.1%). MDS analysis also demonstrates the high divergence between FA tanks samples and homogeneity for the IA samples. This suggests that poor larval survival may not be linked to colonisation by deleterious bacteria but is rather more dependent on the nature of the microbial community present in the water.

SIMPER based analysis reveals that fundamentally the greenwater treatments lead to considerably different communities that may have as much variation between individual tank samples as it does between treatments, especially in the case of the FA tanks. A high proportion of TRFs could not be identified from the clone library data and also suggests greater diversity occurs in the water as compared to what is observed in the larval samples.

2.3.2.4 Comparison of water and larval communities

Using TRFLP analysis bacterial communities were compared between the larval samples and the surrounding water. MDS, ANOSIM and SIMPER analysis results suggests the communities are different. Overall, dissimilarity was high at 84.3% correlating with an R value of 0.91 (p<0.02) (Fig. 2.10A). Several TRFs were more abundant in larval samples including 60-*HhaI* (f), 66-*HhaI* (r), 66-*HinfI* (f), 257-*HaeIII* (f) and 570-*HinfI* (r). Most of these TRFs likely derive from *Roseobacter* clade members and *Nannochloropsis* plastids. Several water sample TRFs [e.g. 81-*HinfI* (f), 228-*HhaI* (r)], were found to be specific to water samples. By comparison pooling water and larvae-derived TRFLP data revealed no significant difference on the basis of treatment-dependent performance (R=-0.11, Fig. 2.10). In the case of individual treatments the CW and IA treatment tanks appear to form rough subsets of the overall FA community diversity.

Table 2.6: SIMPER analysis results for TRFLP profile data obtained from larvae-rearing tank water compared on the basis of survival performance.

TRF	Predicted species	Green water treatment			Treatment Performance:	
		Fresh Algae	Clear Water	Instant Algae	Poor	Good
		% contribution to % similarity:				
60 *Hhal* (f)	*Sulfitobacter/ Roseobacter* clade	-	8.39	4.81	5.84	2.96
61 *HaeIII* (f)	*Methylobacterium*	-	-	-	-	1.49
61 *HinfI* (r)	*Sulfitobacter/ Roseobacter* clade	-	10.75	-	-	-
64 *HaeIII* (f)	-	2.27	5.12	-	3.00	2.65
66 *HaeIII* (f)	-	-	7.38	-	-	-
66 *Hhal* (r)	*Sulfitobacter/ Roseobacter* clade	-	19.14	13.53	7.40	5.83
69 *HaeIII* (f)	*Sphingomonas*	5.57	-	7.82	4.00	7.47
70 *Hhal* (r)	*Methylobacterium/Sphingomonas*	-	7.88	5.28	2.67	6.10
71 *HinfI* (r)	-	-	-	-	-	4.39
74 *Hhal* (r)	-	-	3.20	-	-	2.12
76 *Hhal* (f)	-	-	4.38	-	-	2.03
76 *HaeIII* (r)	-	-	3.71	-	-	1.62
81 *HaeIII* (f)	-	6.11	-	-	-	2.54
81 *HinfI* (r)	*Methylobacterium*	-	19.21	-	13.69	4.50
90 *HaeIII* (f)	-	-	-	-	-	1.78
90 *HinfI* (f)	-	-	11.21	-	-	-
100 *HaeIII* (f)	-	-	-	-	-	1.71
100 *Hhal* (f)	-	-	-	-	-	2.04
100 *HinfI* (f)	*Sphingomonas*	13.59	-	-	-	8.56
113 *HaeIII* (r)	-	3.47	-	-	4.51	2.14
115 *HaeIII* (r)	-	-	-	-	3.27	-
183 *HaeIII* (f)	-	-	-	3.33	-	-
190 *HaeIII* (f)	-	-	-	3.94	-	2.74
197 *HinfI* (f)	*Nannochloropsis* plastids	-	-	-	5.00	-
202 *HaeIII* (r)	-	4.73	8.39	-	7.11	2.72
228 *Hhal* (r)	*Microbacterium*	-	12.68	-	3.89	10.24
241 *HinfI* (r)	-	-	-	4.62	2.08	-
267 *HinfI* (f)	-	4.95	-	-	-	4.21
270 *HinfI* (r)	-	5.54	-	-	-	-
296 *HinfI* (f)	*Sulfitobacter/ Roseobacter* clade	-	-	3.88	2.04	-
326 *HinfI* (f)	*Pseudoalteromonas*	4.90	3.29	-	4.81	-
349 *Hhal* (r)	-	-		24.12	16.40	11.93
366 *HinfI* (r)	*Methylobacterium*	-	-	7.40	1.58	-
461 *HinfI* (f)	-	-	-	-	-	1.24
484 *HaeIII* (r)	*Roseobacter* clade	-	-	23.10	-	1.36
524 *Hhal* (r)	-	-	-	10.56	-	-
563 *HinfI* (r)	Uncultivated alphaproteobacterium	2.76	-	6.11	2.42	3.48
572 *HaeIII* (r)	*Sulfitobacter*	-	-	10.02	-	-
587 *HinfI* (r)	*Pseudoalteromonas*	4.57	-	4.23	1.99	4.83
Similarity%:		23.3	33.8	63.8	23.1	31.8

[a]Tanks are compared on the basis of survival performance.

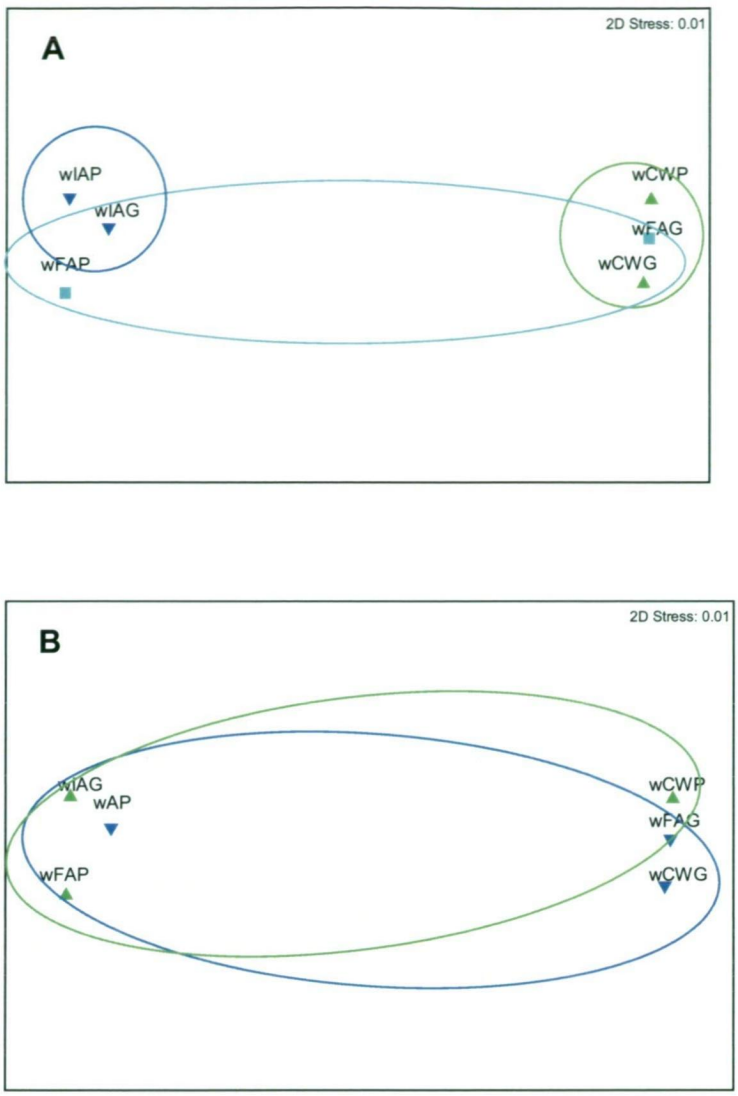

Figure 2.9: MDS plot of pair wise comparisons between 16S rRNA gene-based TRFLP profiles from water samples in which larvae are reared – (A) using instant algae (wIA), clear water (wCW) or (wFA) fresh algae treatments. (B) Data pooled in regards to survival performance (P, poor; G, good performance).

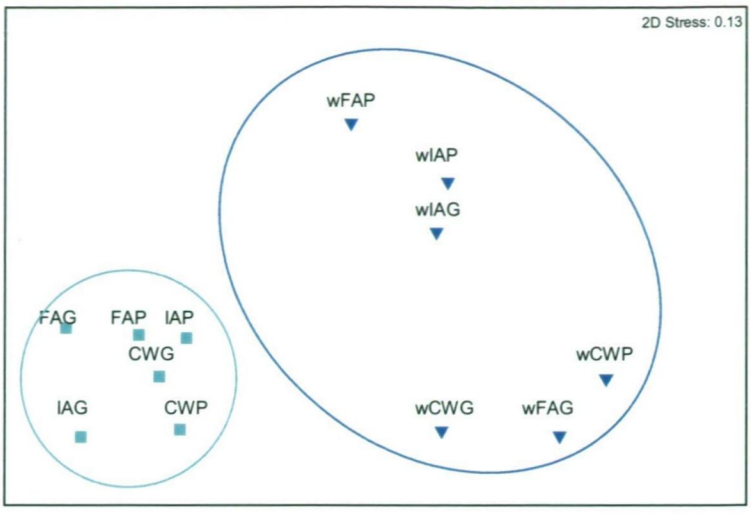

Figure 2.10: MDS plot of green water treatment samples showing a comparison of TRFLP profiles obtained from tank water and larvae samples: ■ water; ▼ larvae.

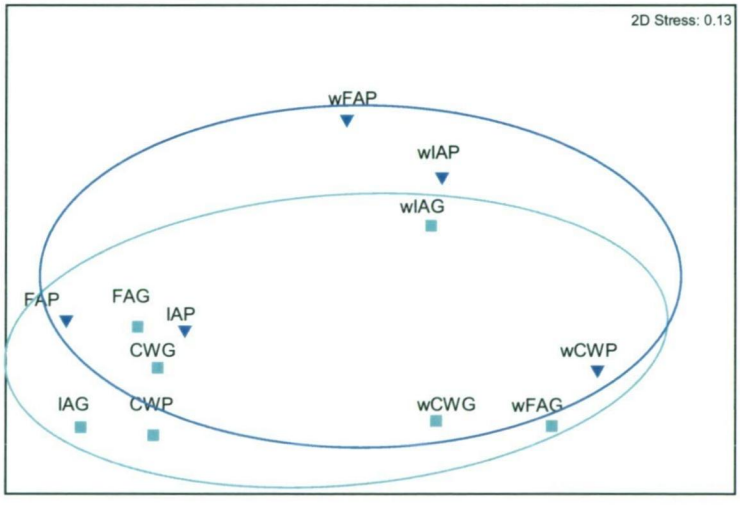

Figure 2.11: MDS plot of green water treatment samples showing a comparison of TRFLP profiles obtained from samples defined on the basis of larval survival performance. Performance: ▼ Poor; ■ Good

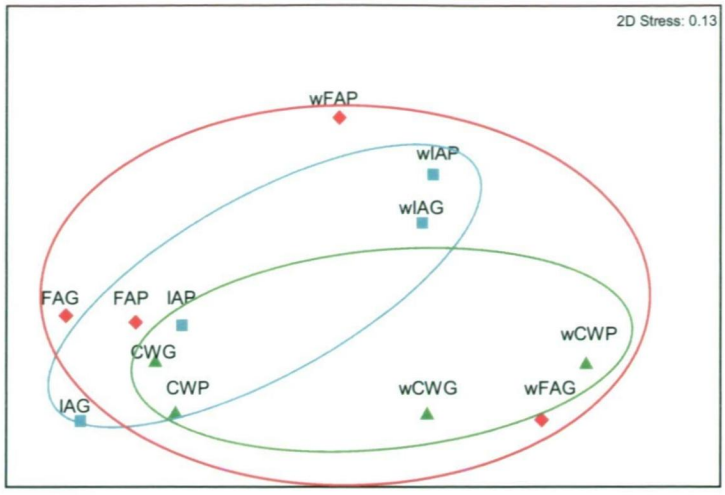

Figure 2.12: MDS plot of green water treatment samples showing a comparison of TRFLP profiles obtained from water samples and larvae samples. Treatments: ▲ Clear Water; ■ Instant algae; ◆ Fresh Algae.

2.4 Discussion

The overall results of this study showed that the microbial community of the 15 days post hatch larvae was relatively low in complexity, in comparison to other microbial environments, such as soils and marine sediment, with majority of phylotypes belonging to class *Alphaproteobacteria*. The bacterial diversity in this study is in general agreement with other studies of larvae microbiota (Jensen et al., 2004; Romero and Navarrete, 2006; Schulze et al., 2006) that show only low diversity occurs and the predominance of proteobacteria. No *Vibrio* spp. were detected; however, *Vibrio* spp. are likely to have been present since in culture based studies they have been isolated, following enrichment using a selective media (see Chapter 3). However, in the *Vibrio* populations are likely too low to be detected in the randomized clone library survey performed here. It has been found that it can be difficult to generate good quality genomic DNA preparations from a number of *Vibrio* spp. (Wong and Kuo, 2006), however this could be due to interfering components derived during extraction of relatively large amounts of biomass.

This low complexity in diversity observed may be a result of the shortcomings of PCR based studies, as reported by Wintzingerode et al. (1997). Other groups may have been missed through selection of the primers used to construct the libraries, such that only the predominant species in a microbial community were detected through the use of clone library construction and the use of TRFLP (Vlasov et al., 1998; Smith et

al., 2007). It has also been suggested that bacteria already present in hatchery environments may influence the composition of subsequent larvae-associated microbiota (Cahill, 1990; Ringo and Olsen, 1999; McIntosh et al., 2008).

Differences in communities suggested by the clone library may have been caused by the different treatments that changed the microbial community structures. However, as the time frame for colonisation is relatively short, the colonisation process could be stochastic in its early phases. This is suggested by the presence of several similar phylotypes that occurred sporadically from sample to sample. This is similar to what is observed during juvenile development of animals and humans, in which heterogeneous communities tend to occur in neonates but with maturation the communities converge towards homogeneity (Palmer et al. 2007). These different colonisation outcomes may have produced an effect that was deleterious to larvae survival; however, based on TRFLP data, no differences in performance in the larvae-associate communities could be observed, either dependent on or independent of the green water rearing approach. In the case of the tank water, survival outcomes could potentially be related to tank water microbial communities (Fig 2.9). This suggests that the deleterious effects on larval survival could be due to microorganisms occurring in the water but not necessarily active in larval fish colonisation.

The predominant species in the larvae-associated microbial community belong to the *Roseobacter* clade (Table 2.2). This is also apparent in the TRFLP data, which shows that the major taxonomic group present was comprised of *Roseobacter* clade members (Table 2.5). The only detected gammaproteobacteria belonged to the genera *Psychrobacter* and *Pseudoalteromonas*, both of which are commonly found in marine samples and are not noted for pathogenicity to fish (Hjelm et al., 2004; Bowman, 2006). It is possible that another potentially antagonistic species may become more prevalent in the absence of typical pathogens like *Vibrio*.

Members of the genus *Microbacterium* have been isolated from marine samples but have never been shown previously to cause problems related to fish health, although they have been found on rare occasions to be associated with animal infections (Funke et al., 1997). It is remotely possible that particular conditions provided in the samples examined in this study allowed for *Microbacterium* strains to become sufficiently abundant to have deleterious effects on the larvae.

As no *Vibrio* spp. were detected, they cannot immediately be painted as an indicator of poor health as has been done in other studies. When comparing data from other studies, it is sometimes difficult to compare culture-based studies to molecular-based studies. Culture-based studies select and enrich for certain bacteria, for example with TCBS agar, which selects for *Vibrio* spp. and has additives (bile salts) that prevent most

other species from growing. This may explain why no *Vibrio* spp. were detected in this study and why they were detected in previous culture-based studies (Battaglene et al., 2006). Battaglene et al. (2006) used selective TCBS to study microbiota associated with striped trumpeter larval cultures, but did not use other media to culture other heterotrophic bacteria. Although it is thought that *Vibrio* spp. are an indicator of poor tank hygiene (Villamil et al., 2003), some *Vibrio* strains have probiotic properties, including, for example, *V. alginolyticus* (Ringo and Vadstein, 1998; Ottesen and Olafsen, 2000; Huys et al., 2001; Makridis et al., 2001).

No concrete reason can be given for why one tank of a treatment performed better than another, but it may be proposed that the conditions that they provided do influence the bacterial community. There is a suggestion that the differences that occur are possibly induced by the initial availability of nutrients and what subsequently occurs during the feeding process (Romero and Navarrete, 2006).

The change in the community when different conditions occur is seen also in the water samples. A more diverse community is present in the seawater while larvae harbour a more restrictive range of microbiota (Jensen et al., 2004). This can perhaps be explained by the bacteria being part of either autochthonous (adherent) or allochthonous (transient) communities. Investigation of the intestinal bacterial community of Atlantic salmon found that when fish were fed two varying diets, the population

changed in regards to autochthonous and allochthonous communities (Ringo and Olsen, 1999; Schulze et al., 2006). As larvae get older, their microbiota also changes, as seen by Jensen et al. (2004), who reported that feeding Atlantic halibut larvae had more complex DGGE profiles than non-feeding larvae. Hansen and Olafsen (1989) proposed that bacteria in the surrounding water mass are involved in the initial colonization of larval fish and dominate subsequent communities in adult fish. The TRFLP results from the current investigation also support this idea, as the dominant species found in the larvae were also detected in the water.

