Seafood molecular biologist: mapping microbial communities in seafood production and processing environments to improve targeting intervention strategies

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Project No. 2008/768

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Non-Technical Summary

Project 2008/768 Seafood molecular biologist: mapping microbial communities in seafood production and processing environments to improve targeting intervention strategies.

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PROJECT OBJECTIVES:

1. Identify specific microbial causes of oyster larval infection, determine microbial species that can reduce their presence and viability, and define environmental conditions that influence their presence in hatchery operations.
2. Define specific spoilage organisms that impact the quality of processed fresh fish, define processing conditions and intervention strategies that influence their presence, and identify microbial species that can competitively exclude spoilage organisms.
3. Characterise the spoilage communities in prawn supply chains.

OUTCOMES ACHIEVED

The first outcome of the suite of projects was the development and publication of a new method to analyse bacterial communities in algal cultures. The 16S rRNA gene is used as a taxonomic marker in microbiology. This gene in algal chloroplasts is similar to the 16S rRNA gene in bacteria. This means that in a mixed algae and bacterial sample the algal DNA is preferentially amplified over bacterial DNA amplified by PCR. We designed a primer that prevents the amplification of the algal DNA allowing the amplification and analysis of the bacterial DNA. This method will be applicable in any research with bacteria and microalgae.

The study of an oyster hatchery in Tasmania is the most extensive study of the microbiology of shellfish hatcheries of which we are aware. The structure of microbial communities in the seawater, algal feed, tank biofilms and larvae themselves and the variation in these communities was examined in relation to the success of larval production. We found that the bacterial communities in all compartments of the hatchery were characteristic of that compartment but varied significantly over time. The water treatment processes in place at the time did not result in a more stable bacterial population in the water that the larvae were exposed to. We did not find a single microbial group that was associated with either the production of healthy larvae or the occurrence of mass mortality. The information on the diversity and variability of the communities present in the hatchery has allowed hatchery managers to take a broader view of water quality than can be provided by presumptive *Vibrio* counts. The hatchery has changed their water treatment program and it is now more consistent and stringent.

The study of Atlantic Salmon has produced evidence that the organisms responsible for the spoilage of Australian modified atmosphere packed Atlantic Salmon are different to the generally accepted northern hemisphere specific spoilage organism *Photobacterium phosphoreum*. This is important because models and tools that relate storage conditions to product quality and shelf-life are based on the characteristics of the spoilage organisms. The model developed in this study is more relevant to the Australian industry than currently available models, and we anticipate that the use of this model will allow producers to make more accurate estimations of bacterial numbers and shelf-life, design better modified atmosphere packages, and plan and monitor cold transport chains.

Bacterial spoilage communities in different prawn fishery and supply chains were identified using the same samples as project 2011/748. The identification of the spoilage bacteria complemented the work on prawn quality in project 2011/748 by providing the microbiological reasons for the observed differences in quality in among the different supply chains.

LIST OF OUTPUTS PRODUCED

The outputs from this set of projects are:

- A database of sequences describing microbial ecology of an oyster hatchery
- A database of sequences describing the spoilage communities of Australian produced Atlantic salmon
- Model for effect of temperature and carbon dioxide on the growth of spoilage bacteria on modified atmosphere Atlantic salmon.
- A database of sequences describing the spoilage communities of Australian wild caught prawns

Four papers were published in peer-reviewed scientific journals and two more are in preparation. The published papers have already been cited in the scientific literature.

1. *Milne D., Powell S.M. 2014. Limited microbial growth in Atlantic salmon packed in a modified atmosphere. Food Control 42:29-33.*
2. *Powell S.M., Chapman C.C., Bermudes M. and Tamplin M.L. 2013. Dynamics of seawater bacterial communities in a shellfish hatchery. Microbial Ecology. 66:245–256.*
3. *Powell S.M., Chapman C.C., Bermudes M. and Tamplin M.L. 2012. Use of a blocking primer allows selective amplification of bacterial DNA from microalgae cultures. Journal of Microbiological Methods 90:211-213.*
4. *Powell S.M. and Tamplin M.L. 2012 Microbial communities on Australian modified atmosphere packaged Atlantic salmon. Food Microbiology 30:226-232.*

In preparation:

1 .Powell S.M., Ratkowsky D.A. and Tamplin M.L. Predictive model for growth of spoilage bacteria on Australian modified atmosphere packaged Atlantic salmon.
2. Powell S.M., Tonkin R., Howieson J. and Tamplin M.L. Changes in sensory quality and microbial communities during storage of wild caught Australian prawns.

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General Introduction

Until the 1990s, when it became possible to access and analyse DNA directly from the environment, the study of microbiology was limited to studying those microbes that were able to be grown in artificial culture. The advent of DNA-based methods provided a way to access and study the enormous diversity of microbes that actually exist. Some fields of microbiology were quicker to take up this technology than others. Microbiological analyses carried out in industrial settings have, although this is changing, remained culture-based because they are standard methods required by regulators. They tend to be technically straight-forward and inexpensive. The goal of the Seafood Molecular Biologist was to apply DNA-based techniques to a range of existing problems within the seafood industries. The three projects developed covered shellfish aquaculture, the processing of Atlantic salmon and supply chains in the wild prawn fishery. Due to the varied nature of these projects, they are treated separately in this report.

1. The role of microbial communities in oyster hatcheries

1.1 Introduction and Background

Bacterial disease is one of the largest constraints on aquaculture production world-wide, with bacteria from the genus *Vibrio* the most frequently reported pathogens (Paillard 2004, Prado 2005). However these bacteria are also found associated with healthy larvae (Elston et al., 2008) suggesting that they are not always pathogenic. Oyster larvae are also sensitive to many common chemicals and toxins at very low concentrations (Dove and O'Connor, 2007) and the complex interaction between larvae, their environment and the bacteria they are exposed to determine whether larvae become diseased or not. This relationship has been described as the disease triangle (McNew, 1960): the interaction between the susceptibility of the host, the virulence of the pathogen and the environmental conditions at the time of infection. Studies that only examine one of these factors may miss important factors that can assist in the prevention of epizootics and the mass mortality of larvae.

Within an oyster hatchery different bacterial communities exist in multiple 'compartments' in the hatchery, each of which is subject to different environmental factors and each of which present different opportunities for the bacteria to interact and affect the larvae. The quality of the water in which the larvae grow is particularly important and is measured by both its chemical and biological characteristics. Hatcheries generally have several treatments in place to reduce the number of potentially pathogenic bacteria in water. This can include filtering at various levels, foam fractionation (to remove proteinaceous material) and UV sterilisation followed by microbial maturation (re-colonisation of the water). These processes will have different effects on bacteria in the water but will not necessarily remove all toxins or other metabolites produced by bacteria and microalgae in the intake water that may still affect larval health. Most of the work that has been reported on the effectiveness of water treatments on larval health has been carried out in finfish rather than shellfish culture (for example Salvesen et al. 1999; van der Meeren et al., 2011).

The algae provided to larvae as food are also a significant route of exposure to bacteria, as larvae directly ingest algae. Algal culture is carried out under strict conditions to limit the growth of potentially pathogenic microbes, however the nutrient-rich environment required to grow the algae provides an ideal environment for bacterial growth as well. The relationship between algae and bacteria has been shown to affect the survival of young larvae (Alabi et

al., 1999; Nicolas et al., 1989). This may be through toxins produced by either bacteria or algae (Brinkmeyer et al., 2000l Croci et al., 2006) or by changing the nutritional value of the algae or through the effect of algae on the bacterial community structure (Lam and Harder, 2007). Understanding the interactions between bacteria and algal cultures is therefore important for understanding and controlling production in shellfish hatcheries where micro algae are the main food source.

Some species of bacteria from the genus *Vibrio* are known to cause disease of oyster larvae. As a result, plate counts for the quantification of *Vibrio* using thiosulfate-citrate-bile salts-sucrose (TCBS) agar are the most common water quality measure carried out in hatcheries. It has been shown however that this medium is not completely selective for *Vibrio* with one study finding that at least 39% of the isolates obtained using TCBS agar were not *Vibrio* (Pfeffer and Oliver 2003). In addition, there is not always a clear link between the numbers of either *Vibrio* or the total culturable bacteria in the water and the occurrence of disease (Bourne et al. 2004, Verner-Jeffereys et al. 2004). The culturable fraction of the marine bacterial population is only between 0.01 and 12% of the total viable population (Ferguson et al. 1984), hence it is possible that important interactions with other organisms are not being detected when only plate counts are used to describe the microbial ecology of a hatchery.

Our goal was to improve understanding of the microbial ecology of an oyster hatchery so that hatchery managers had evidence on which to base disease management decisions. Extensive sampling of all compartments of the hatchery was conducted over a two-year period including comparisons of two facilities run by the same company. To gain a more detailed and thorough picture than previously possible, both standard plate count and molecular (DNA based) methods were used.

1.1.1 Need

Oyster hatcheries are responsible for spawning oysters and raising the larvae to “set” in order to produce spat for oyster farmers. It is vital for the oyster industry, as a whole, that there are reliable supplies of spat. Mass mortality events have been reported in hatcheries world-wide and are a recurrent and serious problem for the industry as they can result in the loss of entire batches of larvae, often after 10 to 12 days of rearing larvae. This mortality is often reported to be due to vibriosis, a disease caused by several species of bacteria from the genus *Vibrio*, because high numbers of *Vibrio* spp. can be isolated from dead animals. However, the role of other bacteria in this process, in terms of both promoting and suppressing disease, has not been thoroughly investigated. To be able to implement strategies to prevent the recurrence of these mass mortality epizootics, it is important that the microbial ecology of the hatchery is more thoroughly understood.

1.1.2 Objectives

The overall objectives of this project were to identify specific microbial causes of oyster larval infection, determine microbial species that can reduce their presence and viability, and define environmental conditions that influence their presence in hatchery operations.

The specific objectives were to:

- develop methods for sampling and measuring microbial communities in all inputs into a shellfish hatchery (intake seawater, tank biofilms, algal feed);
- describe the microbial communities found within shellfish hatcheries;
- determine the variability in microbial communities found in the different compartments of the hatchery; and
- determine if there are particular groups of microbes associated with the occurrence of mass mortality events or production of healthy oyster spat.

1.2 Methods

1.2.1 Sampling site: hatchery description

The commercial *Crassostrea gigas* hatchery was located in Tasmania, Australia, and was studied between September 2009 and May 2011. During this time, some production runs resulted in the production of healthy spat but several ended with a mass mortality event in which over 80% of larvae died.

Seawater used in the hatchery was obtained from a neighbouring bay. The water was subject to various treatments including foam fractionation and filtration (to a minimum of 1 µm) and heating to 26 °C prior to use; this water was referred to as 'clean seawater'. Upon addition of larvae and algae to the tanks, the water was designated 'tank water'. Two types of tanks were used. The batch system consisted of 11,500 L tanks filled approximately 16 h prior to the addition of larvae and used for a period of 48 h before tanks were changed. The flow-through system was conducted in 1,000 L tanks with a continuous flow of clean seawater at a rate of 0.6–3.0 L/min. These tanks were changed every 24 h. During water changes, larvae were collected on 60 µm screens, separated into size classes and culled as required before being placed into new tanks. Tanks were cleaned, chlorinated and allowed to dry for at least 12 h before being filled again.

Larvae were spawned either by strip-spawning or by "natural" spawning using controlled temperature. Eggs and sperm were combined and allowed to fertilise for approximately 1 h at 25 °C before being placed into batch tanks at densities of 30-45 larvae/ml. Some larvae remained in the batch tanks but

some were transferred to flow-through tanks at any age beyond four days depending on operational requirements.

Larvae were fed a mixture of live diatom and flagellate algae species grown on site. Three algal species were used in mixes that changed throughout the production cycle to suit changing dietary requirements. *Chaetoceros calcitrans* was batch-cultured under axenic conditions in autoclaved carboys. *Isochrysis galbana* and *Pavlova lutheri* were grown in a flow-through 500 L bag system in axenic conditions with filtered (1-10 µm) and pasteurised seawater (heated to 80 °C for 1 h). All algae species were grown at 22 °C with nutrient media and aerated with CO_2 enriched (1% CO_2) air. Feed charts were used to determine feed rates based on assessment of larvae size and health and the amount of residual feed in the tanks. The production cycle took at least two weeks and metamorphosis of eyed pediveligers was induced with epinephrine and spat were transferred to a "nursery" if they survived to this stage.

1.2.2 Sampling

Samples were collected in triplicate. Intake seawater was sampled from the intake pipe as it entered the hatchery. Clean seawater used in larval tanks was sampled either directly from the tanks prior to the addition of food and larvae or from the tap used to fill the tanks (when the tanks were not in use). Water from tanks with larvae and food was referred to as 'tank water' samples. Water samples were collected from the top 300 mm of the tanks and filtered through an alcohol-disinfected 60 µm screen to remove larvae. Approximately 500 ml was filtered through a 0.22 µm filter for DNA extraction. Algae were sampled from a composite harvest line from 10-20 upright 500 L bags. Biofilms on the sides of emptied tanks were sampled with sterile swabs. Fertilised egg samples were collected from the bucket in which fertilisation occurred using 20 µm screens. Larvae samples were collected from the top 300 mm of tanks using a PVC pipe with a 60 µm screen on one end. Larvae were washed with sterilised seawater and a 1.0 ml aliquot of the larval suspension transferred into a sterile glass grinding tube (Corningware). Larvae were counted in 3 × 0.1 ml subsamples before the remainder was homogenised until particulates were no larger than 5 µm at their greatest diameter (measured by light microscopy). The homogenate was used for plate counts and the non-homogenised larvae were stored frozen at -18 °C prior to DNA extraction. All other samples were used within 2 h of collection for plate counts and the remainder frozen at -18 °C until DNA extraction.

1.2.3 Bacterial enumeration by plate counts

Viable counts for total heterotrophic marine bacteria and *Vibrio* spp. were carried out using plate counts on marine agar (5 g peptone, 5 g yeast extract, 35 g red sea salt and 15 g agar per litre) and TCBS (Oxoid) agar. Replicate

samples were serially diluted and three dilutions plated out. Plates were incubated at 26 °C for 24 h (TCBS) or 48 h (marine agar) before counting.

1.2.4 Development of blocking primer for algae samples

Initial analysis of the bacterial communities in algal cultures used as larvae feed showed that algal chloroplast DNA was also amplified in the PCR, and thus interfering with the clone library and tRFLP results. To prevent this, a blocking primer was developed following a similar procedure to that in Vesiheim & Jarman (2008). The blocking primer, 907R_block (TGAGTTTCACCCTTGCGAGCG_C3 spacer) was validated by clone library construction which showed that the proportion of algal sequences detected decreased from 70% to 8% (Powell et al. 2012). The blocking primer was added to all PCR reactions that were using DNA extracted from algal cultures as template DNA. The blocking primer was used at 20 times the concentration of the other primers.

1.2.5 Microbial community structure analysis

The structure of the microbial communities throughout the hatchery were examined using a combination of DNA-based techniques. These techniques all used DNA extracted directly from an environmental sample as the basis for the analysis, hence overcoming well-known limitations of culture-based methods. Terminal restriction fragment length polymorphism (tRFLP) produces a characteristic fingerprint of microbial communities that is dependent on the dominant species present. It does not allow identification of bacteria present in a sample but is useful for comparing many samples simultaneously. To identify the bacteria present in a sample from the environmental DNA, previously the best method involved construction and sequencing of clone libraries. In this method, individual fragments of DNA were separated, replicated and then sequenced. The sequences were then compared to databases to determine identity. This method provided good quality, detailed information but was labour intensive and expensive. Over the last five years, new sequencing technologies have become available that generate 10 to 50 times as many sequences. Once this technology (pyrosequencing or next generation sequencing) became economical we have used this method to both generate fingerprints and identify the bacteria present in environmental samples.

DNA extractions.

DNA was extracted from water samples using the RapidWater DNA kit (MoBio) following the manufacturer's directions.

DNA was extracted from biofilm swab samples using the UltraClean Microbial DNA kit (MoBio). Swab tips were placed into a tube and 30 ul of the microbead solution was added and tips were vortexed for 1 min. The

supernatant was transferred to the microbead tubes and the manufacturer's directions followed. A method modified from that of Burke et al. (2009) was used to extract microbial DNA from algal culture samples. Briefly, 0.5 ml of rapid enzyme cleaner (3M Australia) and 1.0 ml of 0.5 M EDTA were added to a 50 ml algal sample. This was incubated at room temperature for 2 h with gentle shaking. This mixture was centrifuged for 30 min at 1000 x g. The supernatant was removed and DNA extracted from the supernatant using the UltraClean 15 kit (MoBio).

DNA was extracted from larvae and egg samples following a protocol modified from Griffiths et al. (2000). Approximately 3-4 mm^3 of eggs or larvae was transferred into 2 ml tubes containing 0.1 g each of 0.1 mm and 1.0 mm zirconia/silica beads. 0.5 ml of hexadecyltrimethylammonium bromide (CTAB, pH 8) extraction buffer was added. An equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added and tubes were bead-beaten at 20 oscillations /s for 1 min. Tubes were centrifuged at 16,000 × g for 5 min at 4 °C. The aqueous top layer was extracted with an equal volume of chloroform: isoamylalcohol (24:1). DNA was precipitated with two volumes of polyethylene glycol (PEG, 30%) with 1.6 M NaCl at room temperature for 2 h. The precipitate was centrifuged at 18,000 × g for 10 min at 4 °C, washed in ice-cold 70% ethanol and resuspended in 10 mM Tris buffer (pH 8).

tRFLP

A “fingerprint” of the overall bacterial community structure was obtained by tRFLP analysis of the 16S rRNA gene (a taxonomic marker). This method is useful for identifying similarities and differences in the microbial community structure between groups of samples but does not allow the identification of any microbes present. tRFLP was carried out as described in Appendix 1.