This investigation found that the water sample TRFs, compared to those of the larvae, were different (R=1, Fig 2.10) but compositionally analogous; that is, certain members of communities co-occurred in both locations but in different proportions. A similar observation was also reported by Smith et al. (2007) who observed that the flora of the outer mucus layer of whiting (*Merlangius merlangus*) is more diverse than that of its mouth and gut. Smith et al. (2007) also saw found that the bacteria associated with whiting had limited similarity to the bacterial community of the surrounding water when analysed using culture-independent approaches, including 16S rRNA gene-based clone libraries and TRFLP. These findings support what has been observed in this investigation, because although larvae and surrounding tank water did share some of the same TRFs, they were in different proportions and some were not present at all in the larvae. This may be explained by the diet of the fish

and that certain bacteria are better suited to colonise the larval gut than others.

Conclusions:

This investigation provided an insight into the bacterial diversity of striped trumpeter using 16S rRNA bacterial clone libraries and TRFLP. It showed that under different green water culture conditions the bacterial diversity appears relatively low. Definite conclusions cannot be drawn about the influences of the bacterial community of the larvae from the information obtained, although the surrounding water seems to have a potential influence on larval survival performance outcomes.

From this initial knowledge of the bacterial diversity of the striped trumpeter and the changes that different rearing conditions bring upon it, further investigations can now be conducted by screening isolated bacteria from these larvae cultures for their probiotic capabilities. Assessment of ways of altering the bacterial communities of live feeds, including *Artemia* and rotifers, and devising methods to track probionts within changing bacterial communities will also be explored.

Chapter 3: Assessment and tracking of bacterial probionts within a striped trumpeter larvae rearing system

3.0 Abstract

Potential probiotic candidates were identified by using antimicrobial *in vitro* plate testing against known pathogenic *Vibrio* species, with six out of 25 isolates tested selected for further testing. In *Artemia* challenge trials, it was determined that *Pseudoalteromonas agarivorans* ST18 and *Aliivibrio fischeri* ST7 had the least effect on *Artemia* survival. To further assess the probiotic capability of strains ST18 and ST7, rotifer and *Artemia* cultures were challenged with pathogenic strain *V. proteolyticus* V760 mixed with strains ST18 or ST7. Strain ST18 was found to have a probiotic effect in that cultures containing both V760 and ST18 were not significantly different from the control system but produced significantly better survival compared to the pathogen-only treatments. To further investigate ST18 and ST7 in a mixed cultured system terminal restriction fragment length polymorphism (TRFLP) analysis was applied to monitor the change in bacterial community. Through tracking probiont strain specific terminal restriction fragments (TRF) the probionts could be distinguished within the microbial community associated with rotifers and appeared to be readily taken up by rotifers. However, in *Artemia* experiments, uptake of the probionts appeared to be less successful.

3.1 Introduction

In Chapter One a review of the literature indicates bacterial probionts may have protective and/or beneficial effects in aquaculture processes. It was also discussed that since rotifers and *Artemia* are common live feeds, they could act as vectors for *Vibrio* spp., many of which are pathogenic (Lopez-Torres and Lizarraga-Partida, 2001). With this in mind, research has been undertaken to explore ways of manipulating the bacterial community associated with rotifers and *Artemia.* The manipulation is first designed to reduce harmful *Vibrio* spp. populations. Once successful the live feeds are fed to the larvae with the knowledge that they contain reduced amounts of harmful bacteria. Also once reduced it is also possible to introduce beneficial bacteria to the live feeds, by adding them during the enrichment stages. By doing this after the live feeds have been reduced in bacterial populations it increases the probability of the beneficial bacteria being incorporated into the live feed (Douillet, 2000).

The aim of Chapter Three was to identify potential probiotic bacteria using previously obtained isolates. This was done by assessing their ability to produce antimicrobial compounds that inhibited growth of known pathogens. These potential probionts were then inoculated into bacteria-reduced rotifer and *Artemia* cultures and survival was assessed. This was achieved by:

1) Assessment of the probiotic properties of 22 bacterial isolates obtained from striped trumpeter against five known fish-pathogenic *Vibrio* strains by employing *in vitro* antimicrobial plate tests.

2) Assessment of four strains of bacteria isolated from striped trumpeter larvae that were shown to have some level of antimicrobial activity against at least one of the five known fish-pathogenic *Vibrio* strains by:

i) Assessing the four candidates for probiotic capacity in rotifer cultures when challenged with a pathogen.

iii) Evaluating probiotic capacity in cultures when challenged with a pathogen in *Artemia* cultures.

iii) Terminal restriction fragments (TRFs) were determined for each potential probiont so that it could be specifically identified when in a mixed community. This allowed an assessment of probiont uptake by the rotifer or *Artemia.*

3.2 Materials and Methods

3.2.1 Bacterial Isolates

A total of 22 bacterial isolates obtained by enrichment from striped trumpeter cultures (Table 3.1) (see Chapter 2, Section 2.2.1) were tested against 5 pathogenic *Vibrio* strains (Table 3.2) that were obtained from Dr Jeremy Carson of the Fish Health Unit of the Department of Primary Industries, Water and the Environment, Launceston, Tasmania. The bacterial isolates obtained from striped trumpeter larvae were sampled from three days post hatch to 15 days post hatch, at two day intervals. The larvae were washed in sterilized sea water (three washes to remove external bacteria) and homogenized, and plated on marine agar media and thiosulfate citrate bile salts sucrose agar (TCBS; Difco) after serial dilutions. After incubation at 25°C for 48 h isolated colonies were

randomly selected and purified on ZoBell's marine agar (Oxoid) plates. All strains were phenotypically identified with the MicroSys ® V48 kit for the identification of *Vibrio* spp. (Carson et al., 2001) and the data matched with the probabilistic identification software for Windows (PibWin) (Bryant, 2004). The five pathogenic strains tested were isolated from culture tanks in which rotifers, *Artemia* or larval fish or rock lobsters had been cultivated at the Marine Resarch Laboratories, Taroona, Tasmania, and undergone mass mortalities (J. Carson personal communication). The culture methods are as described for striped trumpeter samples.

3.2.2 *In vitro* antimicrobial activity assay

Antimicrobial activity was assessed against five known fish-pathogenic bacteria (Table 3.2) using the cross-streak assay desribed by Lemos et al. (1985). The assay was performed by heavily inoculating each isolate onto one third of a marine agar plate and incubating at 25˚C for 7 days. This was done by swabbing and covering one third of the agar with enriched isolates grown for 48 h at 25˚C grown in marine broth. Pathogens were then inoculated as a single streak at right angles to the isolate growth and the plates re-incubated for a further 48 h. Extensive zones of inhibition of a known pathogen were scored as "++", narrow zones of inhibition were scored as "+" and no inhibition was scored as "-" .

3.2.3 Bacterial production

Bacterial strains were grown in marine broth, which was made in the same manner as marine agar, but with the omission of the agar; 250 ml

Schott bottles were filled with 100 ml of marine broth and autoclaved. The broths were loop inoculated with bacteria that had been previously sub-cultured on marine agar for 24 h and then incubated at 25°C for 48 h.

Table 3.1: Species identification of potential probiotic bacteria used in the *in vitro* antimicrobial activity test, all isolates were obtained from striped trumpeter larvae.

Strain no.	MicroSys ID
ST1	*Vibrio alginolyticus*
ST2	*Vibrio alginolyticus*
ST3	*Vibrio anguillarum*
ST4	*Vibrio anguillarum*
ST5	*Vibrio chagasii*
ST7	*Vibrio fischeri I*
ST8	*Vibrio fischeri II*
ST9	*Vibrio ichthyoenteri II*
ST11	*Vibrio penaeicida*
ST12	*Vibrio splendidus I*
ST13	*Vibrio splendidus I*
ST14	Phenon 36
ST15	Phenon 36
ST16	Type 1
ST17	Type 2
ST18	Type 3a
ST19	Type 3b
ST20	Type 5
V4	Phenon 29
V8	Phenon 29
V17	Phenon 59
V52	Phenon 59

Table 3.2: Pathogenic *Vibrio* species used in the *in vitro* antimicrobial activity tests. All were isolated from water or animals that had been identified to have had a bacterial issue that resulted in mortalities.

Strain no.	MicroSys ID	Host species or source
V34	*Vibrio alginolyticus*	Seawater
V568	*Vibrio anguillarum*	Rotifers
V760	*Vibrio proteolyticus*	*Artemia*
V886	*Vibrio alginolyticus*	Rock lobster phyllosoma
V890	*Vibrio harveyi*	Rock lobster phyllosoma

The densities of the cultures were calculated by taking a subsample of the broth and counting populations using a haemocytometer. For the haemocytometer counts the following formula was used:

Total cells counted x (25 x 10^4) x no. of triple ruled squares counted = Number of cells/ml x the dilution factor (if applied).

3.2.4 Rotifer production

Rotifers (*Brachionus plicatilis*) were harvested daily from semi-continuous stock cultures raised on a diet of the microalgae *Nannochloropsis* sp. (Battaglene et al., 2006). Harvested rotifers were rinsed in ozonated seawater and then transferred to 500 ml Schott experimental glass vessels for 12 h enrichment, at 400 rotifers ml^{-1} and 23°C. Rotifers were enriched with AlgaMac (Aquafauna Biomarine, Hawthorne, California, USA) at 0.2 g per million rotifers. AlgaMac was blended with seawater and otherwise added according to the manufacturer's instructions. Aeration and oxygen were provided to maintain dissolved oxygen above 4 mg l^{-1}. Probionts were added to the corresponding rotifer enrichment vessel at the same time as the AlgaMac was added.

3.2.5 *Artemia* production

Decapsulated *Artemia* cysts (E.G. Artemia Systems, INVE, Belgium) were hatched in a 100 l conical tank at 28°C in sea water with vigorous aeration and 24 h light. After 24 h hatched nauplii were rinsed for 5 min with seawater after passing through a 180µm screen filter to remove the hatched and unhatched cysts and then through a 60 µm screen filter to catch the *Artemia* nauplii, which were then placed into a 1 l beaker to a final approximate density of 50 *Artemia* ml^{-1}. The *Artemia* bacterial load was reduced using Sanocare Hatch control (INVE) at a rate of 0.1 ml l^{-1}. Prior to stocking Artemia were also washed in ozonated sea water for 5 min.

3.2.6 DNA and extraction and purification

DNA was extracted from bacterial cells using the same methods set out in Chapter Two Section 2.2.2

3.2.7 PCR amplification of 16S rRNA genes

PCR amplification of the 16S rRNA gene was performed using the Hotstart Taq kit (Qiagen) and universal bacterial primers 10f (5'-GAG TTT GAT CCT GGC TCA G-3') and 907 (5'-CCG TCA ATT CCT TTG AGT TT-3'). Each reaction was a 25 µl reaction mix with 12.5 µl of HotStart mastermix, 1 µl of the forward primer, 1 µl of the reverse primer, and approximately 10 ng of DNA template. A final volume of 25 µl was adjusted with sterile milliQ water. The following thermal cycling program

was used: initial denaturing at 94°C for 15 min, 34 cycles of denaturing for 1 min, annealing at 55°C for 1 min, extension for 3 min: final extension at 72°C for 10 min. The reaction was purified using the Qiagen PCR cleanup kit.

3.2.8 DNA Sequencing and TRFLP analysis

Both DNA sequencing and TRFLP analysis was performed as described in Chapter Two Sections 2.2.6 and 2.2.7.

3.3 Probiotic activity assessment of V52, ST18, V8, ST14, ST7

3.3.1 Experimental design

The experimental vessels were 500ml Schott bottles with aeration inlets and outlets. Each treatment had three replicates. The air inlet had a sterile, 0.2 µm pore size hydrophilic filter in place to stop any airborne contaminants entering and the outlet had sterile 0.2 µm hydrophobic filters to stop bacteria escaping from the cultures. Fig.3.1 provides a schematic of the set up. Oxygen was provided using an oxygen concentrator (Millennium M5, Respironics, Pennsylvania, USA). At the point at which the glass tube entered the lid of the bottle a piece of rubber tube was fitted to make an airtight fit. The cultures were placed into a climate-controlled room set at a 12:12 light:dark cycle and a temperature of 25°C ± 1°C.

To 400 ml of ozonated sea water a total population of 2×10^5 rotifers (500 ml^{-1}) or 2×10^4 *Artemia nauplii* (50 ml^{-1}) was added. Rotifers and

Artemia were enriched in the same process as that used in the TAFI hatchery to mirror the process in a working hatchery, by adding the required amount of AlgaMac as outlined above. Bacterial isolates were inoculated to achieve a final concentration of 5×10^5 cells ml^{-1}, to assess if they have any detrimental effects on the rotifer population. This was determined by plate counts performed on the broth cultures. The broths were homogenized and the calculated amount of broth required to achive the final concentration was taken aseptically and transfered into sterile vials. All experimental units were treated the same with all receiving the same amount of broth by adding uncultured sterilized marine broth to those which needed more and to the controls. As a pathogenic control *Vibrio proteolyticus* was used as it is a known marine pathogen (Vadstein et al., 2004). The cultures were enriched for 14 h after which survival was assessed and samples taken for further analysis. Survival was assessed by taking triplicate 1 ml subsamples and counting them under a dissection microscope. If the rotifers or *Artemia* moved freely and were active, they were considered to have survived. If they were moribund, they were classified as effectively deceased. Samples were taken for TRFLP analysis by separating the rotifers and *Artemia* from the culture water using a 62 µm screen, and washing three times in sterilized water to remove external bacteria resulting in a 500 µl sample. All samples were kept on ice until frozen within 2 h.

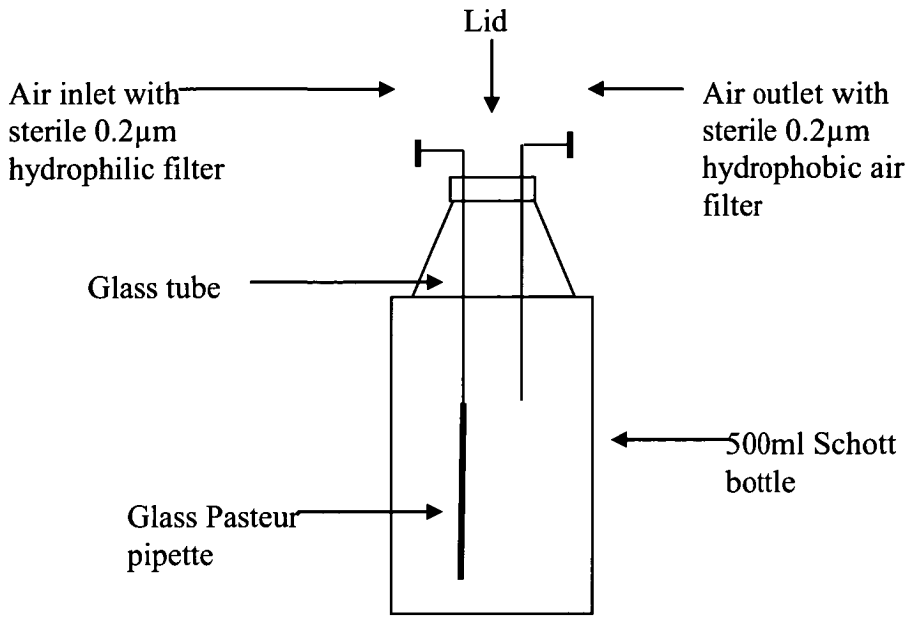

Figure 3.1: Experimental Schott bottle vessel set-up used for survival assessment Experiments.

3.3.2 Assessment of potential probiotic bacteria V52, ST18, V8, ST14, ST7.

Assessment of potential probiotics V52, ST18, V8, ST14, ST7 and effects on rotifer and *Artemia* cultures during the enrichment process was performed through a series of experiments described below. In all experiments survival of the rotifers was assessed after 14 h.

3.3.2.1 Screening of V8, V52 and ST18 on rotifer survival during lipid enrichment (Experiment 1).

The aim of the experiments was to determine if potential probionts had any effect on rotifer survival when added to achieve a final concentration of 5×10^5 cells ml^{-1}. Six different treatments were assessed: 1) a control that had no bacteria added; 2 and 3) pathogen-only containing controls had a known pathogen added, including *V. proteolyticus* V-760, *V. alginolyticus* V-34; 4 to 6) treatments that included isolates V8, V52, and ST18.

3.3.2.2 Screening of ST7, ST14 and ST18 on rotifer survival during lipid enrichment (Experiment 2).

Experiment 2 repeated the first experiment but tested five different treatments. They included: 1) a control that had no bacteria added; 2) a pathogen-containing control with *V. proteolyticus* V-760 added; and 3-5) the probiont-containing treatments including strains ST14, ST18, and ST7 added to achieve a final concentration of 5×10^5 cells/ml.

3.3.2.3 Survival of rotifers when challenged with a known pathogen V-760 and combinations of potential probionts (Experiments 3 and 4)

The aim of the experiments was to determine if potential probionts could reduce the pathogenicity of a known pathogen when added to rotifer cultures. There were six different treatments: 1) a control that had no bacteria added; 2) a pathogen control that *V. proteolyticus* V-760 added, and 3-6) the test probionts (ST7, ST18) were added singularly or as mixtures with the pathogen (ST7 +V-760, and ST18 + V-760). Strain V-760 was added to achieve a final concentration of 5×10^4 cells ml^{-1} (Experiment 3) or 5×10^5 cells ml^{-1} (Experiment 4). The test probionts were added at 5×10^4 cells ml^{-1}.

3.3.2.4 Survival of *Artemia* when challenged with a known pathogen V-760 and combinations of potential probionts (Experiments 5 and 6).