Clone library construction

The egg and algal samples were amplified by PCR using Immomix PCR Mastermix with 0.2 uM of each of the 10F (GAGTTTGATCCTGGCTCAG) and 907R (CCGTCAATTCCTTTGAGTTT) primers. For the algal samples the primer 907R_block was also included as described above. The thermal cycling program was as described for tRFLP. Clean PCR product was cloned using the TOPO-TA cloning kit (Invitrogen) according to the manufacturer's standard protocol. Clones were sub-cultured and placed directly into a PCR reaction with the M13 primers (F: GTAAAACGACGGCCAG and R: CAGGAAACAGCTATGAC). Successful amplifications were precipitated using ethanol and the clean, dry PCR product sent to Macrogen Inc (South Korea) for sequencing. The BLAST algorithm was used to search GenBank for matches with good quality sequences. A phylogenetic tree was constructed in the BioEdit program using the DNADIST and neighbour-joining tree methods. Only sequences of the same region of the 16S rRNA gene

were included in the phylogenetic tree although several other sequences could be identified to phylum level by their closest matches in GenBank.

1.2.6 High throughput amplicon sequencing (pyrosequencing)

To examine the microbial communities present in the water more closely and to see if subtle differences in the bacterial population were correlated with production outcomes, a sub-set of water samples were subject to 16S rRNA gene tag pyrosequencing.

Tag-encoded FLX amplicon pyrosequencing of the V1 and V2 regions was carried out by Research and Testing Laboratories (Lubbock, Texas) using a Roche 454 FLX instrument with titanium reagents and the primers 28F (5'TTTGATCNTGGCTCAG) and 519R (5'GTNTTACNGCGGCKGCTG) as described in Dowd et al. (2008) and Wolcott et al. (2009).

The data were processed using the MOTHUR package (Schloss et al. 2009) available from http://www.mothur.org/. The MOTHUR implementation of Pyronoise (Quince et al. 2009) was used to remove poor quality sequences. The dataset was then trimmed to remove barcode and primer sequences, sequence reads that were less than 200 bp long, sequences with more than one error in the barcode or primer sequence or a homopolymer of 8 bp or more. Each library was screened for chimeric sequences using B2C2 (Gontcharova et al. 2010) with the lower threshold set to 3 and the upper threshold set to 5 taxonomic levels. Good-quality, non-chimeric sequences from all libraries were combined and aligned using ClustalW. Sequences were grouped into OTU at the 3% dissimilarity level using the furthest neighbour clustering method in MOTHUR and a table of how many times each OTU appeared in each library was generated. Any OTUs represented by only one sequence were removed as potentially chimeric sequences not detected in the chimera screening process. Finally, the taxonomic position of a representative sequence from each OTU was assigned using the Classifier tool on the Ribosomal Database Project website (http://rdp.cme.msu.edu) (Cole et al. 2009).

1.3 Results

1.3.1 Method development

The first algal clone library analysed contained a very high proportion of sequences from algal chloroplasts (70%) rather than bacterial and it was decided to design a blocking primer to prevent preferential amplification of algal chloroplast sequences. Another clone library was constructed from this sample but the blocking primer 907R_block was included in the first PCR step. In this library the proportion of algal chloroplasts sequences was reduced to only 8%. However, because the design of the blocking primer was

based on the 16S rRNA sequences from *Isochrysis galbana* chloroplasts (a major component of the algal diet used to feed young larvae), it did not completely block the amplification of all chloroplast sequences. In all libraries one or two sequences from diatoms were observed and in one clone library, a high percentage (23%) of sequences from *Pavlova lutheri* was observed.

1.3.2 Microbial communities in hatchery compartments

Algal feed

A lack of growth on TCBS plates indicated that *Vibrio* spp. were absent (or present in numbers less than 1 CFU ml-1) in the algal cultures. The number of bacteria on marine agar plates varied between 1×10^5 and 1×10^7 CFU ml^{-1}. After the nutrient level in the media used to grow the algae was decreased, there was a significant decrease in the total number of bacteria present from an average of 5.2×10^6 (SE 1.1×10^6) CFU ml^{-1} to 9.1×10^5 (SE 3.4×10^5) CFU ml^{-1} (Students t-test $p < 0.001$).

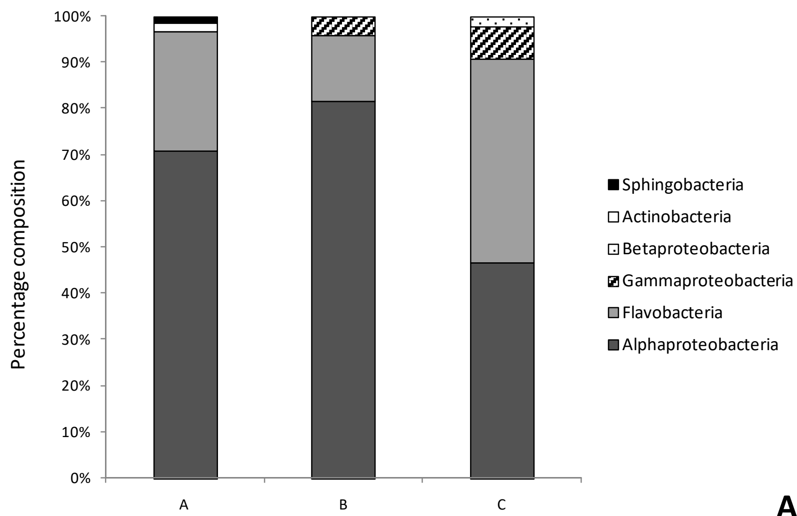
Three clone libraries were constructed from algae samples to identify the dominant bacteria present. The level of nutrient supplied to the algal cultures was reduced during the study, resulting in three sample types: high nutrient + mass mortality (A), low nutrient + healthy spat (B) and low nutrient + mass mortality (C). A comparison of the composition of each clone library is shown in Figure 1.1 by class (Fig. 1.1A) and by genus (Fig. 1.1B). Library B (low nutrient + healthy spat produced) was more diverse than the other two libraries with a species richness of 31 phylotypes compared to 23 in library A and 18 in library C. All three libraries were dominated by bacteria from the class Alphaproteobacteria. There were twelve different genera present in libraries A and C and sixteen in library B. Three genera were present in all libraries: *Martelella*, *Sphingopyxis* and *Phaeobacter*. Three genera (*Sulfitobacter*, *Erythrobacter* and *Citromicobium*) were present in both libraries A and B and five genera were only present in one library. Gammaproteobacteria were only found in the two libraries from algal cultures supplied with a low level of nutrients (B and C). Different genera from the class Flavobacteria were also present in all three libraries but were a greater proportion of the libraries in algal feed from runs that suffered a mass mortality event (A and C) than in the algal feed from the run that produced healthy spat (B). However, despite consisting of a smaller proportion of the library, clone library B contained a greater diversity of Flavobacteria (4 phylotypes) than the other libraries (2 phylotypes each). The sequences from these clone libraries were deposited in GenBank under accession numbers JF707640- JF707766.

Intake and clean seawater

The most thorough description of the microbial communities present in both the intake and clean seawater was provided by the high throughput pyrosequencing of a small set of samples collected between October 2009 and January 2011 (Table 1.1). All samples were dominated by bacteria from the class Alphaproteobacteria (Figure 1.2). The classes Flavobacteria and Gammaproteobacteria were the next most numerous in all samples except for December 2009 and January 2010 (early and late summer 2009) in which the Cyanobacteria were more numerous. Several classes were present as a small (less than 2%) fraction of each library and for clarity these were grouped together as "Other Classes". This group included Actinobacteria, Deltaproteobacteria, Betaproteobacteria, Verrucomicrobiae, Spirochaetes, Acidobacteria, Sphongobacteria, Planctomycetacia and Bacilli; these groups were present in nearly every sample but in comparatively low numbers.

Table 1.1: Samples used for pyrosequencing analysis,

Sample	Type	Season	Production Outcome
October 2009	Intake and clean seawater	Spring	Mass mortality
December 2009	Intake seawater	Early summer	Healthy spat
January 2010	Intake seawater	Late summer	Mass mortality
September 2010	Intake and clean seawater	Early spring	Healthy spat
November 2010	Intake seawater	Early summer	No larvae present
January 2011	Intake seawater	Late summer	Healthy spat



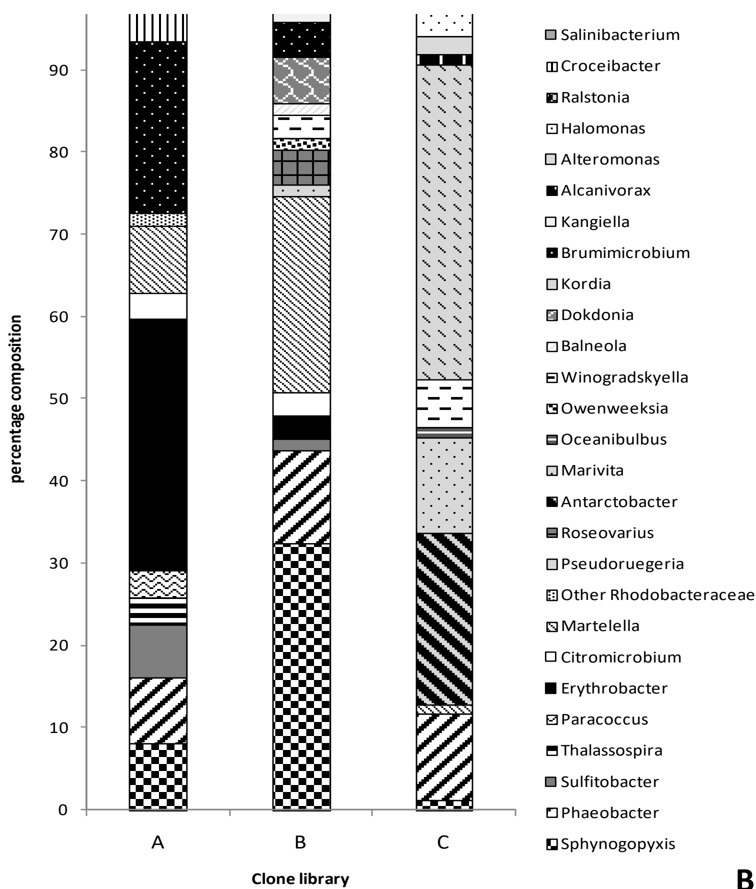


Figure 1.1: Comparison of microbial communities in algal cultures based on clone library analysis of samples indicated in Figure 2. Graphs show percentage composition of each library based on class-level (A) or genus-level (B) identification of sequences. Library A = high nutrient level, mass mortality event, library B = low nutrient level, healthy spat produced, library C = low nutrient level, mass mortality event.

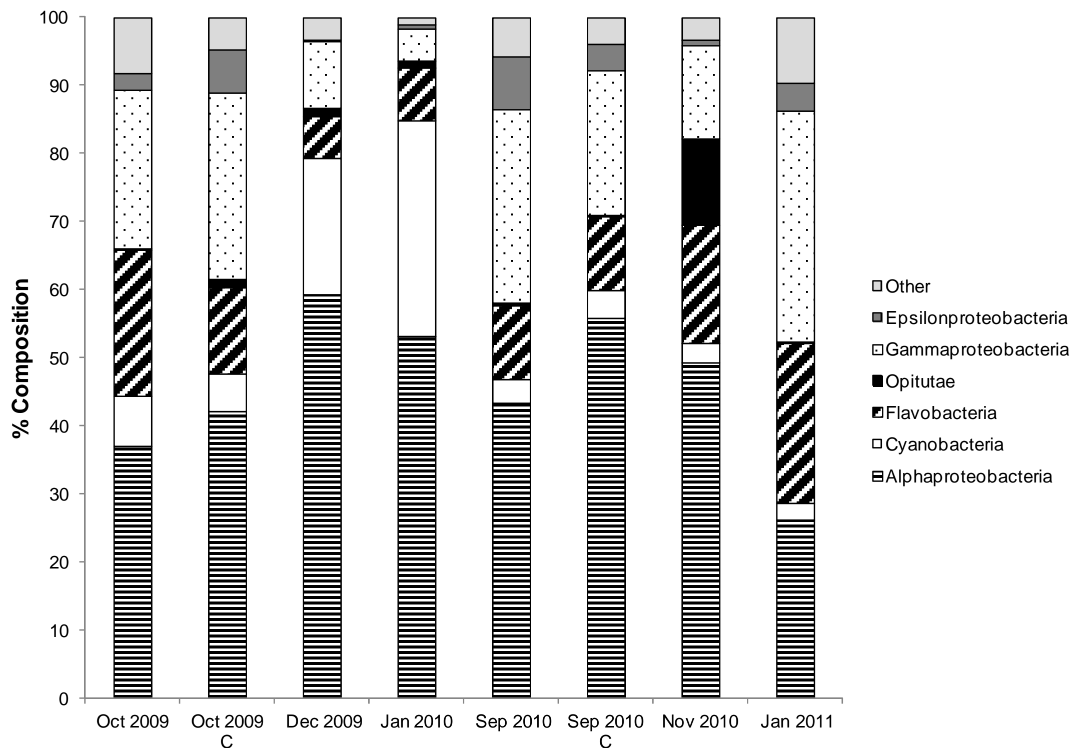


Figure 1.2: Bargraphs showing average percentage composition of bacterial communities in seawater samples based on pyrosequencing and aggregation of the data to class level. The group “other classes” contains the Actinobacteria, Deltaproteobacteria, Betaproteobacteria, Verrucomicrobiae, Spirochaetes, Acidobacteria, Sphongobacteria, Planctomycetacia and Bacilli. All samples are intake seawater except those labelled “C” which indicates clean seawater.

Eggs
As initial attempts to extract DNA from egg samples were not successful, samples were plated onto marine and TCBS agar and isolates tentatively identified by partial sequencing of the 16S rRNA gene. The isolates belonged to the genera *Staphylococcus*, *Moritella*, *Pseudoalteromonas*, *Tenacibaculum* and *Vibrio*. The *Vibrio* spp. isolates grouped with the *V. splendidus* and *V. lentus* groups.

By using a combination of methods, DNA was extracted from an egg sample and a clone library constructed. Seventy-four sequences belonging to 30 different phylotypes in seven phyla were retrieved (see Table 1.2). The Bacteroidetes and Actinobacteria are the most numerous and, along with the Alphaproteobacteria, the most diverse classes. Several phylotypes, including the two dominant phylotypes (one Bacteroidete and one Actinobacterium) have been associated with diatom blooms (eg GenBank accession number FN433392 (unpublished data)). This may reflect the fact that the broodstock oysters were sourced from an open farm environment where they were exposed to the ocean rather than from tanks within the hatchery. The Betaproteobacterial clones are associated with fish, both as gut organisms in healthy fish and associated with fungal disease in fish (GenBank accession number DQ226068 (unpublished data)).

The strains isolated by culture were from different genera compared to those observed in the clone library. This may be because samples used for culturing and for cloning were collected on different days, or it may reflect the culture conditions used to obtain the isolates. Three different *Vibrio* spp. strains were isolated that may have originated in the broodstock oysters or from the water from which the oysters were taken. The lack of *Vibrio* spp. sequences in the clone library suggests that they are not a dominant part of the microbial community found in the eggs.

Table 1.2: Diversity of egg clone library sequences within each phylum.

Phylum	**Number of phylotypes**	**Number of sequences in each**
Gammaproteobacteria	2	1,1
Firmicutes	3	6, 1, 1
Betaproteobacteria	2	7, 1
Alphaproteobacteria	6	1,1,1,1,1,1
Bacteroidetes	8	11, 5, 3, 1, 1, 1, 1, 1
Actinobacteria	7	15, 4, 2, 2, 1, 1, 1
Planctomycetes	2	3, 2

1.3.3 Variability in the microbial communities in the hatchery

Temporal variability in clean seawater (within and between production runs)
The clean seawater used in the hatchery for larval rearing was sampled over three different production runs in the 2009-2010 production season. The variability in the bacterial communities found in the clean seawater (i.e. prior to addition or larvae of feed) was examined both within and between runs using tRFLP (Figure 1.3). A comparison of the similarity of the microbial communities to each other is visualised as a non-metric multidimensional scaling plot (MDS) in which more similar samples are located next to each other, the closely together the more related. The MDS plot clearly shows that the bacterial community present in September 2009 was different to that present in December 2009 and January 2010. The latter two runs were less different to each other than to September 2009.

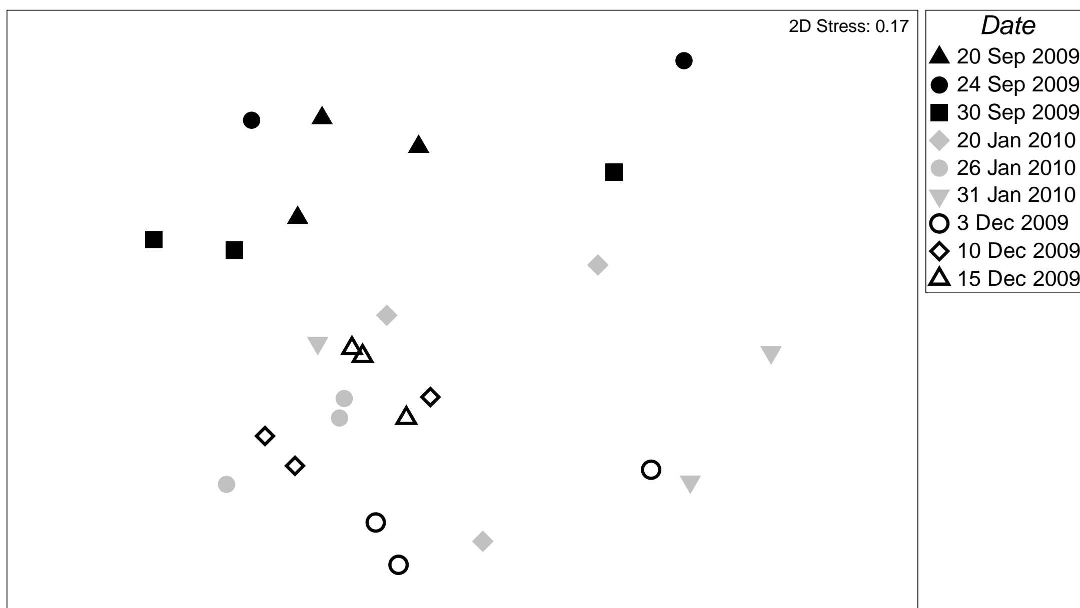


Figure 1.3: MDS plot showing similarity among bacterial communities in the clean tank water during three different production runs visualised on an MDS plot. Black symbols represent September 2009, open symbols December 2009 and grey symbols January 2010. The different shaped samples represent different days within each run. The December 2009 production run was the only one to result in the production of commercial quantities of healthy spat. ANOSIM values for differences between days and runs are given in Table 1.3.