The aim of the experiment was to determine that potential probionts had on *Artemia* cultures when the known pathogen V-760 and the test probionts are added at the same time in order to assess whether the probionts can reduce the effect of the pathogen. The experiment consisted of 1) a control that had no bacteria added; 2) a pathogen-containing control in which *V. proteolyticus* V-760 was added, 3-4) treatments in which the test probionts (strains ST7, ST18) were added singularly; 5-6) and added with strain V760 as done in rotifer Experiments 3 and 4. Experiments 5 and 6 were except involved different initial bacterial loading (to achieve a final concentration of 5×10^5 cells ml^{-1} for Experiment 5 and 5×10^4 cells ml^{-1} for Experiment 6).

3.3.3 TRFLP analyses

TRFLP was analyzed as outlined in Chapter Two, section 2.2.7. In addition TRFLP analysis was performed on selected strains ST7, ST14, ST18, V52, V34, V760, V8, (Tables 3.1 and 3.2) so that their TRFs could be determined experimentally (not just *in silico*), thus enabling them to be potentially identified within a mixed population sample. PCR pseudo-TRFs can be formed with the left over primer bases and thus give false peak readings. Which is why it is advised to perform virtual digestion of corresponding sequence data (i.e. clone library-derived sequenes). It is also important to design the primers being used correctly, to optimise primer levels in the TRFLP PCR thermocyling process, and to take account of PCR run-to-run variations (Schutte et al., 2008) thus minimising pseudo-TRF artifacts. By using SIMPER analysis (Primer v.6) an estimation of the average contribution to the total profile peak area of the probiont-derived TRFs within the sample was obtained.

3.3.4 Statistical analyses

Statistical analysis was performed by one-way analysis of variance (ANOVA). For all tests a significance level of $P<0.05$ was adopted. Tukey's post hoc test was used to compare means. Homogeneity of variance was evaluated using residual plots. The statistical package SPSS V.10 was used.

3.4 Results

3.4.1 *In vitro* antimicrobial plate results

Using *in vitro* antimicrobial plate tests it was found that five of the 22 species had some antimicrobial activity against the test pathogens (Table 3.3). Strains ST7, ST18, V8 and V52 exhibited the most inhibition. All of the five strains were found to inhibit *V. alginolyticus* (V-34) with ST18 also inhibiting *V. proteolyticus* (V760). Strain ST18 was seen to have the most inhibition across the test pathogens having an effect on all but *V. harveyi* (Table 3.3). A typical *in vitro* antimicrobial plate is shown in Fig. 3.2.

Table 3.3: Results of the *in vitro* antimicrobial plate test.
++ indicates strong inhibition + indicates slight inhibition and – indicates no inhibition seen on test pathogen growth.

	Test Pathogens:				
Bacterial Isolate	*V. algino-lyticus* (V34)	*V. anguillarum* (V568)	*V. proteo-lyticus* (V760)	*V. algino-Lyticus* (V886)	*V. harveyi* (V890)
ST1	-	-	-	-	-
ST2	-	-	-	-	-
ST3	-	-	-	-	-
ST4	-	-	-	-	-
ST5	-	-	-	-	-
ST7	++	-	+	-	-
ST8	-	-	-	-	-
ST9	-	-	-	-	-
ST11	-	-	-	-	-
ST12	-	-	-	-	-
ST13	-	-	-	-	-
ST14	+	-	-	-	-
ST15	-	-	-	-	-
ST16	-	-	-	-	-
ST17	-	-	-	-	-
ST18	++	+	++	+	-
ST19	-	-	-	-	-
ST20	-	-	-	-	-
V4	-	-	-	-	-
V8	++	-	+	-	-
V17	-	-	-	-	-
V52	++	-	-	-	-

Figure 3.2: Photograph of an *in vitro* antimicrobial plate showing inhibition of *Vibrio* test pathogens by putative probiont isolate ST18. A maximum inhibition score of "++" was recorded against streaks of strains numbered 4 and 2. Strain streaks 1 to 5, are *V. anguillarum* (V568), *V. alginolyticus* (V34), *V. harveyi* (V890), *V. proteolyticus* (V760) and *V. alginolyticus* (V886), respectively.

Seventeen isolates had no detectable activity against any of the *Vibrio* test pathogens (Table 3.3). Of the pathogens examined *V. alginolyticus* (V34) showed a tendency to be more readily inhibited.

3.4.2 Identification of putative probiont isolates

Putative probiont isolates not identifiable with the MicroSys system (strains ST14, ST15, ST16, ST17, ST18, ST19, ST20, V4, V8, V17, and V52; Table 3.2) were identified using 16S rRNA gene sequencing. The following strains were grouped as glucose non-fermenters: ST16, ST17, ST18, ST19 and ST20. Strains V4, V8, V17, and V52 were identified as *Vibrio* spp. by MicroSys but these could not be given a species name as the data obtained could not be matched with any known members of the family *Vibrionaceae.*

Table 3.4 shows the 16S rRNA gene sequencing results determined by GenBank BLAST matches (http:/www.ncbi.nlm.nih.gov/Blast (Altschul et al., 1997). Strain ST7 is likely a strain of *Aliivibrio fischeri* (Urbanczyk et al. 2007). ST14 was most likely a strain of *Vibrio penaeicida* (99% similarity to the type strain LMG 19663^T). Strain ST18 was likely a strain of *Pseudoalteromonas agarivorans* (>99% similarity to type strain KMM 255^T). Strain V8 was found to group in the *V. splendidus/V. tasmaniensis/V. lentus* complex of strains (99% similarity to *Vibrio* sp. V004 isolated from *Latris lineata*; Gudkovs et al, unpublished).

Table 3.4 Comparison of MicroSys system identifications to 16S rRNA gene sequence data results. The five isolates with potential as probionts are highlighted in bold.

Strain no.	MicroSys ID	IDS	Host	Closest 16S rRNA gene sequence match (GenBank accession number):	% similarity
ST1	*V alginolyticus*	0.99906	Striped trumpeter	*Vibrio alginolyticus* strain UQM 2770 (AY264938)	99
ST2	*V alginolyticus*	0.99888	Striped trumpeter	*Vibrio alginolyticus* strain SR1 (DQ269211)	98
ST3	*V anguillarum*	0.99999	Striped trumpeter	*Vibrio anguillarum* (EF467287)	99
ST4	*V anguillarum*	0.99984	Striped trumpeter	*Vibrio anguillarum* strain MHK11 (EF091706)	99
ST5	*V chagasii*	0.99703	Striped trumpeter	*Vibrio pomeroyi* (AJ491290)	99
ST7	***V fischeri I***	**0.99967**	**Striped trumpeter**	***Allivibrio fischeri* strain VFISC2 (AY780014)**	**99**
ST8	*V fischeri II*	1.00000	Striped trumpeter	*Aliivibrio fischeri* strain 082205 (DQ174503)	99
ST9	*V ichthyoenteri II*	1.00000	Striped trumpeter	*Vibrio ichthyoenteri* (AJ437192)	99
ST11	*V penaeicida*	1.00000	Striped trumpeter	*Enterovibrio norvegicus* (AJ437193)	97
ST12	*V splendidus I*	0.99715	Striped trumpeter	*Vibrio tasmaniensis* strain 562 (AY620964)	99

ST13	*V splendidus I*	0.99964	Striped trumpeter	*Vibrio splendidus* (AJ874367)	99
ST14	**Phenon 36**	**0.99927**	**Striped trumpeter**	***Vibrio penaeicida* (AJ437191)**	**99**
ST15	Phenon 36	0.99992	Striped trumpeter	*Enterovibrio norvegicus* (AJ437193)	99
ST16	Type 1	0	Striped trumpeter	*Marinomonas aquimarina* (AJ843079)	99
ST17	Type 2	0	Striped trumpeter	*Marinomonas aquimarina* (AJ843079)	99
ST18	**Type 3a**	**0**	**Striped trumpeter**	***Pseudoalteromonas* sp. P11-B-12 (EU016154)**	**99**
ST19	Type 3b	0	Striped trumpeter	*Pseudoalteromonas agarivorans* (AB049728)	99
ST20	Type 5	0	Striped trumpeter	*Enterovibrio norvegicus* (AJ437193)	99
V4	Phenon 29	0.99995	Striped trumpeter	*Vibrio splendidus* (AJ874367)	98
V8	**Phenon 29**	**0.99995**	**Striped trumpeter**	***Vibrio* sp. (DQ146970) (*V. splendidus* strain complex)**	**99**
V17	Phenon 59	1.00000	Striped trumpeter	*Aliivibrio fischeri* (DQ090767)	97
V52	**Phenon 59**	**1.00000**	**Striped trumpeter**	***Aliivibrio fischeri* (DQ090767)**	**97**
V34	*V. alginolyticus*	0.99994	Seawater	*Vibrio alginolyticus* (DQ269211)	98

V568	*V. anguillarum*	1.00000	Rotifers	*Vibrio anguillarum* strain MHK11 (EF091706)	99
V760	*V. proteolyticus*	1.00000	Artemia	*Vibrio proteolyticus* (AF513463)	98
V886	*V. alginolyticus*	0.99787	Rock lobster phyllosoma	*Vibrio alginolyticus* strain SR1 (DQ269211)	97
V890	*V. harveyi*	1.00000	Rock lobster phyllosoma	*Vibrio harveyi* (AY967728)	99

3.4.3 Determination of the probiotic capabilities of possible candidates on two live feeds - Rotifer and *Artemia* challenge experiments.

Results from Experiment 1 and 2 indicated that the controls with no bacteria added survived significantly better (75±2%) ($F=19.713$, $Df=5,12$ $P<0.001$) than other treatments (Figure 3.3 and 3.4). Addition of strain ST18 lead to significantly better survival (63 ± 2%) than the other isolates (Fig. 3.3). Experiment 2, adding ST14 significantly reduced survival (43 ± 4%) ($F= 156.288$, $Df=5,12$ $P<0.001$) .

Experiments 3 and 4 confirmed that adding ST18 had no significant impact on rotifer survival (Figs 3.5 and 3.6). Results from Experiment 3 where lower inoculums of bacteria were added indicated that adding both ST18 and ST7 together provided significantly better survival than ST7 added alone but was not significantly different from the control in which probiotics were not added ($F= 32.198$, $Df =4,10$ $P<0.001$) (Fig.3.5) while adding ST18 or ST7 to cultures challenged with V-760 significantly improved survival compared to the negative controls in both experiments ($F=33.854$ $Df=5,12$ $P=<0.001$), $F=42.11$ $Df=5,12$ $P=<0.001$) (Figs 3.5 and 3.6).

Experiments 5 and 6 confirmed that the addition of ST18 and ST7 to *Artemia* showed no significant difference to the control ($F=63.129$ $Df=5,12$ $P<0.001$). When added with V-760 it improved survival ($F=42.612$ $Df=5,12$ $P<0.001$) compared to treatments that received only strain V-760 (Figs 3.7 and 3.8).

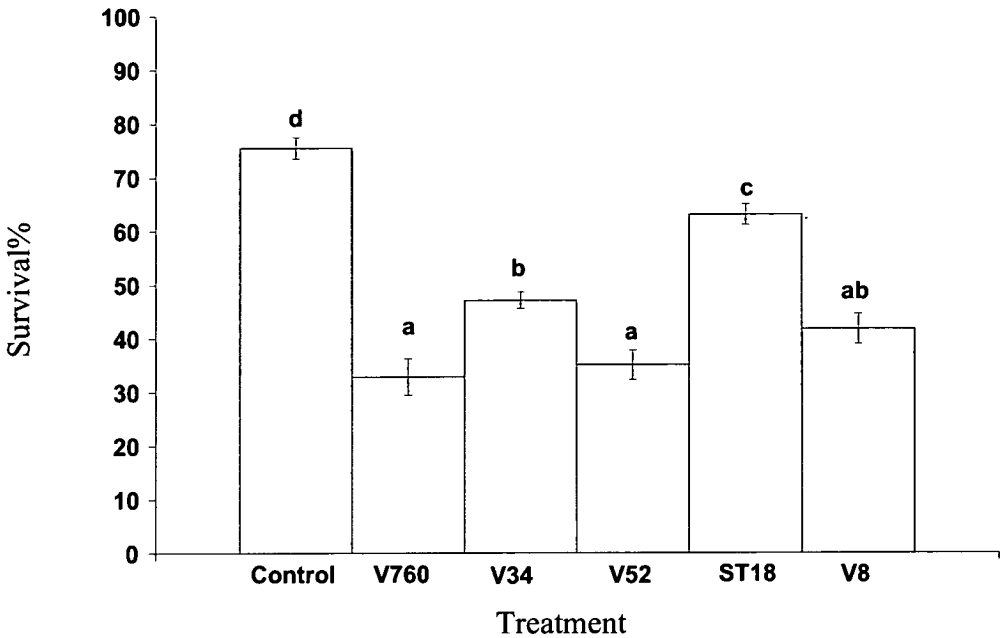

Figure 3.3: Mean survival (± standard deviation) of rotifers when challenged with *V. proteolyticus* V760, V. alginolyticus V34, and isolates V52, ST18 and V8, over a 14 h enrichment period. Bacterial cell concentrations used were 5×10^5 cells ml^{-1}. Columns sharing the same letter are not significantly different ($P < 0.05$).

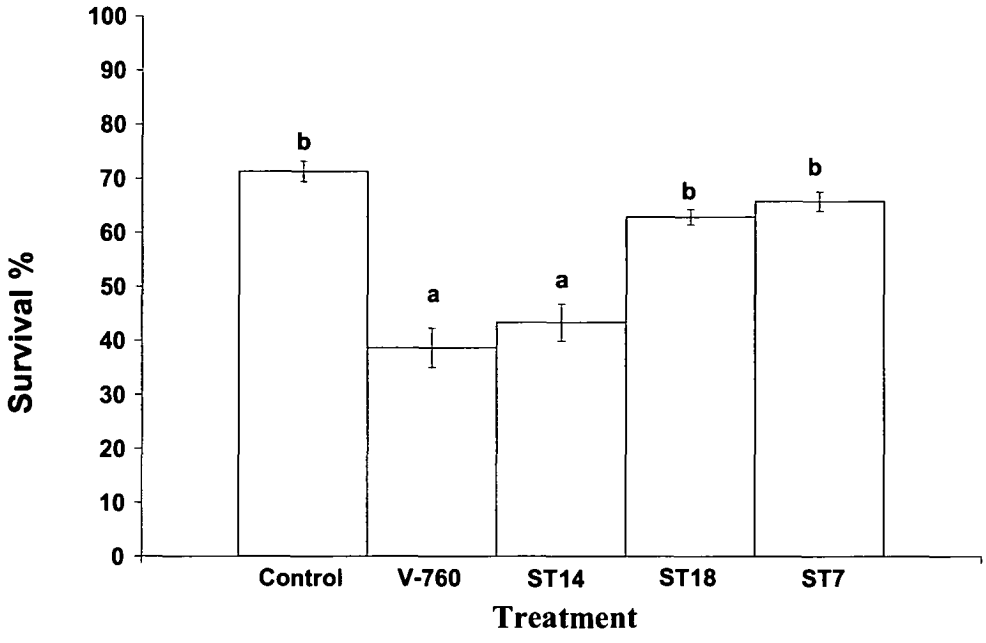

Figure 3.4: Mean survival (± standard deviation) of rotifers when challenged with *V. proteolyticus* V-760, ST14, ST18, and ST7, over a 14 h enrichment period. Bacterial cell concentrations used were 5×10^5 cells ml^{-1}. Columns sharing the same letter are not significantly different ($P < 0.05$) in mortality between each treatment.

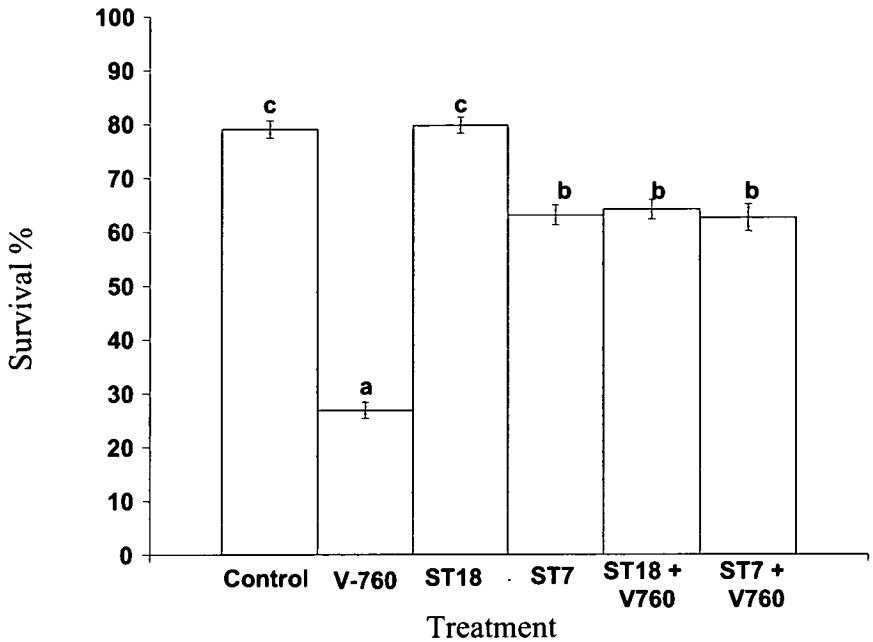

Figure 3.5: Mean survival (± standard deviation) of rotifers when challenged with *V. proteolyticus* V-760, ST18, ST7 over a 14 h enrichment period. Bacterial cell concentrations used were 5×10^4 cells ml^{-1}. ST18 + V-760 and ST7 + V-760 had both the bacteria isolates added at the same time at a concentration of 5×10^4 cells ml^{-1} for each isolate. Columns sharing the same letter are not significantly different ($P < 0.05$) in survival among treatments.