A nested ANOSIM test accounting for multiple days within each production run, showed that the production runs were significantly different to each other, (global test R = 0.572, p = 0.004, pair-wise comparisons in Table 1.3) and also that the different days were significantly different to each other (R = 0.398, p= 0.001). When each run was examined separately, the days within each run were generally, but not always, different to each other (Table 1.3). This indicates that although there was significant variation in the bacterial community structure on a day-to-day basis, the variation on a scale of weeks to months (among production runs) was greater. The difference between the R-values (0.572 for among production runs compared to 0.398 for amongst days) supports this.

Table 1.3 ANOSIM values describing the strength of the dissimilarities among bacterial communities found in the clean water used in different production runs and on different days within each run as measured by tRFLP. Bold groups indicate a significant difference.

Comparison	**R-value (strength of dissimilarity)**	***p* value (significance)[a]**
Between Runs		
Sept 2009 – Dec 2009	0.889	0.1
Sept 2009 – Jan 2012	0.667	0.1
Dec 2009- Jan 2012	0.370	0.1
Within September 2009 run		
day 1 – day 2	0.667	0.1
day 1 – day 3	0.370	0.1
day 2 – day 3	0.001	0.4
Within December 2009 run		
day 1 – day 2	0.519	0.1
day 1 – day 3	0.630	0.1
day 2 – day 3	0.556	0.1
Within January 2010 run		
day 1 – day 2	0.481	0.1
day 1 – day 3	0.148	0.3
day 2 – day 3	0.481	0.1

[a] Due to the number of replicates taken on each day, the minimum *p* value possible was 0.1, hence results with a *p* value of 0.1 are considered significant.

Effect of water treatment on variability in water communities
The difference between the intake seawater and the clean seawater was investigated at three-monthly intervals over a one-year period using tRFLP (Figure 1.4). Although the intake seawater and clean seawater samples did not cluster separately, within each month the two types of water samples were significantly different to each other (two-way crossed ANOSIM R=0.519, p = 0.003). Each month is also significantly different to each other (two-way crossed ANOSIM, global R = 0.667, p = 0.001; pairwise comparisons in Table 1.4). These results indicate that despite the water treatment processes in place, the temporal variability in the bacterial communities in the intake seawater is still present in the treated clean seawater used in the hatchery.

The effect of the water treatment processes was examined further using high throughput amplicon sequencing of intake and clean seawater collected on two different occasions. The pyrosequencing data showed that the difference between the intake and clean seawater samples resulted from the diversity of the clean water samples being less than that of the corresponding intake water samples (Table 1.1). SIMPER analysis of the presence/absence transformed data at the level of genera showed that much of the difference between the intake and clean water was that certain genera were present more often in intake seawater samples than clean water samples. The ten genera contributing most to the difference between the two groups were: *Lutimonas, Aureispira, Propionigenium, Maritimimonas, Plesiocystis, Roseovarius, Desulfotalea, Ilumatobacter, Formosa* and *Agromonas.*

Table 1.4 Results of a two-way crossed ANOSIM with values describing the strength of the dissimilarities of the microbial communities among different months across intake / clean seawater type groups. All comparisons were significantly different.

Comparison	**R-value (strength of dissimilarity)**	***p* value (significance)**
August 2009 – November 2009	0.923	0.01
August 2009 – February 2010	0.852	0.01
August 2009 – April 2010	0.944	0.01
November 2009–February 2010	0.444	0.02
November 2009 – April 2010	0.667	0.01
February 2010 – April 2010	0.759	0.01

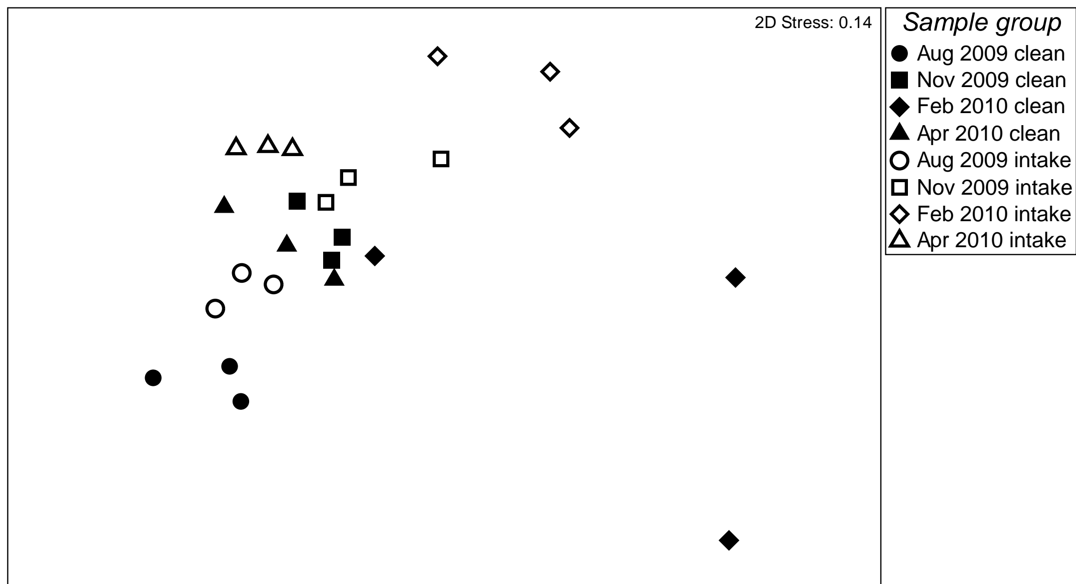


Figure 1.4: MDS plot showing similarities among bacterial communities in the intake seawater (open symbols) and clean seawater (black symbols) over several months: August 2009 (circles), November 2009 (squares), February 2010 (diamonds) and April 2010 (triangles). ANOSIM tests showed a significant difference between intake and clean seawater (R = 0.519, *p* = 0.003) and among months (see Table 1.4).

Variability in communities in algal feed

The overall bacterial community structure was examined using tRFLP (Figure 1.5). This figure shows that the samples clustered by month and by the nutrient level in the algal cultures (high or low). Analysis of similarity tests (ANOSIM) showed that the difference between the high and low nutrient groups was significant (p = 0.001) and strong (R = 0.499). ANOSIM tests also showed that each month was significantly different to the others (Table 1.5), although the months from the low nutrient group were not as different to each other as they were to the high nutrient group (R value of 0.259 compared to 0.499 or 0.594).

The tRFLP analysis shows that the bacterial communities in the algal cultures were dynamic with subtle changes occurring on almost a daily basis. When the communities were analysed by ANOSIM for differences between days, 86% of days that occurred within the same month were different to each other ($p < 0.05$) whilst all comparisons of days from different months were significantly different ($p < 0.05$).

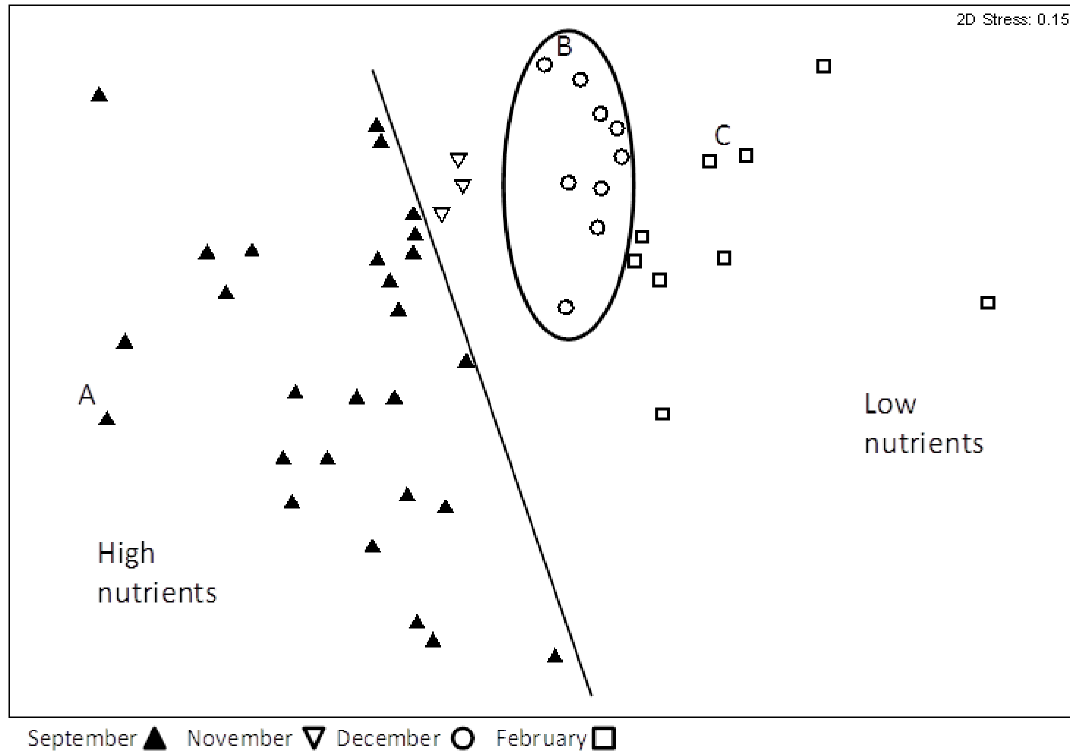


Figure 1.5: Comparison of microbial community structure in algal cultures by tRFLP. The MDS shows the similarity of the communities in each sample; the closer points are to each other, the more similar. Symbols indicate the month that samples were collected. Samples taken when the nutrient supplied to the algae was at the “high” level are on the left of the line and samples from “low” nutrient levels on the right. The circled samples were taken from a run that ultimately produced healthy spat, whereas all others were from runs that suffered mass mortality events. A, B and C are the samples that were used for constructing clone libraries (Figure 1.1).

Table 1.5 Results of ANOSIM tests comparing the strength of the dissimilarity between groups of bacterial communities in the algal feed. Significant differences are highlighted in bold.

Groups	**R**	***P***
High nutrient and low nutrient	**0.499**	**0.001**
Healthy spat and mass mortalities	0.034	0.32
Low nutrient only: healthy spat and mass mortalities	**0.182**	**0.015**
September and December	**0.449**	**0.001**
September and February	**0.594**	**0.001**
December and February	**0.259**	**0.002**

Communities in different compartments of hatcheries
The similarity between the microbial communities in the different components (larvae, algal feed cultures, clean water, tank water and biofilm) of both the Bicheno and Pipeclay Lagoon hatcheries were compared to each other (Figure 1.6). This figure clearly shows that the two hatcheries contained different microbial communities (ANOSIM R = 0.208, p = 0.001).

The microbial communities in the different components of each hatchery were also compared. The similarities of the communities in each hatchery component are shown as MDS plots in Figure 1.7. It can be seen that the different components were distinct (significantly different) from each other within both hatcheries (see Table 1.6 for ANOSIM values) except for the larvae and egg samples that were not different. However, the MDS plot shows that some components were more similar to each other than others. For example, the communities in the two types of water samples, clean water and tank water, were more similar to each other than to the communities in the algal cultures or larvae. The communities in the larvae appeared to be more similar to the algal cultures than the water or biofilm cultures.

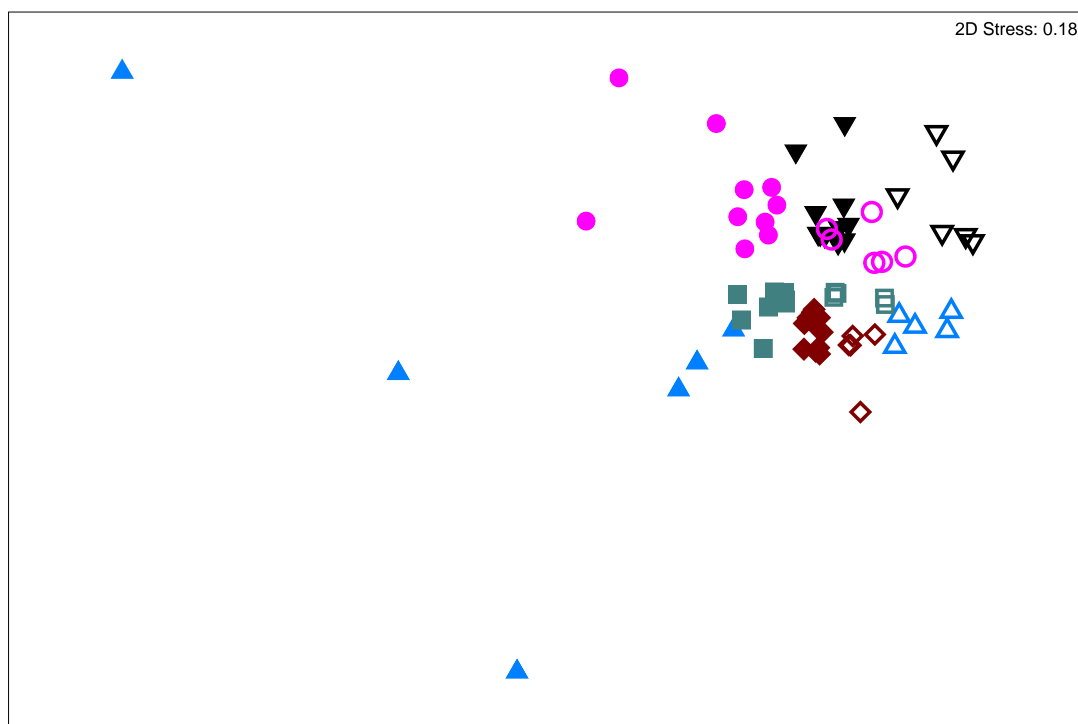
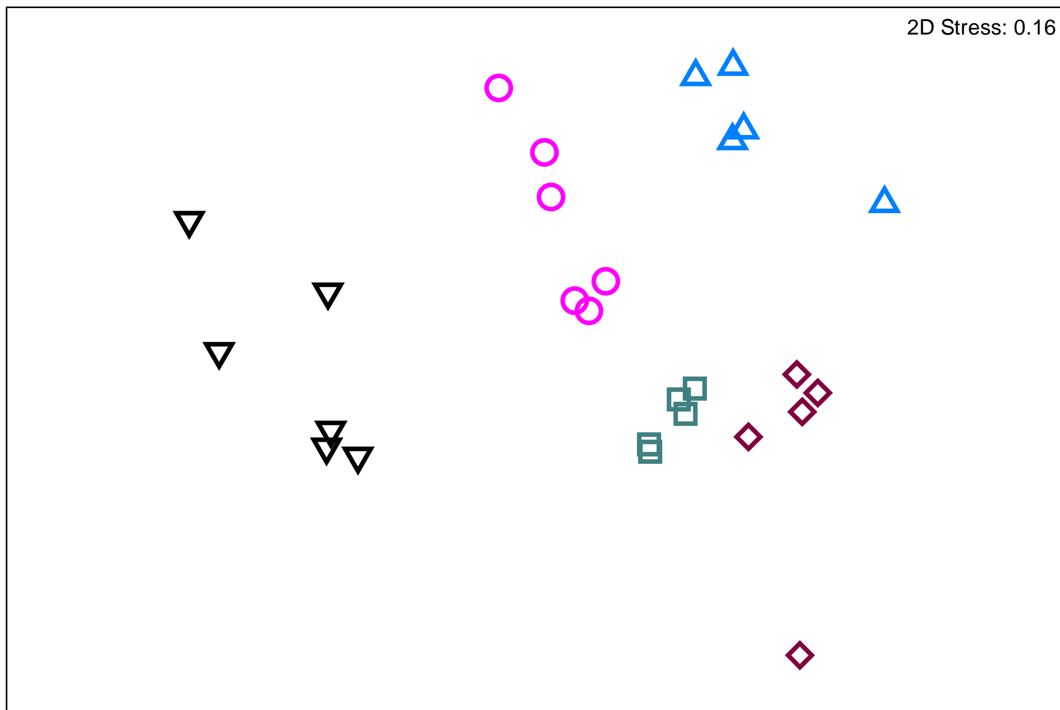


Figure 1.6: MDS plot of the similarity in microbial communities found throughout the Bicheno (open symbols) and Pipeclay Lagoon (solid symbols) hatcheries. The different components shown are larvae (upwards triangle), algal feed (downward triangle), tank water (square), clean water (diamond) and tank biofilm (circle).