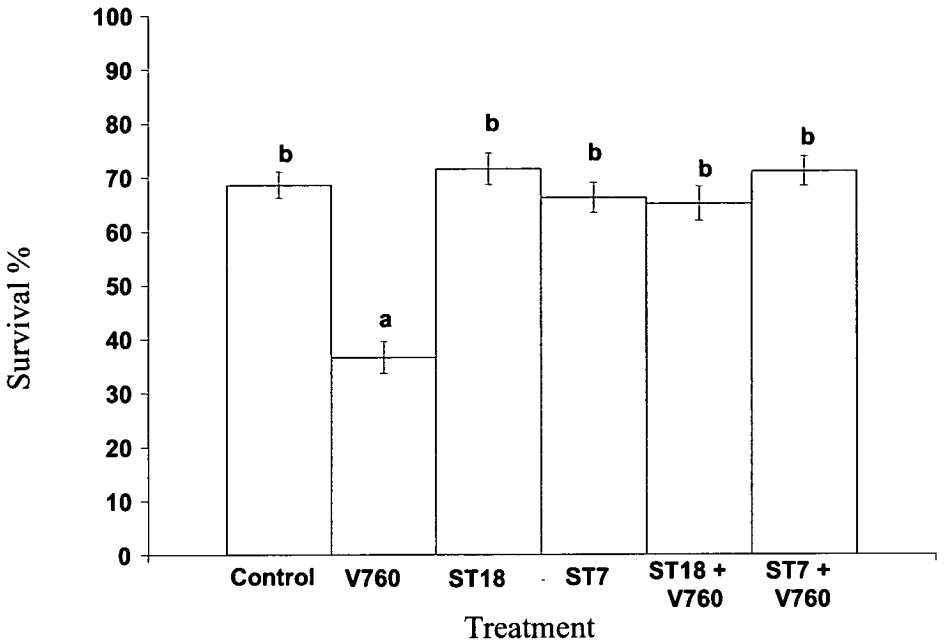

Figure 3.6: Mean survival (± standard deviation) of rotifers when challenged with *V. proteolyticus* V-760, ST18, ST7 over a 14 h enrichment period. Bacterial cell concentrations used were 5×10^5 cells ml^{-1}. ST8 + V760 and ST7 + V760, had both the bacterial isolates added at the same time at a concentration of 5×10^5 cells ml^{-1} for each isolate. Columns sharing the same letter are not significantly different ($P < 0.05$) in mortality between each treatment.

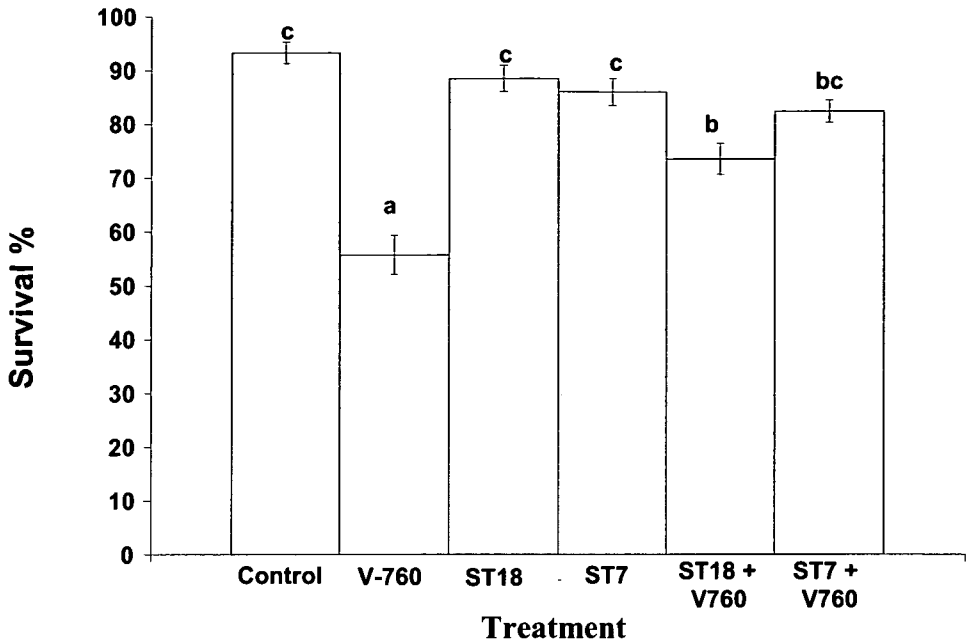

Figure 3.7: Mean survival (± standard deviation) of *Artemia* when challenged with *V. proteolyticus* V-760, ST18, ST7 over a 14 h enrichment period. All initial bacterial cell concentrations used were 5×10^5 cells ml^{-1}. Columns sharing the same letter are not significantly different ($P < 0.05$).

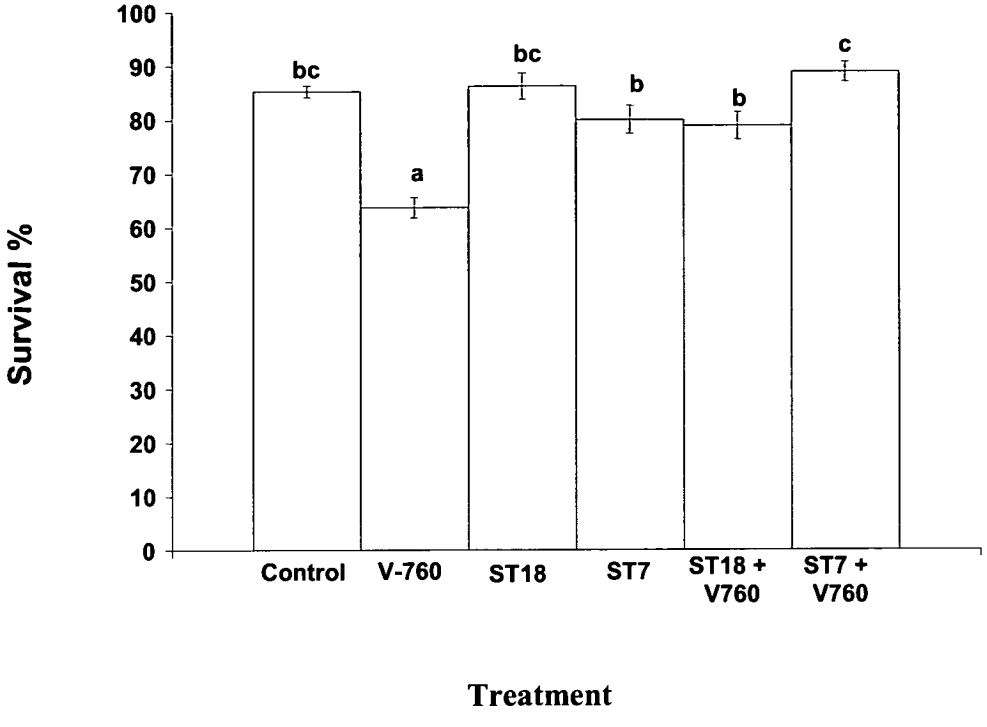

Figure 3.8: Mean survival ± SE of *Artemia* when challenged with *V. proteolyticus* V-760, ST18, ST7 over a 14 h enrichment period. All initial bacterial cell concentrations used were 5×10^4 cells ml^{-1}. Different letters indicate significant difference ($P < 0.05$) in mortality between each treatment for individual days (n=3).

Table 3.5: TRFs representing putative probiont strains utilising restriction endonucleases enzymes *HaeIII*, *HinfI* and *HhaI* determined using TRFLP

	HaeIII	
Strain:	**Forward fragments:**	**Reverse Fragments:**
ST14	-	190
ST18	64	508
ST7	84	-
V52	84	-
V34	78, 84	190
V760	78, 84	190
V8	78, 84	-
	HinfI	
Strain:	**Forward fragments:**	**Reverse Fragments:**
ST14	71, 172, 338	270
ST18	104	270
ST7	268	270,
V52	328	270, 588
V34	72, 338	270, 587
V760	72, 338	270, 587
V8	-	270
	HhaI	
Strain:	**Forward fragments:**	**Reverse Fragments:**
ST14	179, 226,	350, 549
ST18	60	366
ST7	68	177
V52	179, 226,	349
V34	475	349, 579
V760	179, 226,	579, 475
V8	178	349

3.4.4 TRFLP analysis and TRF tracking results

The MDS plot derived from the TRFLP data for probiotic assessment Experiments 1 and 2 (Figs 3.9 and 3.10) shows that all samples have some overlap with the control bacterial community in which putative pathogens or probionts were not added. The addition of strains V760, V34, V52, V8, and ST14 resulted in TRFLP profiles not significantly different to the control sample ($p>0.7$, Figs 3.9 and 3.10). ANOSIM values, however indicated the treatment in which strain ST18 is added alone to rotifer cultures (at 5×10^5 cells/ml) was most consistently different to the control as well as the other treatments ($R = 0.185$ to 0.667, significance $p<0.3$, and only 28% similar to the control profile). Using SIMPER analysis it was possible to track ST18 and detect it through the observation of distinctive TRFs (Table 3.5) amongst the other TRFs present. Strains V760, V34 and V52 could not be readily tracked (Table 3.6) as their TRFs were not distinct from each other and thus TRF information could not be used for confident identification with any of these strains. The same identification problem was also observed in the other Experiments using these isolates. It was found that with addition of a bacterial isolate to the rotifers, the overall similarity between the replicates increased. The controls with no addition of bacteria were only 27% similar to each other, while the addition of V760, V52, ST18, V8 and V34 increased the similarity between the replicates (53, 43, 42, 44.2 and 25% respectively). Table 3.6 indicates which TRF distinct to ST18 that they were detected and the percent contribution they represent within the overall profile. It is possible other related members of *Pseudoalteromonas* or *Aliivibrio* may be

present in the samples, however ST18 or ST7 distinct TRFs were not detected in the controls suggesting the populations are below the sensitivity level of the TRFLP method to detect. When strains ST18 and ST7 were added at higher numbers (5×10^5 cell ml^{-1}, Experiment 2) TRFLP profiles obtained were significantly different ($R > 0.78$) and were only 18-21% similar to the control. ST18 and ST7 could also be more readily identified through the observation of distinct TRFs, which showed increased contributions to the total peak area (Table 3.7).

The same trend in similarity between treatments increasing with addition of isolates ST18 was also seen in Experiment 3 with the control profiles being 42% similar. This similarity increased when other isolates were added to about 46-57%. When added in combination with V760 fewer differences were observed in the profiles than when they were added independently (Fig. 3.11, Table 3.8). When a lower population of V760 was added a more marked difference was apparent ($R=0.30$, $P<0.1$). The effect of adding an order of magnitude more bacterial cells, as examined in Experiment 4, made no specific difference to profiles. However ST7 and ST18 additions created distinct TRFLP profiles while sole V760 additions did not differ from controls (Fig. 3.12, Table 3.9). It was observed that the control and V760 were 53% similar, while ST18 and ST18+V760 were 63% similar; by comparison ST7 and ST+V760 were 48% similar, resulting in clustering of these groups (Fig. 3.12). TRF tracking data for ST18 and ST7 were in the same general range, though TRF abundances were

approximately halved when they are added with strain V760 (Tables 3.8 and 3.9) which is possibly indicative of competition between strains for uptake into rotifers.

In Experiments 5 and 6 the addition of V760, ST7 and ST18 to *Artemia* had a different response to what was observed for rotifers. In general the strain differences were not largely different to the controls with SIMPER similarities mostly ≥ 50%. This may be due to a higher level of bacteria in the *Artemia* samples at the beginning of the experiments. TRFLP patterns of the treatment in which V760 was added at 5×10^4 was not different from the control (R=0.07, P=50%, Fig. 11; 73.6% similar). However, when added at higher populations a greater difference was observed (R=0.26, P<0.1). In the case of sole additions of strains ST18, ST7 or V706, differences to the control were not significant (Tables 3.10, 3.11) with similarity between 50-60%. The TRFLP profiles were significantly different to the profile of the treatment in which ST18 was added with V706 suggesting together the strains more significantly influence the overall community. Large variances were observed between replicates, especially for the additions of ST7 in combination with V706, suggesting ST7 populations may have fluctuated widely between treatments and replicates making separation of differences difficult. ST18 and ST7 unique TRFs were detected using SIMPER (Tables 3.10, 3.11), but only occurred in small numbers, which could be due to the higher abundances of other bacterial species present.

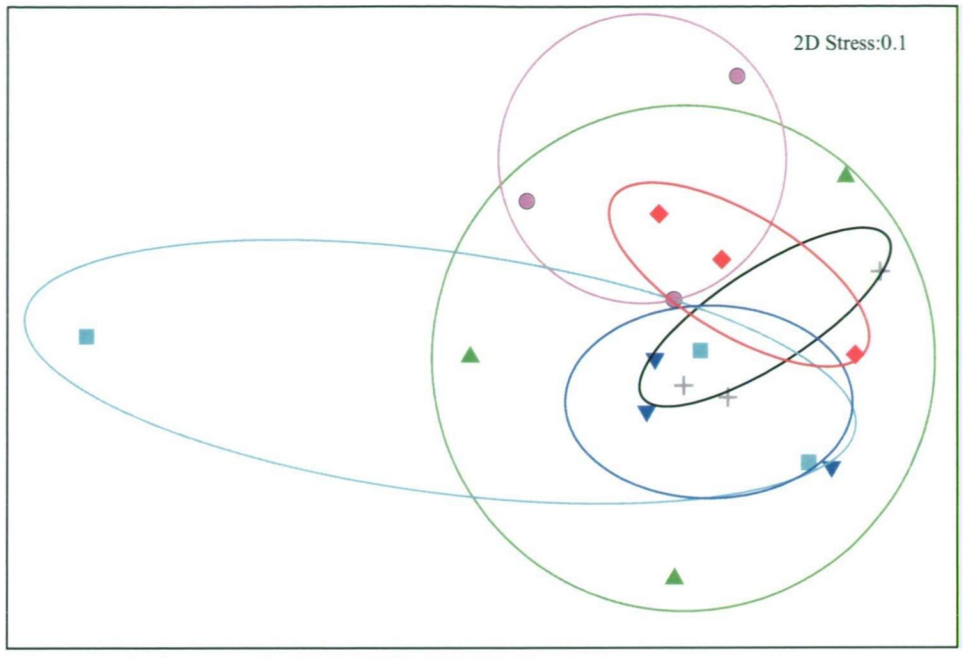

Figure 3.9: MDS plot of TRFLP profiles of bacterial communities associated with rotifers challenged with different bacterial strains added at 5×10^5 cells ml^{-1} and incubated over a 14 h period (Experiment 1): (1) ▲ Control, no bacteria added; (2) ▼ *V. proteolyticus* V760; (3) ■ V. *alginolyticus* V-34; (4) ◆ strain V52; (5) ● strain ST18; and (6) + strain V8.

Table 3.6: Summarized SIMPER output utilised for tracking of probiont strain ST18 through detection of distinguishing TRFs for ST18 within the rotifer challenge treatment experiment (as shown above in Fig. 3.9).

Treatment		SIMPER Similarity (%):	Distinguishing TRFs (% contribution to % similarity):					
			ST7	ST18:				
			177 *Hhal*(r)	60 *Hhal*(f)	64 *HaeIII*(f)	104 *HinfI*(f)	366 Hhal(r)	508 HaeIII(r)
1	Control	26.66	-	-	-	-	-	-
2	V760	53.46	-	-	-	-	-	-
3	V34	24.57	-	-	-	-	-	-
4	V52	42.95	-	-	-	-	-	-
5	ST18	42.04	-	8.53	5.17	4.50	-	5.23
6	V8	44.20	-	-	-	-	-	-

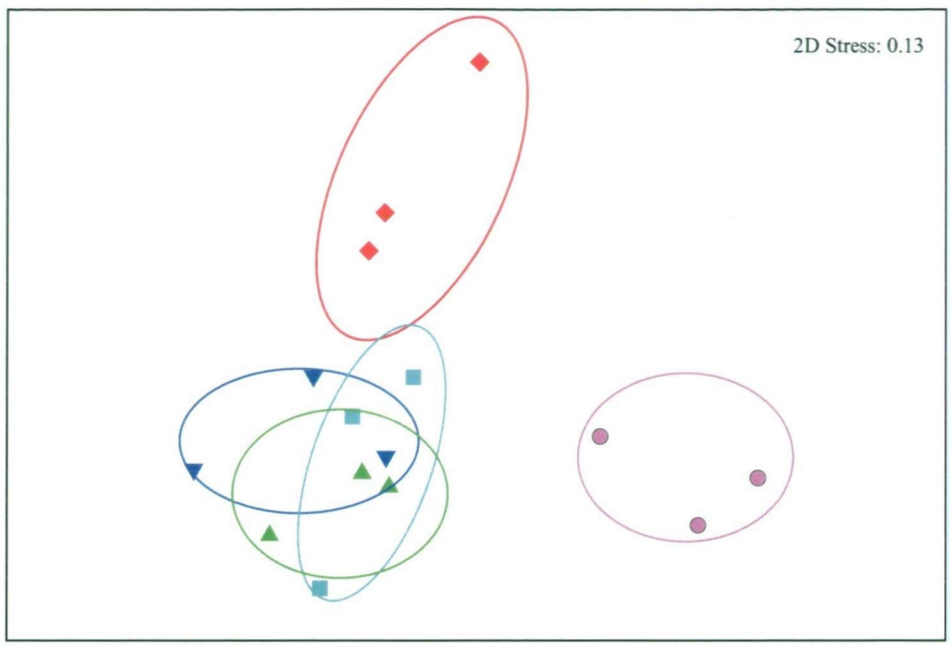

Figure 3.10: MDS plot of TRFLP profiles of bacterial communities associated with rotifers challenged with different bacterial strains added at 5×10^5 cells ml^{-1} and incubated over a 14 h period (Experiment 2): (1) ▲ Control, no bacteria added; (2) ▼ *V. proteolyticus* V760; (3) ■ strain ST14; (4) ◆ strain ST18; (5) ● strain ST7.