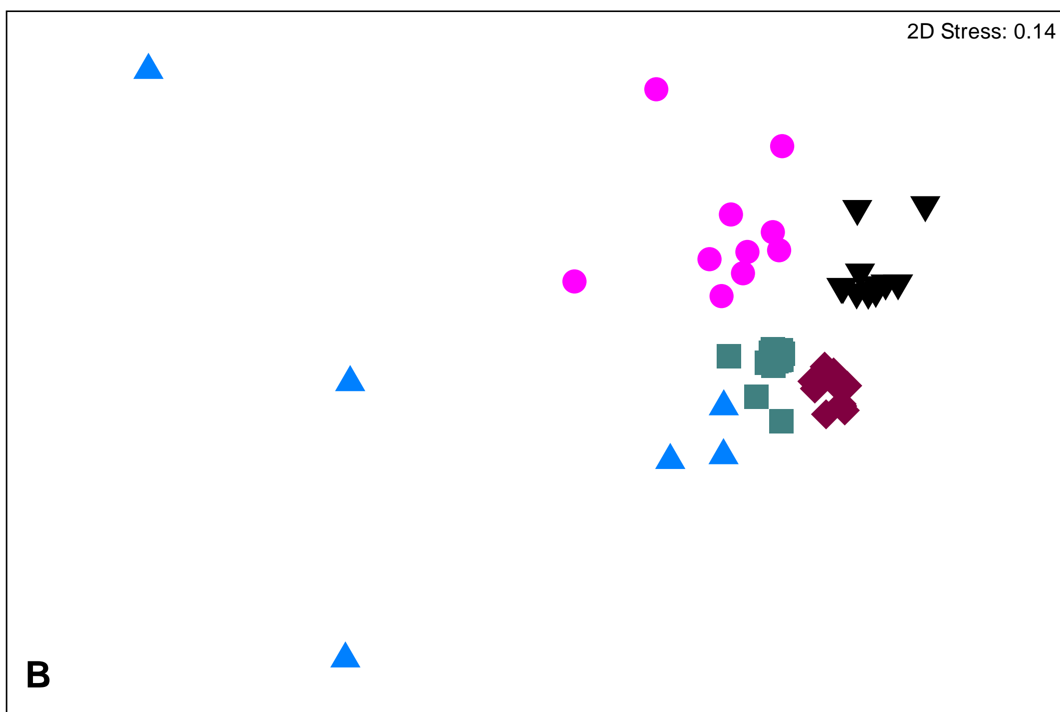


Figure 1.7: MDS plot showing the similarity in the microbial communities found throughout the Bicheno (A) and Pipeclay Lagoon (B) hatcheries. The different components shown are larvae (upwards triangle), algal feed (downward triangle), tank water (square), clean water (diamond) and tank biofilm (circle).

Table 1.6 ANOSIM test comparing strength of dissimilarity among microbial communities in each component of the hatcheries (all comparisons were significant).

Components compared	R statistic	*p* value
Within Bicheno hatchery		
larvae – biofilm	0.605	0.002
larvae – tank water	0.844	0.008
larvae – clean water	0.860	0.008
larvae – algal feed	1.0	0.002
biofilm – tank water	0.563	0.004
biofilm – clean water	0.760	0.002
biofilm – algal feed	0.906	0.002
tank water – clean water	0.524	0.008
tank water – algal feed	0.997	0.002
clean water – algal feed	1.0	0.002
Within Pipeclay Lagoon hatchery		
larvae – biofilm	0.675	0.001
larvae – tank water	0.789	0.001
larvae – clean water	0.819	0.001
larvae – algal feed	0.784	0.001
biofilm – tank water	0.683	0.001
biofilm – clean water	0.749	0.001
biofilm – algal feed	0.718	0.001
tank water – clean water	0.826	0.001
tank water – algal feed	0.960	0.001
clean water – algal feed	0.984	0.001

1.3.4 Correlation between microbial communities and production outcomes

Algae

The circled samples in Figure 1.6 are those taken during the only run that produced healthy spat. Although they clustered together in the middle of the plot, there was no clear differentiation between these samples and those from runs that suffered mass mortalities (ANOSIM R = 0.034, $p = 0.32$). However, when only the samples from the low nutrient runs were included, there was a significant difference between the run producing healthy spat and the run that suffered mass mortality (ANOSIM R = 0.182, $p < 0.05$).

Water

The tRFLP analyses of both bacterial and eukaryotic microbes indicated that there was no significant difference between the communities present when mass mortality events occurred and when healthy spat were produced (Figures 1.3 and 1.8, respectively).

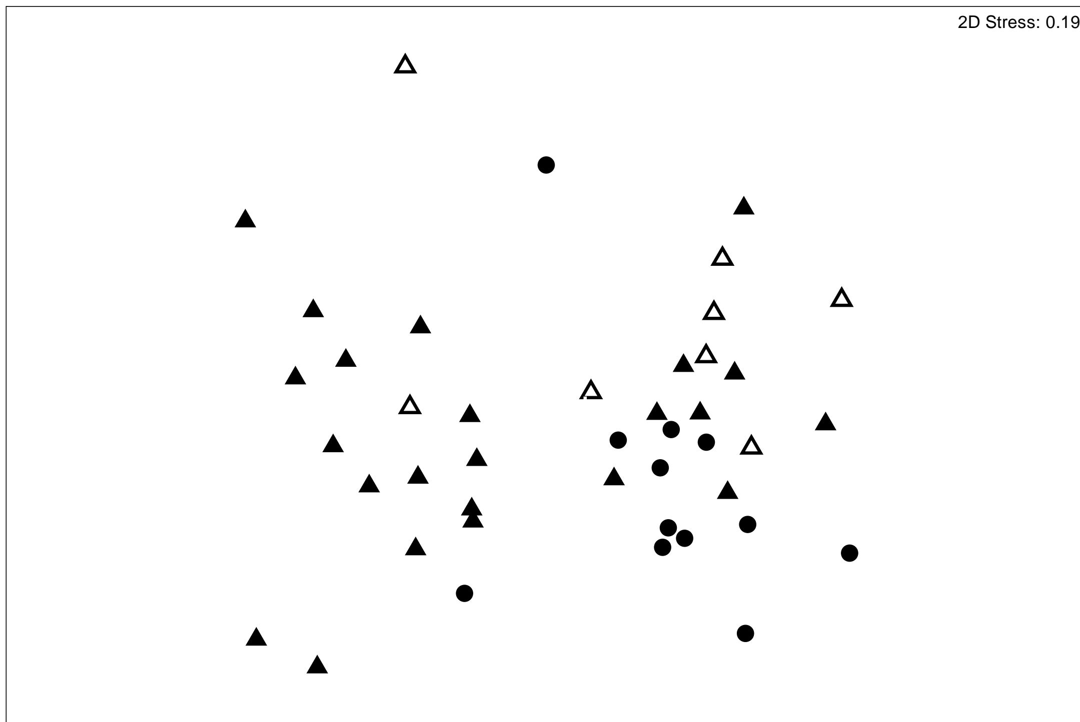


Figure 1.8: MDS plot showing the similarity in the microbial eukaryotic communities found in the intake (circles) and clean (triangles) seawater. White symbols were taken during production runs that resulted in healthy spat, black symbols were taken during production runs that experienced a mass mortality.

This observation was examined in more detail with high throughput amplicon sequencing. The samples did not cluster together based on whether healthy spat were produced or mass mortality events occurred, and a one-way ANOSIM including all samples showed no significant difference between the two groups (R = 0.08, $p = 0.18$). A nested ANOSIM test (date within outcome) on the intake seawater samples again showed that the bacterial communities present on different dates were significantly different (R = 1, $p = 0.02$) but there was no significant different between the communities present when healthy spat were produced compared to the occurrence of mass mortality events (R = -0.25, $p = 0.9$).

The December 2009 and January 2010 samples were temporally close together (early and late in the same summer season) but had different production run outcomes. Both these groups had a greater proportion of Cyanobacteria present compared to the other time points (Figure 1.9). Statistical analysis using the SIMPER routine in Primer6 showed that the differences between December 2009 and January 2010 was the presence of several cyanobacterial Group IIA OTU in January 2010 (mass mortality event)

that were absent in December 2009 (healthy spat produced). There were also several OTU from the Gammaproteobacteria and Alphaproteobacteria that were more common in December 2009 than January 2010 including the genera *Leucothrix*, *Thalassobacter*, *Roseovarius*, and *Halomonas*.
When all samples (including clean seawater) were included in the analysis, some OTU from the Group IIA group of Cyanobacteria Family II were again more common in samples from production runs ending with mass mortality events. In addition, OTU from the genera *Glaciecola*, *Polaribacter*, *Pseudomonas* and *Leeuwenhoekiella* were also more prevalent when this occurred. An OTU from the genus *Leucothrix* was more prevalent in samples from production runs that produced healthy spat. When only the intake seawater was included in the analyses, the result was much the same: several OTU from the cyanobacterial group IIA, one *Pseudomonas* and one *Polaribacter* OTU were more common when mass mortalities occurred. Gammaproteobacterial OTU from the genera *Leuothrix, Listonella* and *Glaciecola* were more numerous in the production runs producing healthy spat.