Table 3.7: Summarized SIMPER output utilised for tracking of probiont strain ST18 through detection of distinguishing TRFs for strains ST7 and ST18 within the rotifer challenge treatment experiment (as shown above in Fig. 3.10).

Treatment		**SIMPER Similarity (%):**	**Distinguishing TRFs (% contribution to % similarity):**					
			ST7	**ST18:**				
			177 *Hhal*(r)	**60 *Hhal*(f)**	**64 *HaeIII*(f)**	**104 *Hinfl*(f)**	**366 Hhal(r)**	**508 HaeIII(r)**
1	Control	41.98	-	-	-	-	-	-
2	V760	45.15	-	-	-	-	-	-
3	ST14	46.77	-	-	-	-	-	-
4	ST18	55.00	-	12.76	-	-	15.87	15.87
5	ST7	57.29	20.07	-	-	-	-	-

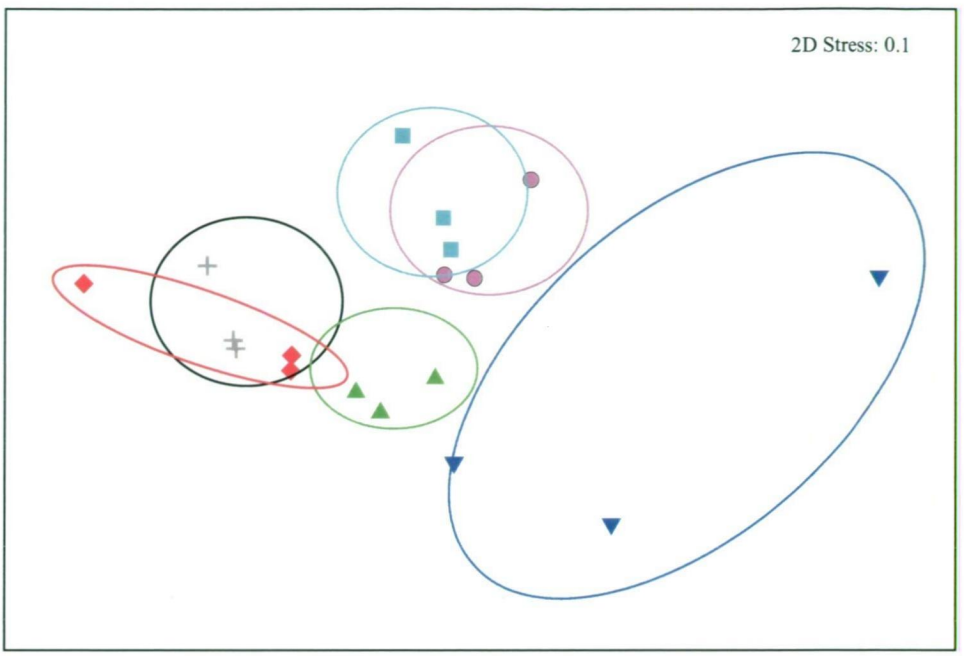

Figure 3.11: MDS plot of TRFLP profiles of bacterial communities associated with rotifers challenged with different bacterial strains added at 5×10^5 cells ml^{-1} and incubated over a 14 h period (Experiment 3): (1) ▲ Control, no bacteria added; (2) ▼ *V. proteolyticus* V760; (3) ■ strain ST18; (4) ◆ strain ST7; (5) ● strains ST18 + V760; (6) + ST7 + V760.

Table 3.8: Summarized SIMPER output data utilised for tracking of probiont strains through detection of distinguishing TRFs for strains ST7 and ST18 within the rotifer challenge treatment experiment (as shown above in Fig. 3.11).

Treatment		**SIMPER Similarity (%):**	**Distinguishing TRFs (% contribution to % similarity):**					
			ST7:	**ST18:**				
			177 *Hhal*(r)	**60 *Hhal*(f)**	**64 *HaeIII*(f)**	**104 *HinfI*(f)**	**366 HhaI(r)**	**508 HaeIII(r)**
1	Control	46.05	-	-	-	-	-	-
2	V760	39.52	-	-	-	-	-	-
3	ST18	50.52	-	23.23	14.13	-	10.90	22.02
4	ST7	56.40	16.77	-	-	-	-	-
5	ST18 + V760	42.19	-	11.90	11.48	-	7.67	12.96
6	ST7 + V760	51.56	9.93	-	-	-	-	-

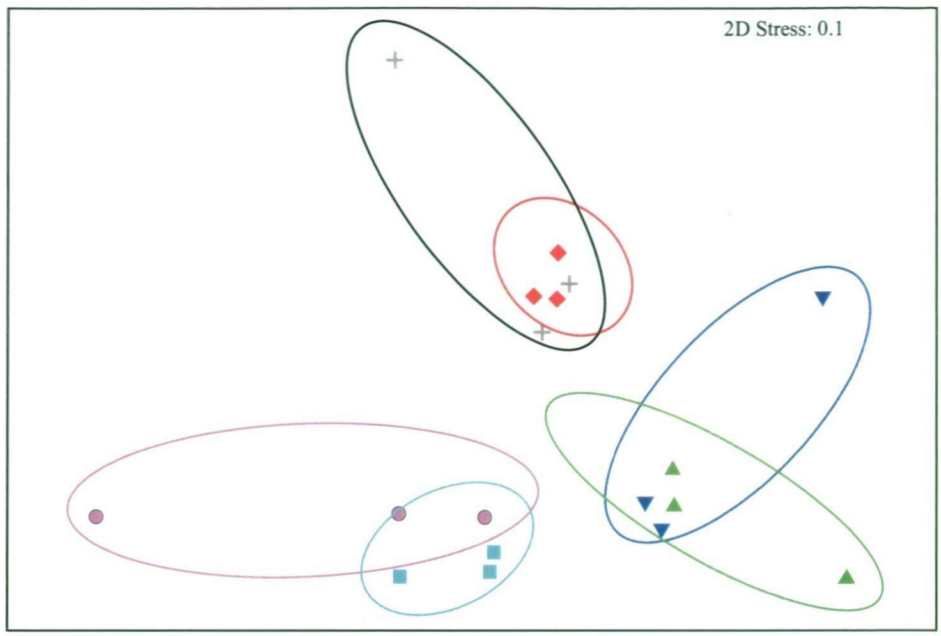

Figure 3.12: MDS plot of TRFLP profiles of bacterial communities associated with rotifers challenged with different bacterial strains added at 5 × 10^4 cells ml^{-1} and incubated over a 14 h period (Experimentt 4): (1) ▲ Control, no bacteria added; (2) ▼ *V. proteolyticus* V760; (3) ■ strain ST18; (4) ◆ strain ST7; (5) ● strains ST18 + V760; (6) + ST7 + V760.

Table 3.9: Summarized SIMPER output data utilised for tracking of probiont strains through detection of distinguishing TRFs for strains ST7 and ST18 within the rotifer challenge treatment experiment (as shown above in Fig. 3.12).

Treatment		**SIMPER Similarity (%):**	**Distinguishing TRFs (% contribution to % similarity):**					
			ST7:	**ST18:**				
			177 *Hhal*(r)	**60 *Hhal*(f)**	**64 *HaeIII*(f)**	**104 *HinfI*(f)**	**366 HhaI(r)**	**508 HaeIII(r)**
1	Control	60.04	-	-	-	-	-	-
2	V760	44.23	-	-	-	-	-	-
3	ST18	56.54	-	13.17	-	19.94	-	5.29
4	ST7	67.04	15.97	-	-	-	-	-
5	ST18 + V760	43.15	-	13.98	-	19.50	-	13.77
6	ST7 + V760	55.57	13.23	-	-	-	-	-

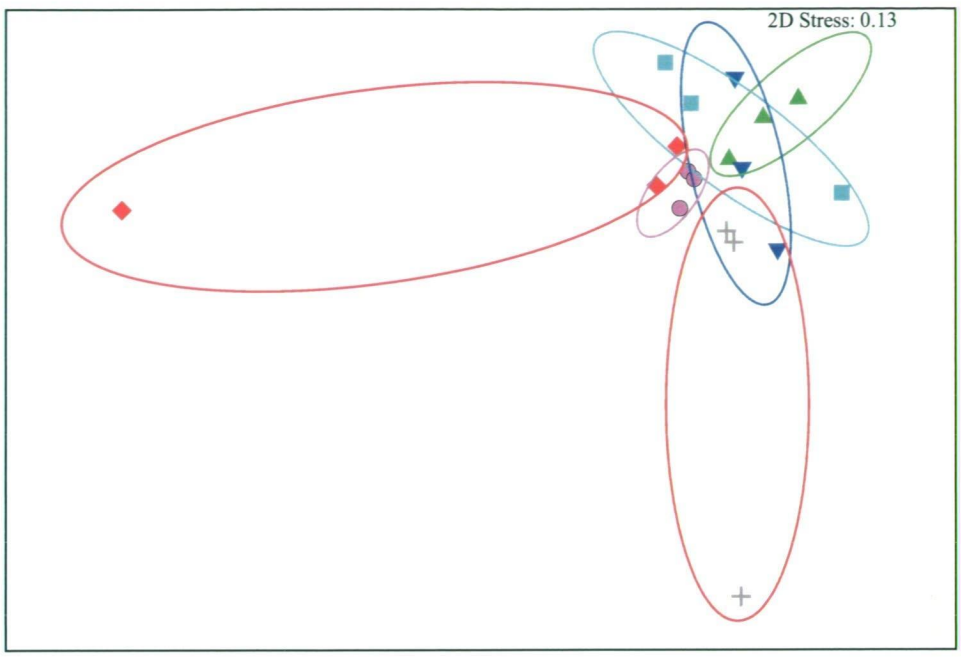

Figure 3.13: MDS plot of TRFLP profiles of bacterial communities associated with *Artemia* challenged with different bacterial strains added at 5×10^5 cells ml^{-1} and incubated over a 14 h period (Experiment 5): (1) ▲ Control, no bacteria added; (2) ▼ *V. proteolyticus* V760; (3) ■ strain ST18; (4) ◆ strain ST7; (5) ● strains ST18 + V760; (6) + ST7 + V760.

Table 3.10: Summarized SIMPER output data utilised for tracking of probiont strains through detection of distinguishing TRFs for strains ST7 and ST18 within the *Artemia* challenge treatment experiment (as shown above in Fig. 3.13).

Treatment		**SIMPER Similarity (%):**	**Distinguishing TRFs (% contribution to % similarity):**					
			ST7:	**ST18:**				
			177 *HhaI*(r)	**60 *HhaI*(f)**	**64 *HaeIII*(f)**	**104 *HinfI*(f)**	**366 HhaI(r)**	**508 HaeIII(r)**
1	Control	79.01	-	-	-	-	-	-
2	V760	69.30	-	-	-	-	-	-
3	ST18	66.26	-	-	-	-	2.53	-
4	ST7	40.02	-	-	-	-	-	-
5	ST18 + V760	82.51	-	-	-	-	4.14	1.06
6	ST7 + V760	68.61	-	-	-	-	-	-

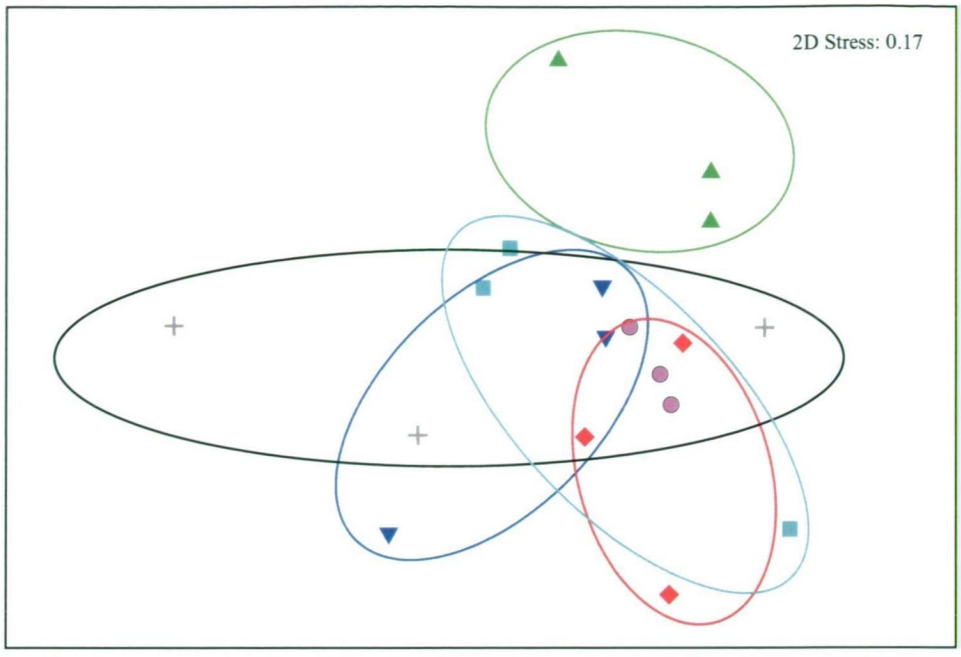

Figure 3.14 MDS plot of TRFLP profiles of bacterial communities associated with *Artemia* challenged with different bacterial strains added at 5×10^4 cells ml^{-1} and incubated over a 14 h period (Experiment 6): (1) ▲ Control, no bacteria added; (2) ▼ *V. proteolyticus* V760; (3) ■ strain ST18; (4) ◆ strain ST7; (5) ● strains ST18 + V760; (6) + ST7 + V760.

Table 3.11: Summarized SIMPER output data utilised for tracking of probiont strains through detection of distinguishing TRFs for strains ST7 and ST18 within the *Artemia* challenge treatment experiment (as shown above in Fig. 3.13).

Treatment		**SIMPER Similarity (%):**	**Distinguishing TRFs (% contribution to % similarity):**					
			ST7:	**ST18:**				
			177 *HhaI*(r)	**60 *HhaI*(f)**	**64 *HaeIII*(f)**	**104 *HinfI*(f)**	**366 HhaI(r)**	**508 HaeIII(r)**
1	Control	64.57	-	-	-	-	-	-
2	V760	59.23	-	-	-	-	-	-
3	ST18	55.81	-	-	-	-	-	-
4	ST7	61.39	-	-	-	-	-	-
5	ST18 + V760	85.34	-	-	-	-	-	2.49
6	ST7 + V760	43.86	-	-	-	-	-	-

3.5 Discussion

Vibrio proteolyticus has been previously recorded as a pathogen of both rotifers and *Artemia* (Puente et al., 1992; Vandenberghe et al., 1999; De Schrijver and Ollevier, 2000; Verschuere et al., 2000). For example, Verschuere et al. (2000) found that a strain of *V. proteolyticus* (CW8T2) at 3×10^6 cells ml^{-1} killed 80 % of *Artemia* within two days. Isolates ST18 and ST7 demonstrated probiotic characteristics when added individually or mixed together. These two isolates were identified as *Aliivibrio fischeri* and *Pseudoalteromonas agarivorans*.

Verschuere et al. (2000) suggested that a probiont may remove toxic metabolic substances that could otherwise adversely affect the growth and survival of the *Artemia*. Furthermore, they suggested that bacteria that are well adapted to the conditions prevailing in intensive *Artemia* culture (as used here) may also be able to prevent the proliferation of opportunistic bacteria. Given the rapidity with which *V. proteolyticus* V-760 was able to increase in numbers and to affect both *Artemia* and rotifers, it appears to be an effective opportunistic colonising bacterium.

TRFLP analysis of the homogenized rotifers and *Artemia* demonstrated that the background bacteria detected in the controls had no obvious effect on the survival of the rotifers or *Artemia*, as the mortality was only high in the pathogen controls where no putative probiont was added. Even though efforts were made to reduce the background bacteria as much as possible, it was observed from the

TRFLP data, using SIMPER analysis, that the use of ozone reduced the bacterial microbiota diversity (data not shown). It is likely that *Vibrio* spp. are part of the rotifer- and *Artemia*-associated microbiota and are able to survive this decontamination step (Tolomei et al., 2004). From the TRFLP analysis, clear shifts in community structure away from the controls could be observed when strain ST18 was added. When ST18 was challenged with *V. proteolyticus* V706, it out-competed the pathogen, resulting in a community shift to be more similar in structure to the sole ST18 treatment than the sole *V. proteolyticus* V706 treatment. Based on the TRFLP profiles, strain ST18 also generated the largest changes in the initial starting profiles. This suggests that it both persisted and competed well for the 14 h treatment period and that other *Pseudoalteromonas* strains were not a major community component in the control rotifer samples. In *Artemia* samples, fewer differences were observed and a lower abundance of specific TRFs, suggesting that existing *Pseudoalteromonas* strains or alteromonads were present. This makes ST18 specifically more difficult to differentiate from the background community TRFs.

The ability of ST18 to protect rotifers and *Artemia* against a challenge from pathogen *V. proteolyticus* V760 could result from its ability to: 1) inhibit colonisation and growth of strain V760; 2) inhibit pathogenicity of V760 through destruction of secreted toxins or by preventing their expression; 3) improve the immune response of the *Artemia*; 4) or, a combination of mechanisms (Kesarcodi-Watson et al., 2008). As the numbers of *V. proteolyticus* V760 did not

appear to vary much between treatments with or without the putative probiont, it is unlikely that the probiont inhibited colonisation by *V. proteolyticus* V-760 *per se*. This implies that the action of the probiont was directed towards inhibiting the virulence factor expression in *V. proteolyticus* V-760.

Lately, quorum-sensing has been demonstrated as a mechanism for controlling virulence factor expression in some *Vibrio* spp. (Defoirdt et al., 2004) and (Bassler, 2002). Interference in quorum-sensing (i.e., due to the activity of enzymes that cleave the signal compounds, such as acylated homoserine lactones or furanone-derivatives) may reduce virulence expression e.g. of *V. harveyi* in *Penaeus monodon* (Manefield et al., 2000). Whether or not interruption of quorum-sensing in *V. proteolyticus* by ST18 can explain the improved survival of rotifers and *Artemia* that was observed remains to be determined. The data from the current study supports the notion that selected naturally occurring bacteria can be encouraged to dominate the normal microbiota of rotifers and *Artemia* and can protect them from subsequent pathogen challenge. Combined with decontamination procedures (Tolomei et al., 2004) and sound husbandry, this suggests a useful way of reducing striped trumpeter larval mortality when rotifers and *Artemia* are used as live feed. The rotifers and *Artemia* can be used as a vector to introduce desirable strains into the host microbiota instead of allowing entry of pathogens (Lopez-Torres and Lizarraga-Partida, 2001). The strength of this colonisation approach is that the bacteria need only be added once or infrequently. In contrast, other approaches such as using *Bacillus* spp.

require the organism to be added continually, as they often do not colonise the larvae effectively (Vine et al., 2006).

Conclusion

Of the 22 isolates obtained from striped trumpeter culture systems, five isolates ST7, ST14, ST18, V8 and V52 showed some antimicrobial activity against known marine animal pathogens. *Pseudoalteromonas* sp. ST18 and *Aliivibrio fischeri* ST7, when inoculated at 5 x 10^4 cells ml^{-1} and 5 x 10^5 cells ml^{-1}, respectively, were found to cause the least mortality in rotifers and *Artemia.* In addition, they protected *Artemia* against a challenge by a virulent strain of *V. proteolyticus*, which could cause >60% mortality of rotifers within 14 h and approximately 50% mortality of *Artemia* within 24 h. Survival of rotifers and *Artemia* when exposed to the strains ST7 and ST18 were not significantly different from the control. Furthermore, survival of rotifers and *Artemia* in mixtures of ST18 and *V. proteolyticus* V760 and ST7 *V. proteolyticus* V760 was significantly greater than in *Artemia* exposed only to *V. proteolyticus* V760 alone.-Improved survival of *Artemia* required the presence of isolate ST18 or ST7 or a mixture of both. Through TRFLP analysis, it was possible to detect the probionts in the rotifers and to a lesser extent *Artemia* by observation of distinctive TRFs.

The practical use of probionts ST7 and ST18 is further explored in Chapter Four.

Chapter 4: Addition and tracking of probionts to yolk sac and first-feeding striped trumpeter larvae

4.0 Abstract

The protective capacity and most effective delivery mode of putative probionts *Pseudoalteromonas agarivorans* ST18 and *Aliivibrio fischeri* ST7 was investigated in the rearing of yolk sac and first-feeding striped trumpeter (*Latris lineata*) larvae. In these experiments 4500, larvae were randomly stocked into 24, 300 l black hemispherical fiberglass tanks at 1 dph and held under static conditions for 5 days after which 300% daily water changes was applied. Terminal restriction fragment length polymorphism (TRFLP) was used to monitor the changes in bacterial community. The addition of strain ST18 to yolk sac larvae showed no significant reduction in survival (70 ± 6%) versus a control group (83 ± 5%) reared without potential probionts being added. The addition of strain ST7 with and without strain ST18 was found to be more disadvantageous (58 ± 7% and 55 ± 8% survival respectively). By tracking distinct 16S rRNA-derived TRFs, strain ST18 was specifically detected in treatments where it was added by both bioencapulsation and by direct addition. When strain ST18 was added directly to the water it resulted in decreased survival, due to the high bacterial load and possibly potential oxygen demand. The introduction of ST18 to the larvae bioencapsulated in rotifers resulted in the introduction of comparatively smaller numbers of bacteria that did not compromise the growth of the developing larvae.

4.1 Introduction

There has been a realization that throughout development, fish intestinal microbiota is strongly dependent upon the characteristics of the aquatic environment and this has consequences for fish health and productivity. There is therefore a need to investigate the influences and modifications both induced by changes in the local environment during development, as well as those influenced through the introduction of probiotics. It is necessary to better understand how probiotics, either directly released into the surrounding water or administered within the diet, alter larval fish microbiota, thus allowing us to determine the mode of action that most effectively introduces the probiont to fish larvae (Nikoskelainen et al., 2003; Vine et al., 2006). Studies involving the use of beneficial bacteria in aquatic production systems have focused on increased performance, measured in terms of improvements in survival and growth. This can come about by boosted disease resistance in the animals, as well as increased stress tolerance. Such improvements have been observed in some cases after just a single species probiotic treatment; for example, the whiteleg shrimp (*Litopenaeus vannamei*), Indian prawn (*Fenneropenaeus indicus*) (Ziaei-Nejad et al., 2006; Wang, 2007), Indian carp (*Labeo rohita*), and red drum (*Sciaenops ocellatus)* (Ghosh et al., 2003).

Microbial probionts have been defined previously in this thesis (Chapter Three). Probiotics may inhibit the colonization of the harmful bacteria through competitive exclusion, for example by out-competing for attachment sites and nutrients. They

may also use other mechanisms such as secretion of antimicrobial compounds that may inhibit other bacterial growth or through interference of quorum sensing by enzymatic cleavage of autoinducer molecules (Olsen et al., 2000). Probiotic research has centered on two major strategies: bioremediation (modification of an established flora to one that is supportive of the host) and biocontrol (use of a specific microorganism to minimize the impact of a specific pathogen) (Verschuere et al., 2000). Methods for selecting potential probionts have been generally based on *in vitro* tests, such as the test used in Chapter Three, or predominance in gut microbiota in fish groups that come from high survival and better performance (Makridis et al., 2005; Planas et al., 2006; Vine et al., 2006). Challenge tests can be applied to determine the ability of probiotic bacteria to prevent disease, as outlined in Chapter Three (Section 3.2.4). They can also be used to determine if the bacterial isolate is harmful in any way to live feeds. The selection of potential probionts was conducted in Chapter Three (Section 3.3.2) and the best candidates were isolates *Aliivibrio fischeri* ST7 and *Pseudoalteromonas* sp. ST18. TRFLP also revealed that it is possible to track these isolates in culture and during enrichment of both rotifers and *Artemia*, using the isolate's distinctive fingerprint TRFs (Table 3.5). The next step in assessing the potential efficacy of the identified probionts was to test them in larval culture through the ability to bioencapsulate the potential probiont within rotifers and *Artemia*.

Within this chapter two major questions are explored:

1) What is the effect of adding potential probionts to striped trumpeter yolk sac larvae, either singularly or in combination?

To answer this question, strains ST7 and ST18 were introduced to yolk sac larvae held under static clear water conditions, with the aim of modifying the microbial community of both the yolk sac larvae and the surrounding water. Assessment of larval performance when reared with different combinations of both probionts was undertaken.

2) What is the effect of adding combinations of potential probionts using different strategies, either through rotifer bioencapsulation, direct addition to the culture water or both? These additions would occur when the larvae are in a phase of active rotifer feeding, from 6 to 15 days post-hatch (dph).
To answer this question, the performance of the feeding larvae was assessed under a series of different treatments, using TRFLP analysis to track the uptake. In this case, the changes in the microbiota of larvae and the surrounding water in association with the treatment were investigated.

4.2 Material and Methods

4.2.1 Culture of yolk sac larvae

Eggs were collected from a female striped trumpeter broodstock by strip-spawning, and fertilized with the milt of four males. Fertilized eggs were incubated and hatched as previously described (Bransden et al., 2004). Larvae (4500) were randomly stocked into 24, 300 l black hemispherical fiberglass tanks at 1 dph. Larvae were held under static conditions in seawater from 1 to 5 dph. A photoperiod of 18 h light: 6 h dark was used throughout the experiment, produced by a computerized halogen light source (~11 μmol s^{-1} m^{-2} at the water surface) with a gradual fade in and fade out. Dead larvae were siphoned twice daily and deducted from the original numbers stocked to estimate survival in each tank.

4.2.1.1 Experiment 1: Effect of potential probionts on yolk sac larvae

The aim of this experiment was to determine the effect that the potential probionts ST18 and ST7 have on non-feeding yolk sac larvae. The null hypothesis was that were would be no significant difference in survival of the yolk sac larvae over the six days in which they rely on endogenous food reserves. A further aim was to determine if the uptake and changes in the bacterial communities of the larvae could be explored using the capabilities of TRFLP analysis to track the isolates.

Four treatments were assessed, each with six replicate tanks. The control treatment consisted of rearing larvae in seawater with no added bacteria. The second treatment involved addition of live bacterial cultures of both strains ST7 and ST18 at a final concentration of 5×10^{-5} cells ml^{-1}. The third and fourth treatments involved individual addition of ST7 or ST18 at a final concentration of 5×10^{-5} cells ml^{-1} each.

Initial samples of 50 yolk sac larvae were siphoned from each tank at stocking to determine the size and condition of the larvae. TRFLP analysis was also undertaken on these samples to gain a baseline assessment of the bacterial community. At 1, 3 and 6 dph further larval and water samples were taken for TFRLP. Larvae (n=50) were removed from each of the experimental tanks using a siphon and anesthetized in 0.06% 2-phenoxyethanol as described in Battaglene et al. (2006). Of these larvae twenty, were examined using an Olympus SZ microscope to determine morphormetric indices, including standard length, swim bladder inflation, grey gut and condition. Samples of a further 20 larvae were analysed by TRFLP analyses. At the same time 50 ml water samples for TRFLP analysis were removed with a beaker (assigned to each tank) and placed into 50 ml Falcon tubes and frozen at -20°C.

4.2.2 Culture of feeding larvae

Egg collection, incubation and yolk sac rearing is as described in section 4.2. At 6 dph internal 390 µm mesh screens were placed into the centre of each tank to

allow seawater outflow and removal of rotifers, algae and incoming seawater overnight. A 'greenwater' environment was then provided with live *Nannochloropsis oculata* at a turbidity level of 3 Nephelometric Turbidity Units (NTU) (HACH 2100 portable turbidity meter). Each morning the various feed treatments were added to each tank at a rate of 8.8 l min^{-1} (total 30 l) from the 80 l reservoirs accompanying each individual tank. The algal suspensions or seawater had been acclimated to tank temperature in the reservoirs overnight and provided with aeration. Tanks remained static during the light phase with gentle aeration (200 ml min^{-1}) (Shaw, 2006). All tanks were supplied with live rotifers (*B. plicatilis*) enriched on Algamac 2000 (Aquafauna Biomarine, USA) from 6 dph, as described by Battaglene et al. (2006). Enriched rotifers were supplied at 10 ml^{-1} each morning following probiont enrichment as described below in section 4.2.3. Surface skimmers were used from 8-15 dph to remove surface oil and promote swim bladder inflation (Trotter et al., 2003). Water quality was measured daily. Temperatures ranged from 15.5 – 16.5°C, salinity 34.1-34.7 ‰, pH 7.9-8.2 and dissolved oxygen was greater than 90% saturation. Larvae were spot siphoned to determine mortality levels and counted daily up to the conclusion of the experiment (15 dph) when all live larvae were removed and counted to determine final survival. The final survival tally was determined by a series of volumetric counts on each tank. Prior to counts heavy aeration was applied to the tanks so that the larvae were distributed evenly.

4.2.2.1 Experiment 2: Effect of potential probionts added directly to the culture water, to the enriched rotifers or a mixture of both

The aim of this experiment was to determine the effect that the potential probionts ST18 have on first-feeding larvae. The null hypothesis was that there would be no significant difference in survival of the larvae over the seven days compared to the control reared larvae

Four treatments were assessed each with 6 replicate tanks:

Treatment 1: control rotifers enriched with Algamac 2000 and fresh algae (*Nannochloropsis oculata*) greenwater culture at 3 NTU.

Treatment 2: ST18 enriched rotifers plus ST18 added to the culture water to achieve a concentration of 5×10^{-5} cells ml^{-1}.

Treatment 3: ST18 enriched rotifers but not added to the culture water.

Treatment 4: ST18 added to the culture water to achieve a concentration of 5×10^{-5} cells ml^{-1}.

Fifty larvae were siphoned and water samples taken from each of the treatments as described in section 4.2.1.1. Sampleing took place at 6, 10 and 13 dph.
An initial sample of 20 larvae and a 50 ml water sample were taken from the incubator before stocking to assess the existing bacterial community using TRFLP. All sampling took place in the morning prior to feeding.

4.2.3 Rotifer enrichment

Rotifer enrichment was as described in Section 3.2.4 but modified by the rotifers being transferred to 200 l vessels for 12 h enrichment, at 400 rotifers ml^{-1} and 23°C. Rotifers were enriched with AlgaMac at 0.3 g per million rotifers according to the manufacturer's instructions. Aeration and oxygen were provided to maintain dissolved oxygen above 4 mg l^{-1} in the enrichment vessels. Probionts were added to the corresponding rotifer enrichment vessel at the same time as the AlgaMac was added, to achieve a concentration of 5 x 10^{-5} cells ml^{-1}. At the end of the enrichment period rotifers were rinsed and cleaned with ozonated seawater and counted using a Coulter counter.

4.2.4 Bacterial preparation

Bacterial strains were grown as in Chapter Three, section 3.2.3

The bacterial inoculums were grown in 2000 ml broth and shaken daily, the final volume was standardized for each tank so that they all received the same amount of broth being added, the short fall if any was made up using sterilized marine broth.

4.2.5 DNA extraction

Extraction of DNA was done as in Chapter Two Section 2.2.2

4.2.6 16S rRNA gene PCR.

PCR conditions was performed as in Chapter Three Section 3.2.7

4.2.7 TRFLP sample preparation

Preparation for TRFLP was performed as Chapter Two Section 2.2.6

4.2.8 TRFLP data analysis

TRFLP analysis was done as in Chapter Two Section 2.2.7

4.2.9 Survival Statistical analyses.

Survival statistical analyses was performed as in Chapter Three Section 3.3.4

4.3 Results

4.3.1 Determination of the probiotic capabilities of possible candidates on yolk sac larvae

The inclusion of strain ST7 to the culture water resulted in significantly reduced survival of 55 ± 8% compared to 83 ± 5% survival in the controls (Fig. 4.1). The addition of ST18 resulted in 70 ± 6% survival. A combination of ST18+ST7 resulted in 58 ± 7% survival. Only the addition of ST7 was significantly different to the control (Fig. 4.1).

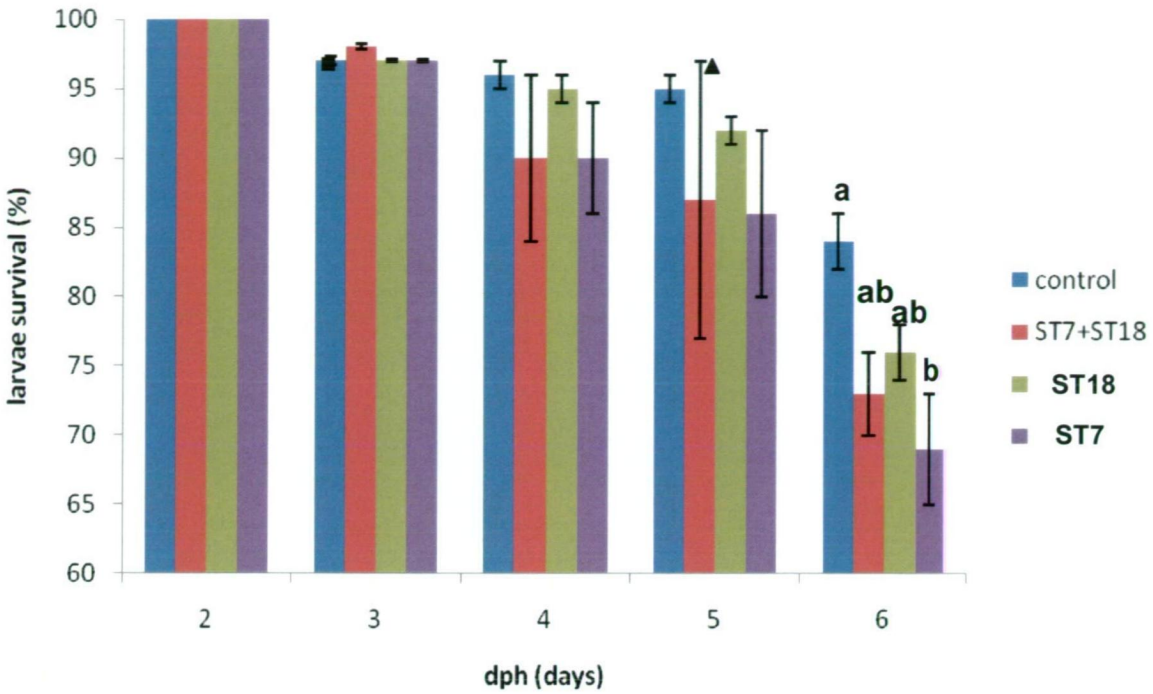

Figure 4.1: Yolk sac larvae survival over the time for Experiment 1 (Section 4.2.1.1). Values for the 6 dph samples not sharing a common letter are significantly different ($P<0.05$).

Table 4.1: Yolk sac larvae Experiment 1 (see section 4.2.1.1) showing larval size and survival and water physicochemical parameters.
[a,b,c]Values within rows sharing a common letter indicates statistically significantly differences (P>0.05) determined using one-way ANOVA.

Treatment		Control	ST7+ST18	ST18	ST7
Age		1DPH			
Standard length	mm	5.1±0.03			
Survival	%	100.0			
pH		8.1±0.0			
DO	%	90±1			
Temperature	°C	15.4±.0.1			
Age		2DPH			
Standard length	mm	-	-	-	-
Survival	%	100[a]	100[a]	100[a]	100[a]
pH		8.14±0.1[a]	8.1±0.0[b]	8.2±0[ab]	8.1±0.01[b]
DO	%	81.65±1.2[a]	74.6±0.6[b]	73.1±0.7[b]	76.7±1.61[b]
Temperature	°C	15.3±.0.1[a]	15.38±.0.16[a]	15.4±0.02[a]	15.4±0.02[a]
Age		3DPH			
Standard length	mm	-	-	-	-
Survival	%	97 ± .0.3[a]	98 ± 0.2[a]	97 ±0.1[a]	97 ± 0.1[a]
pH		8.1±.0.1[a]	8.0±0.0[ab]	8.0±0.0[b]	7.9±0.0[b]
DO	%	86.1±0.8[a]	77.0±1.1[ab]	75.2±0.7[b]	72.4±4.0[b]
Temperature	°C	16.1±0.1[a]	16.1±0.1[a]	16.1±0.1[a]	16.0±0.0[a]
Age		4DPH			
Standard length	mm	-	-	-	-
Survival	%	96 ± 1 [a]	90 ± 6[a]	95 ± 1[a]	90 ± 4[a]
pH		8.1±0.0[a]	7.9±0.0[b]	7.9±0.0[b]	7.9±0.0[b]
DO	%	86.2±0.9[a]	73.9±2.1[b]	71.9±0.9[b]	69.2±4.1[b]
Temperature	°C	16.2±0.0[a]	16.2±0.0[a]	16.2±0.0[a]	16.2±0[a]
Age		5DPH			
Standard length	mm	-	-	-	-
Survival	%	95 ± 1[a]	81 ± 10[a]	92 ± 1[a]	86 ± 6[a]
pH		8.1±0.0[a]	7.9±0.0[b]	7.9±0.0[b]	7.9±0.1[b]
DO	%	85.5±1.31[a]	73.1±1.9[b]	74.8±1.15[b]	67.9±5.1[b]
Temperature	°C	16.2±0[a]	16.2±0[a]	16.2±0[a]	16.2±0[a]
Age		6DPH			
Standard length	mm	5.3±0.1[a]	5.3±0.1[a]	5.3±0.1[a]	5.3±0.1[a]
Survival	%	83 ± 5[a]	58 ± 7[ab]	70 ± 6[ab]	55 ± 8[b]
pH		8.3±0.1[a]	7.9±0.1[b]	7.9±0.1[b]	7.9±0.3[b]
DO	%	83.6±2.2[a]	73.4±3.1[ab]	75.8±1.5[ab]	68.8±4.5[b]
Temperature	°C	16.2±0[a]	16.2±1[a]	16.2±2[a]	16.2±3[a]

4.3.2 TRFLP analysis of yolk sac larvae (Experiment 1)

The MDS plot derived from the TRFLP data obtained from yolk sac larvae at 1, 3 and 6 dph was inconclusive when all replicates were presented due to the overwhelming similarity between groups (data not shown). The only significant change found was between the treatments and the initial larval sample taken from the incubator before stocking (TRFLP profiles, treatment 1, R=0.45; treatment 2, R=0.22; treatment 3, R=0.30; and treatment 4, R=0.52). There were no significant differences in treatment samples between sampling days. Similar patterns were observed for the tank water TRFLP profiles. For the water samples there were no significant differences in profile patterns with time and only very slight changes were apparent in comparison with the initial sample (R values <0.10).

After addition of the ST7, ST18 and ST7 and ST18 in combination at 1 dph it was possible to track both strains the culture water and the larvae through the presence of their distinctive TRFs using SIMPER analysis. In both water and larvae samples ST18 and ST7-derived TRFs were not detected in the control samples (Table 4.2).

When ST7 and ST18 were added in combination within the larval samples ST18 was only detected at 6 dph, however ST7 was detected at all sample times and made a greater contribution to average similarity, which was albeit rather low. In water samples, however ST18 appeared to be much more abundant while ST7

was not detected. When added singularly, ST7 was detectable in larvae at 4 and 6 dph but again was not detected in the water samples. ST18, when added singularly on the other hand was readily detected in both larval and water samples, though in the case of larvae, the abundance appeared to decline considerably by dph 6.

Based on the TRFLP data ST18 thus appears to become a major component of the microbial community in the tank water, however direct uptake of the probionts appeared to be limited. It was observed that the inoculation of bacteria at the yolk sac larvae stage provided no boosting of larval survival and direct addition generally resulted in slightly reduced survival, however, this apparent reduced survival appeared to be attributable to dissolved oxygen availability.

Table 4.2: Summarised SIMPER output data in which TRFLP analysis was used to track probionts ST7 and ST18 in treatments in which the strains were added directly to tanks holding 1 dph yolk sac larvae. Samples analysed included larval and water samples.

Treatment	Dph	Sample	Similarity%	ST18 TRFs			ST7
				104 *HinfI* (f)	366 *HhaI* (r)	508 *HaeIII* (r)	268 *HinfI* (f)
				Contribution to average similarity (%):			
Control	2	larvae	10.61	-	-	-	-
Control	4	larvae	24.49	-	-	-	-
Control	6	larvae	12.60	-	-	-	-
Control	2	Water	37.27	-	-	-	-
Control	4	Water	55.91	-	-	-	-
Control	6	Water	45.72	-	-	-	-
ST7+ST18	2	larvae	7.62	-	-	-	10.65
ST7+ST18	4	larvae	5.22	-	-	-	15.45
ST7+ST18	6	larvae	9.71	-	2.43	-	11.24
ST7+ST18	2	Water	48.86	-	10.36	15.95	-
ST7+ST18	4	Water	44.66	-	11.28	10.78	-
ST7+ST18	6	Water	48.78	-	-	14.07	-
ST7	2	larvae	6.36	-	-	-	-
ST7	4	larvae	23.57	-	-	-	5.64
ST7	6	larvae	3.14	-	-	-	3.95
ST7	2	Water	53.73	-	-	-	-
ST7	4	Water	46.69	-	-	-	-
ST7	6	Water	50.54	-	-	-	-
ST18	2	larvae	12.65	26.03	10.29	-	-
ST18	4	larvae	22.41	14.39	7.29	-	-
ST18	6	larvae	13.88	-	5.23	-	-
ST18	2	Water	34.72	8.12	-	19.24	-
ST18	4	Water	56.36	14.63	-	18.60	-
ST18	6	Water	62.60	20.61	-	24.93	-

4.3.3 Determination of the probiotic capabilities of possible candidates on first feeding larvae (Experiment 2)

The larval response to the introduction of ST18 enriched rotifers, ST18 to the water, or both in combination held under greenwater conditions was found to accelerate between 10 to 13 dph (Fig. 4.2). At 13 dph, survival in treatments in which ST18 was provided bioencapsulated in rotifers were not significantly different to the controls ($F=6.099$, $df=20,3$ $P=0.06$) with the control having the best survival of 87.5 ± 0.4% while the ST18/rotifer addition was similar at 84.4 ± 1.9%. The combined addition of ST18 in the form of rotifer bioencapsulation and direct addition to the tank water was the worst performing treatment with only 70.9 ± 3.3% larvae survival occurring at 13 dph (Fig.4.2, Table 4.3) while direct water addition alone provided more intermediate survival outcomes.

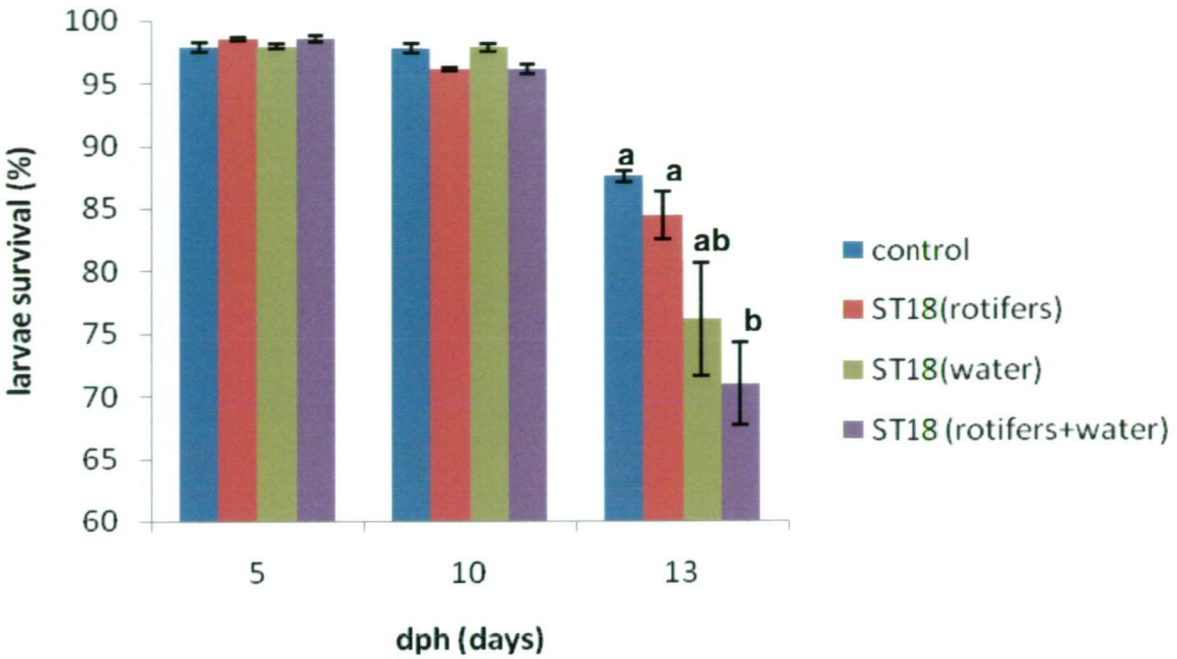

Figure 4.2: Survival percentage over the time of the Experiment 2 during first feeding. Different letters above the 13 dph results represent significant differences occurring between treatments (P=0.05).

Table 4.3: First feeding larvae Experiment 2 (see section 4.2.2.1) showing outcomes for larval size and health and water chemical parameters.

Age	**2 dph**			
Treatment	**Initial state**			
Average size (mm)	4.8 ± 0.1			
Swim bladder inflation	-			
Grey gut	-			
pH	8.1 ± 0.1			
Dissolved oxygen (%)	99.1 ± 0.6			
Salinity (‰)	34.8 ± 0.1			
Temperature (°C)	15.7 ± 0.1			
Survival (%)	100			
Age	**5 dph**			
Treatment	**Control**	**ST18r + ST18 w**	**ST18r**	**ST18w**
Average size (mm)	5.1 ± 0.1	5.1 ± 0.1	5.1 ± 0.1	5.1 ± 0.1
Swim bladder inflation	-	-	-	-
Grey gut	-	-	-	-
pH	8.1 ± 0.1	8.1 ± 0.1	8.1 ± 0.1	8.1 ± 0.1
Dissolved oxygen (%)	99.1 ± 0.6	98.3 ± 0.6	98.8 ± 0.5	98.9 ± 0.6
Salinity (‰)	34.7 ± 0.1	34.7 ± 0.2	34.7 ± 0.1	34.7 ± 0.1
Temperature (°C)	16.2 ± 0.1	16.2 ± 0.1	16.2 ± 0.1	16.2 ± 0.1
Survival (%)	97.9 ± 0.4	98.5 ± 0.2	98.7 ± 0.1	97.9 ± 0.2
Age	**10 dph**			
Treatment	**Control**	**ST18r + ST18 w**	**ST18r**	**ST18w**
Average size (mm)	6.1 ± 0.1[a]	5.7 ± 0.1[b]	6.1 ± 0.1[a]	5.1 ± 0.1[b]
Swim bladder inflation	79.1 ± 3.7[a]	43.3 ± 4.5[b]	82.5 ± 3.5[a]	36.6 ± 4.4[b]
Grey gut	90 ± 2.7[a]	42.5 ± 4.5[b]	85 ± 0.1[a]	20.8 ± 3.8[c]
pH	8.2 ± 0.1	8.2 ± 0.1	8.2 ± 0.1	8.2 ± 0.1
Dissolved oxygen (%)	108.8 ± 0.6	107.0 ± 1.4	108.5 ± 0.6	107.3 ± 1.4
Salinity (‰)	34.9 ± 0.1	34.9 ± 0.2	34.9 ± 0.1	34.9 ± 0.1
Temperature (°C)	15.8 ± 0.1	15.8 ± 0.1	15.8 ± 0.1	15.8 ± 0.1
Survival (%)	97.7 ± 0.4	96.1 ± 0.4	98.1 ± 0.1	97.8 ± 0.3
Age	**13 dph**			
Treatment	**Control**	**ST18r + ST18 w**	**ST18r**	**ST18w**
Average size (mm)	6.9 ± 0.1[a]	6.1 ± 0.1[b]	6.9 ± 0.1[a]	6.1 ± 0.1[a]
Swim bladder inflation	98.3 ± 1.2[a]	77.5 ± 4.8[c]	95.8 ± 1.8[ab]	86.7 ± 3.1[bc]
Grey gut	97.5 ± 1.4[a]	77.5 ± 3.2[b]	99.2 ± 0.8[a]	79.2 ± 3.3[b]
pH	8.2 ± 0.1	8.2 ± 0.1	8.2 ± 0.1	8.2 ± 0.1
Dissolved oxygen (%)	107.5 ± 0.4	108.4 ± 0.5	107.7 ± 0.5	108.1 ± 0.4
Salinity (‰)	34.7 ± 0.1	34.7 ± 0.2	34.7 ± 0.1	34.7 ± 0.1
Temperature (°C)	15.8 ± 0.1	15.7 ± 0.1	15.7 ± 0.1	15.7 ± 0.1
Survival (%)	87.5 ± 0.4[a]	70.9 ± 3.3[b]	84.4 ± 1.9[a]	76.1 ± 4.5[b]

[a, b, c]Where significant interactions between factors occur a one-way ANOVA analysis of all treatments is provided. Values within rows sharing a common superscript do not significantly differ (P>0.05).

A similar result was observed for other larvae quality features including swim bladder inflation and presence of grey gut (Table 4.3). Therefore, the addition of the potential probiont appears to be best done through introducing it to the larvae via bioencapsulation within rotifers.

4.3.4 TRFLP analysis for Experiment 2

The MDS plot derived from the TRFLP data for probiotic assessment experiment was inconclusive when all replicates were presented in an MDS due to the overwhelming similarity between groups. No changes could be seen and are therefore not presented. The only significant change found was when comparing the treatments to the incubator TRFLP baseline profiles: treatment 1, R=0.22; treatment 2, R=0.37; treatment 3, R=0.1; and treatment 4, R=0.15. It was also found that during the time of the experiment that all treatments were similar to each other on each sampling day and as a whole shifted in a similar manner resulting in the clustering of sampling days together with no differences observable. For the water samples it was found that there was no significant difference throughout the trial and even comparing 13 dph to the initial incubator water profiles there were only moderate changes (R=0.19-0.33). When assessing the total difference between the larval fish TRFLP profiles to that of the water it was found that they were significantly different at all time points sampled (global R=0.67).

Tracking strain ST18 was successfully done using TRF data in both the water and the larvae samples. For the experiments in which ST18 is applied to striped trumpeter larvae by bioencapsulation in rotifers the tracking of strain ST18 could be achieved at all sampling time points (Table 4.4) while it was not detected in the controls. In the larvae-derived TRFLP profiles it was seen that ST18 was only detected in the larvae fed enriched rotifers while the inclusion of ST18 to the water directly indicated ST18 appeared to be detectable in larvae 10 and 13 dph (Table 4.4). In addition there seems to be some suggestion that ST18 relative abundance appears to decline over time in the tank water (Table 4.4).

The similarity between replicates of treatments in both the larvae and water samples also increased over time as it did in the previous experiment and was seen to be highest at 13 dph in the ST18 addition treatments. The fact ST18 had become a major component of the microbial community in the replicates was further emphasized by the large abundance recorded in larvae samples (Table 4.4). Through this experiment not all TRFs of ST18 were detected using SIMPER based analysis and this may be due to biases occurring during the TRFLP analysis. The TRFLP profile data is a compilation of replicates of three separate restriction enzyme digestions and likely results in greater representation of certain TRFs compared to others in individual samples.

Table 4.4: Tracking of strain ST18 using TRF data during the rotifer bioencapsulation experiment applied to striped trumpeter larvae.

Treatment	Dph	Sample	Similarity%	ST18 TRFs		
				60 HhaI (f)	104 HinfI (f)	508 HaeIII (r)
				% contribution to % similarity:		
rotifers + algal feed	5	larvae	19.47	-	-	-
rotifer + ST18	5	larvae	75.53	10.99	62.33	28.75
Control	2	larvae	11.11	-	-	-
Control	5	larvae	34.11	-	-	-
Control	6	larvae	15.55	-	-	-
Control	10	larvae	17.29	-	-	-
Control	13	larvae	40.55	-	-	-
Control	2	water	70.17	-	-	-
Control	5	water	56.53	-	-	-
Control	6	water	74.98	-	-	-
Control	10	water	48.87	-	-	-
Control	13	water	58.22	-	-	-
ST18 (water + rotifers)	6	larvae	26.95	4.3	11.2	-
ST18 (water + rotifers)	10	larvae	32.48	11.1	27.5	-
ST18 (water + rotifers)	13	larvae	54.57	13.2	15.3	21.0
ST18 (water + rotifers)	5	water	53.51	-	-	-
ST18 (water + rotifers)	6	water	49.33	12.2	5.6	-
ST18 (water + rotifers)	10	water	51.87	9.3	2.5	16.9
ST18 (water + rotifers)	13	water	38.64	47.4	-	11.3
ST18 (rotifers)	6	larvae	24.82	6.1	5.9	17.2
ST18 (rotifers)	10	larvae	10.47	15.2	33.3	39.6
ST18 (rotifers)	13	larvae	13.61	22.2	48.2	9.2
ST18 (rotifers)	5	water	48.32	-	-	-
ST18 (rotifers)	6	water	55.37	-	-	-
ST18 (rotifers)	10	water	64.48	-	-	-
ST18 (rotifers)	13	water	39.27	-	-	-
ST18 (water)	6	larvae	14.85	-	-	-
ST18 (water)	10	larvae	24.08	-	-	17.8
ST18 (water)	13	larvae	21.87	-	-	22.2
ST18 (water)	5	water	52.10	-	-	-
ST18 (water)	6	water	52.59	10.1	7.5	13.5
ST18 (water)	10	water	39.34	1.5	5.5	-
ST18 (water)	13	water	66.85	5.3	-	-

4.4 Discussion

A combination of bacterial strains that complement each other and occupy different niches within the gut environment could result in an enhancement or prolongation of the desirable effects on the host immune response and health (Timmerman et al., 2004; Panigrahi and Azad, 2007). The present results indicate that during the yolk sac period the treatments with ST18 and ST7 proved to be unfavourable. The reduced dissolved oxygen (DO) levels observed on addition of the probionts to the tank water (Table 4.1) occurred possibly due to the bacterial inoculums being too high for the static system being investigated. The DO levels within the tank declined in the treatments in which the bacterial probionts were added (Table 4.1). Treatments 2 and 4 had the lowest DO levels of 73.4% and 68.9% of saturation respectively (Table 4.1) which coincided with lowest survival levels (58 ± 7% and 55 ± 8%). A bacterial bloom was also visible to the eye in the form of a floating mat. The water had slightly increased acidity with pH dropping from 8.1 to 7.9 (Table 4.1). The lower DO level may have stressed the larval fish and contributed to their lower survival and growth (Vine et al., 2006). . The inoculated bacteria also proliferated in the ozonated water possibly due to lack of other competitive bacteria. An improved balance in probiont populations may result in better outcomes for larval survival. The problem with striped trumpeter is they do best in very static conditions especially as yolk sac larvae and water exchange is difficult (Shaw 2006). Static

conditions promote bacterial proliferation especially with the addition of nutrient rich rotifers.

The sole addition of strain ST7 was found to be disadvantageous to the culturing of striped trumpeter yolk sac larvae, while the addition of strain ST18 was shown to be less harmful. Strain ST7 is a member of the genus *Aliivibrio* and is related to *Vibrio* spp. that have been tested as probiotics in earlier studies (Gatesoupe, 1999; Makridis et al., 2001; Vine et al., 2006) but there is some criticism of their use as probiotics due to the possibility of them becoming virulent after prolonged application (Vine et al., 2006). The negative impact of strain ST7 was partially mitigated by co-addition of ST18 suggesting an interaction could have been occurring between the strains. This interaction could be in the form of strain ST18 producing an antimicrobial compound that inhibited strain ST7. The ability of strain ST18 to inhibit *Vibrio* strains due to a diffusible antimicrobial compound was observed in Chapter Three (section 3.4.1). It is possible that this antimicrobial compound was also actively produced during the current experiments. Whether production is a consequence of mechanisms such as quorum-sensing, in which a minimum population is needed before antimicrobial production is triggered, requires further investigation. Strain ST18 belongs to the genus *Pseudoalteromonas*, which has been frequently reported to produce a range of antimicrobial compounds (Bowman, 2007). The genus *Pseudoalteromonas* is common in marine environments and has

been seen to comprise the microbiota of rotifers and *Artemia* (McIntosh et al., 2008, Bowman, 2007). It has been previously reported before that both antibacterial and antifouling activities are present in marine *Pseudoalteromonas* (Holmstrom et al., 1999). It is therefore it is possible that ST18 produces a probiotic effect through its release of water soluble compounds that inhibit or influence the growth of various bacterial species, including various pathogenic *Vibrio* spp.

In first-feeding larvae it was seen that the addition of the probiont via enriched rotifers was the most promising mode of addition. When ST18 was added to both the water and through rotifers it resulted in decreased survival, possibly due the number of bacteria present being too high. This is surprising as water was exchanged daily (300% exchange) and other studies have shown that the addition of probionts directly to the water have no detrimental effect (Makridis et al., 2000, Planas et al., 2006, McIntosh et al., 2008). These differing results may be due to the bacterial species being used and also differences among fish species. Striped trumpeter has previously been shown to be sensitive to bacterial infections and perform best in bacteria reduced conditions (Battaglene et al 2006; Battaglene and Cobcroft 2007). Thus the introduction of strain ST18 to the larvae bioencapsulated in rotifers may have resulted in the introduction of comparatively smaller numbers of bacteria that did not compromise the growth of the developing larvae.

It was encouraging that it was possible to track the probionts through the use of TRFLP analysis. By tracking distinct 16S rRNA-derived TRFs, strain ST18 was specifically detected in treatments where it was added by both bioencapsulated and by direct addition. Thus by successfully tracking strain ST18 greater confidence is provided for its biological impacts in the experiments. The TRFLP data also interestingly indicated that strain ST18 did not change the bacterial community of the larvae substantially (as suggested by ANOSIM data). This is the first study to the author's knowledge where TRFLP has been used for this type of application. In an aquaculture setting TRFLP has been used previously to assess the difference between the composition of bacteria associated with whiting mucus and the surrounding water (Smith et al., 2007). Other studies have also used TRFLP to help explain temporal bacterial changes in seawater (Hewson et al., 2003). No previous studies have used TRFLP to actively track a known bacterial isolate within an experiment environment before. It may be plausible to conclude that ST18 may release substances that do not substantially reduce the number of most bacteria present but may inhibit specific species from increasing to too high a population and/or inhibit their ability to produce pathogenic effects, for example proteolytic degradation of secreted enzymes or toxins.

It has been reported widely that one of the main modes of action and beneficial effects of probiotics in aquaculture organisms is enhancement of nutrition of host species through the production of supplemental digestive enzymes and higher growth and feeding efficiency, prevention of intestinal disorders and pre-digestion of anti-nutritional factors present in the ingredients (Verschuere et al., 2000). However, in aquaculture, probiotics can be administered either as a food supplement or as an additive to the water (Moriarty, 1998). In the current study we administered strain ST18 both in live food and water separately and live food with water in the same environment, thus, it was clearly determined where probiotics colonized and worked effectively in terms of growth, and survival in different environments (by live food and/or water). At the doses tested the most effective way to administer probionts appears to be via live food due to colonization observed within larvae. This is in agreement with Suzer et al. (2008) who found similar results when administering probionts to gilthead sea bream (*Sparus aurata*) via addition to water or lives feeds, and saw that the best results were obtaining using live feed treatments. It has also been seen that through addition of probionts to the water or live feeds, the resulting colonization of the larvae gut may not be the amount expected. Planas (2006) found that when a *Roseobacter* sp. probiont was introduced to turbot larvae directly or through enriched rotifers, it did not colonise the gut in high numbers but was found in substantial numbers in the water. It was also seen in this study that the presence of ST18 was detected on both the water and the larvae. These

experiments did not quantify the exact amount present but through occurrence of the TRF signal and the fact that ST18 TRFs were more readily detected in the water thus were possibly present in the water at higher numbers than in the larval gut.

Some of the proposed mechanisms for the probiont activity include greater survival, growth, viability or adhesion to mucosal surfaces of one species in the presence of another species, the production of different enzymes or other proteins, the creation of a probiotic niche and additive/synergistic effects of strain specific properties (Vine et al 2006). It must be taken into account that prior inactivation of probiotic bacteria does not necessarily result in the loss of adhesion to intestinal mucus, although it may depend on the bacterial strain and the inactivation method used. Other studies have postulated probiotic modes of action to be dependent on interactions between probionts and pathogens in the digestive tract, such as competition for space or nutrients, or production by the probiotic of growth-inhibiting metabolites (Balcazar et al., 2006). Therefore, the possibility of an increased adhesion of one of the assayed bacteria in the presence of the other cannot be ruled out.

Conclusions

This study showed that the addition of *Pseudoalteromonas* sp. ST18 at the yolk sac larvae stage was not significantly different from that of the control and that during first feeding the best mode of addition of ST18 was through enriched rotifers. Through the use of TRFLP strain ST18 could be tracked and identified *in situ*. The experimental set up for these experiments resulted in the questions posed in the introduction being successfully answered, however many new questions have arisen during the course of the study. These unknown factors potentially set out future directions for research. The following Chapter Five will discuss further directions that may increase our knowledge in this field of research.

Chapter 5: Outcomes and Future Directions

The intention of this study was to investigate and develop a robust process that could identify and culture probionts in larval fish cultures focusing on these major aims:

1) Identification of the bacterial communities in striped trumpeter larval cultures through the use of 16S rRNA gene-based clone libraries and TRFLP analysis.

2) Determination of the bacteria that possess probiotic activity by using *in vitro* antimicrobial plate tests and assessment of possible candidates on live feeds and development of the use of TRFLP to enable tracking of the probionts in a mixed species environment.

3) Exploration of the best mode of action to introduce the probiont to the larvae. The tracking of the probionts in an experimental striped trumpeter larvae system was to be achieved using TRFLP to match the probiont's unique TRFs.

The first phase investigated and provided an insight into the bacterial diversity of striped trumpeter larvae using 16S rRNA gene-based clone libraries and TRFLP analysis. It showed that under the three different rearing conditions that the bacterial diversity was limited and varied considerably between samples. With this information we cannot draw any definite conclusions that the bacterial community of the larvae and the surrounding

water were influencing larval survival performance. Through the investigation of larvae-associated microbiota using 16S rRNA clone libraries it was also observed that the phylogenetic distribution of the clones was found to include four major taxonomic groups. They were, in order of abundance class *Alphaproteobacteria* (42%), the chloroplasts of the algae *Nanochloropsis granulata* (38%), phylum *Actinobacteria* (11%) and class *Gammaproteobacteria* (10%). The overall results of the study showed that the microbial community of the 15 dph larvae was occurring randomly at this early stage. The bacterial diversity was in general agreement with other studies of larvae microbiota (Jensen et al., 2004; Romero and Navarrete, 2006). No *Vibrio* spp. were observed, however, *Vibrio* strains were likely present in low numbers, as in culture-based studies they were isolated following enrichment in a selective medium. The population density of *Vibrio* was too low to be detected in the randomized clone library survey.

Through the use of TRFLP analysis, microbial communities in the rearing sea water were more diverse than were present in the larvae. This could be due to the bacteria being comprised of both autochthonous (adherent) and allochthonous (transient) sub-populations. As the Atlantic halibut larvae become older their microbiota also changes, as seen by Jensen et al. (2004), finding that feeding Atlantic halibut larvae had a more complex DGGE profiles then with non-feeding larvae. The microbial community at 15 dph is clearly in a transient developmental phase with dominant species

found in the larvae as well as in the surrounding water. A similar change through time is seen in mammals and both age and diet have been found to affect the gut microbiota (Mozes et al., 2008). It has been observed for example that by giving rat pups a higher energy diet it resulted in the gut microbiota being modified leading to an obesity phenotype (Mozes et al., 2008). Once gut microbiota are established at an older age the community becomes relatively stable (Jensen et al., 2004, Mozes et al., 2008). Therefore it is vital if any changes are to be made it must be done during this transition time of establishment in the juvenile phases.

The second phase of this study was the assessment of probiotic bacteria and was undertaken with the aim of ultimately being able to alter the bacterial communities associated with striped trumpeter larvae live feeds that typically include rotifers and *Artemia*. The end goal was to produce positive benefits, such as increased growth rates, reduction of deformities, increased health of larvae and ultimately better survival.

Through the screening of isolated bacteria using an antimicrobial agar plate assay, five candidate strains were further assessed (strains V52, ST18, V8, ST14 and ST7). Of these strains all but strain ST18 were *Vibrio* spp. Strain ST18 belonged to the genus *Pseudoalteromonas*. All strains showed some antimicrobial activity when screened against known *Vibrio* pathogenic strains. Isolates ST18 and ST7, identified as *Aliivibrio fischeri*, were found to

cause the least mortality in rotifers and *Artemia* and protected the *Artemia* when challenged with a virulent strain of *V. proteolyticus*. Results suggest that probiont *Pseudoalteromonas* sp. ST18 successfully competes with larvae pathogen *Vibrio proteolyticus* and interferes with its pathogenesis (see below). Rotifer and *Artemia* survival experiments were consistent with this result. The conclusion was that isolate ST18 showed potential as a probiont for rotifers and *Artemia* feed cultures and to potentially diminish the growth of *Vibrio* in these cultures. The reduced growth of the *Vibrio* in these cultures may be due to ST18 having out-competed the *Vibrio* through production of water soluble antimicrobial compounds.

Approaches were also explored that would ensure that the potential probiotic isolates were successfully delivered to the larval fish. Delivery of potential probionts was tested by using probionts added directly to the seawater and by enriching live feeds with the probionts. TRFLP profiling analyses were also used to see if the probionts could be readily detected during the survival experiments. Using probiont-unique TRFs it was possible to detect the probionts in both the rotifer and *Artemia* experiments. It was also possible to observe changes that the isolates made to the overall microbial communities structure associated with rotifers and *Artemia,* and was assessed using non-parametric ordination of the TRFLP profile data. Here it was seen that when the probionts were added the community shifted to one becoming dominated

by ST18 while in the absence of ST18 TRFs typical of *Pseudoalteromonas* spp. were never detected (Chapter Three section Three).

The mechanism for the protection conferred by probiotic strain ST18 could include : 1) inhibition of colonisation and growth of the pathogenic strain by efficient competition for nutrients and/or by the production of inhibitory secondary metabolites, a common feature of the genus *Pseudoalteromonas* (Bowman 2007); 2) inhibition of the pathogenicity of V760 through destruction of secreted toxins or by preventing their expression by interference with quorum sensing that may activate pathogenicity factors; 3) stimulation of the immune response of the *Artemia*; or 4) a combination of the aforementioned mechanisms.

In the study it was found that the addition of bacteria (at 10^4-10^5 cells ml) to the yolk sac larvae had the undesirable effect of dissolved oxygen levels falling to critical levels. This was likely due to direct bacterial activity. Even with this phenomenon it was still possible to determine that the larvae still acquire the probionts within their gastrointestinal tracts.

During the first feeding experiment it was found that there were no significant differences between the control and the enriched rotifers treatment indicating the probionts did not have any unexpected deleterious affects on the larvae. It was encouraging to find that it was also possible to track the probionts

through the direct use of TRFLP analysis by identifying their unique TRFs. In the TRFLP experiments ST18 was only detected in the treatments where it was added, further proving that TRFLP is a useful way to tracking a bacterial species within a mixed microbial community.

In the third phase of the study probiont ST18 was administered to striped trumpeter larvae in live feed, by direct addition to tank water and by a combined live feed/direct addition approach. It was clearly determined which probiont's addition approach colonized and worked most effectively in terms of growth and survival of the larvae. The data strongly indicated that the most effective approach involved live feed additions, and is advantageous possibly due to colonization of the probiont within rotifers and subsequent transfer to larval fish following digestion.

Studies concerning the use of beneficial bacteria in aquatic production systems have focused on increased performance measured by survival, boosted disease resistance in animals, and increased capabilities under stress usually after receiving a single species probiotic treatment (Kesarcodi-Watson et al., 2008). The use of probiotics shows increasing promise as a strategy that can be used to reduce hatchery mortality and promote larval rearing performance. It has been successfully applied in crustacean and marine fish hatcheries (Gomez-Gil et al., 2000; Panigrahi and Azad, 2007; Kesarcodi-Watson et al., 2008). However, the diversity of species raised in

hatcheries and the specifics of the bacterial interactions means that there is no universal solution to problems associated with fish health.

Striped trumpeter are reared in a very controlled manner, with water quality being monitored and the water being ozonated or UV treated and thus it can be thought that any addition of bacteria to this 'clean' environment may incur problems (Battaglene and Morehead, 2006; Battaglene and Cobcroft, 2007). This was demonstrated in the experiments where the probiotic bacterial strains were added directly to the water. It may be concluded that the mortality rate was higher here as the larvae had no other bacteria present and are relatively sensitive to impacts on water quality. With this in mind, further studies into the effectiveness of the probiont are needed including an appraisal of protective capacities under a range of conditions. These could include experiments introducing the probiont to the larvae that are cultured in non-treated water. In practical terms, if this is achieved it could be simultaneously shown that the probiont is safe and effective. In the case of the specific studies performed in this thesis, the experiments would benefit from running for longer periods in order to determine if changes in the larval microbiota are stable. And also there is the need to better define the numbers of starting probiont cells to be added to the tanks, as it is possible much smaller inocula will yield the same affect, especially if added periodically.

As with all scientific studies many more new questions arise while trying to answer other questions. This area of research must be further explored to expand the understanding of the interactions of bacteria in marine animals and in order to better define their potential benefits and detriments, especially in the context of intensive aquaculture systems. For example, in relation to this study, it is important to gain a greater understanding of the way strain ST18 interacts in inhibiting pathogenic activity and its specific effects on fish larvae.

Gaining knowledge into the bacterial diversity of the striped trumpeter and the changes that different rearing conditions bring upon it warrants further investigation. This could involve more targeted isolation and screening of bacteria associated with larvae cultures to assess potential beneficial roles in larval growth and possible probiotic capabilities. Answering these questions would provide useful underpinning knowledge for the application of probionts in aquaculture systems.

There are so many questions to ask and research to be performed in regards to this research. As Claude Levi-Strauss said "*The scientific mind does not so much provide the right answers as ask the right questions.*"

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