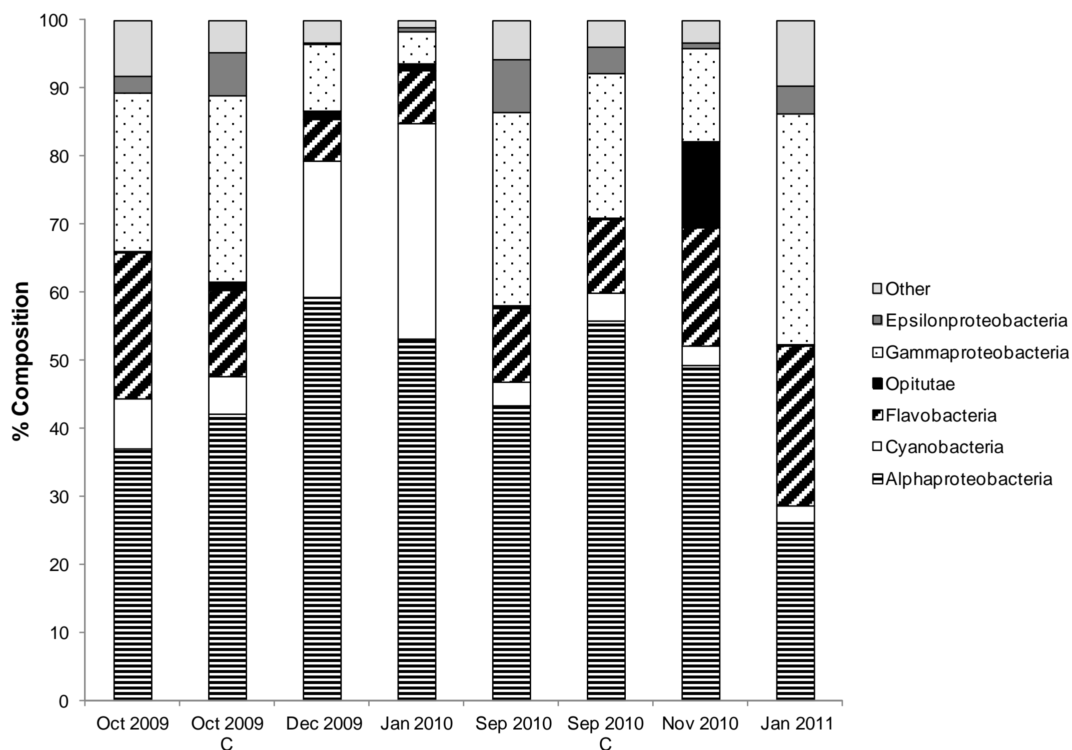


Figure 1.9: Bar graphs showing average percentage composition of bacterial communities in seawater samples based on pyrosequencing and aggregation of the data to class level. The group “other classes” contains the Actinobacteria, Deltaproteobacteria, Betaproteobacteria, Verrucomicrobiae, Spirochaetes, Acidobacteria, Sphongobacteria, Planctomycetacia and Bacilli. All samples are intake seawater except those labelled “C” which indicates clean seawater.

1.4 Discussion

1.4.1 Microbial communities in the hatchery

The bacteria found throughout the hatchery were the general types expected to be in a marine system. Alphaproteobacteria, Flavobacteria, Gammaproteobacteria were the main phyla detected and Cyanobacteria were also common in some water samples. Alphaproteobacteria and Gammaproteobacteria are common in marine ecoysystems (for example see Anderson et al., 2010 or Gilbert et al., 2012) with some studies finding that Gammaproteobacteria become more numerous during disease outbreaks (Jorquera et al., 2004; Nakase et al., 2007). The algal cultures were dominated by Alphaproteobacteria with three genera, *Martelella*, *Sphingopyxis* and *Phaeobacter*, present in all the clone libraries that were sequenced. When the nutrient level supplied to the algal cultures was reduced, the Gammaproteobacteria became more numerous although the Alphaproteobacteria still dominated the cultures. We also observed that the clone library from the algal feed in the one production run that produced healthy spat was more diverse that the other two with some genera of Alphaproteobacteria (*Pseudorugeria* and *Roseovarius*) only appearing in this library.

Although only a small percentage of the total bacterial population, *Vibrio* are considered to be the most important genus of bacteria in aquaculture facilities. We detected *Vibrio* using selective TCBS plates in water and larval samples but not in the algal feed. It should be noted that when isolates from the plates were identified, not all isolates from TCBS plates were actually *Vibrio* spp. The pryosequencing analysis found only 50 *Vibrio* sequences in a total of 12 425 reads in the intake and clean seawater showing that *Vibrio* were a very small portion of the community in the water used in the hatchery. However, this does not mean that they do not cause disease: neither 16S rRNA sequencing nor growth on TCBS plates provide any indication of pathogenicity, or potential infection levels.

Each compartment of the hatchery contained a distinctive microbial community (Figures 1.6, 1.7 and Table 1.6). Some communities were more similar to each other – for example clean seawater and tank water – but the overall structure was always different. The two facilities operated by the company in different locations also contained distinct microbial communities (Figure 1.6). Considering that there were some differences in procedures at the two facilities and that the intake water into each facility was different, it is not surprising that the microbial communities that developed were also different. The bays from which water were sourced were very different: one was more estuarine and subject to algal blooms, one was next to a small slip yard, water intake pipes were at different depths, and once collected, water

was subjected to different types of treatment. There were also differences in the production of microalgae for larval feed. This may have affected the microbial communities both within the algal feed and in the tank water as the tank water communities are a combination of the bacteria in the intake water, the algal feed and those excreted from the larvae. This analysis shows that there are complex relationships between the different compartments in the hatchery including the larvae themselves as they are exposed to bacteria from all these compartments.

1.4.2 Variability in the microbial communities in the hatchery

The microbial communities throughout the hatchery were very dynamic and changed on several scales: between different sampling days, different production runs and seasonally. This was explored in detail for the algal feed, and the intake and clean seawater.

It is significant that although there was a difference between the intake and clean seawater, the intake and clean seawater samples from different days were more different to each other than the intake and clean seawater were to each other on the same day (Figure 1.4). This means that the natural variation in seawater is still present in the clean (treated) seawater used in the hatchery and the treatments in use do not produce a more stable community (hence environment) for the oyster larvae. The strongest filtration used in the hatchery was 1 µm, which is still too large to retain bacterial cells although particle-associated bacteria may have been removed. Foam fractionation is not designed to remove bacterial cells from water although it does reduce and change the food available for bacterial growth. Variability in natural seawater is a well known phenomenon (for example Anderson et al., 2010; Fuhrman et al., 2006; Gilbert et al., 2012). One six-year study observed repeated seasonal patterns in the bacterial community structure that were related to environmental variables. In our tRFLP study it is interesting to note that the August 2009 samples (Figure 1.4, Table 1.4), which were the only samples collected in winter when the water temperature was significantly colder, were most different to other months. The pyrosequencing study, which compared samples from similar times over two years, found that the two spring samples were very similar to each other but that the summer samples were different in each year. This may have been due to variations caused by the East Australia Current that can extend as far south as Tasmania in summer but not always to same extent.

Temporal variability was also observed in the algal feed. This was confounded by the fact that the level of nutrients supplied to the algal was adjusted during our sampling. There were significant differences in both the number (by plate count) and the types (by tRFLP) of bacteria present when a low level of nutrient was supplied compared to a high level of nutrients (Figure 1.6, Table

1.5). Differences were also observed (for low levels of nutrients only) between different months (Table 1.5).

1.4.3 Correlation of community structure with production outcomes
In this study we found no correlations between the production of healthy spat (or the occurrence or mass mortality events) and the bacterial community structure in the intake seawater, clean seawater, algal feed or biofilms. tRFLP detects broad changes in the overall structure of microbial communities but does not have the resolution to detect subtle changes such as the presence (or absence) of only one or two species present at very low levels. Pyrosequencing has a greater ability to detect species that are present as a small percentage of the total population but even with this method we did not find a consistent trend of species that were only present when disease occurred. We did observe that a particular group of Cyanobacteria, associated with the genus *Synechococcus*, were more abundant when mass mortality events occurred. Some *Synechococcus* are known to produce toxins that are highly toxic to invertebrates such as *Artemia* (Martins et al., 2007; Frazao et al., 2010). It could be that these cyanobacteria are producing a toxin, (the water treatment that was in place at the time of this study would not have removed toxins), or it is possible that increased numbers of this group indicate a change in the conditions in the water that is also affecting the oyster larvae.

Although the ANOSIM analysis of the tRFLP results (Table 1.5) seems to indicate that there was a significant difference in the bacterial communities present when mass mortality occurred compared to when healthy spat were produced, this result is confounded by the fact that only one run produced healthy spat. The strength of the dissimilarity – or difference – between the two (as measured by the R value) is lower than the R value for the comparison between different months. Therefore, we cannot say with confidence whether the difference between the communities present when healthy spat were produced was related to the production outcome or simply to the normal variability in the communities.

1.4.4 Experimental design considerations
Although one of the strengths of this project was that it was carried out in a commercial hatchery and therefore subject to all the same factors as commercial larvae production, the fact that production requirements were more important than research weakened the research significantly. Replication was difficult to achieve hence it was difficult draw specific conclusions. We could conclude that the water treatment processes in place did not result in a more stable bacterial community but because these treatments often changed we were unable to determine if specific treatments (for example filtration at different levels or protein fractionation) had a

particular effect. Lack of replication and control has also been a significant factor hindering publication of this work in the scientific literature.

1.5 Benefits and adoption

This research has provided Shellfish Culture with detailed information on the bacterial communities found throughout their hatcheries. They have an improved understanding of the diversity and variability of the bacteria present and why they are difficult to control.

All staff at the Bicheno hatchery attended an informal meeting to explain these results in November 2010. By providing this information and relating it to observations that the staff made during larval production the aim was to raise their awareness of the reasons for and importance of hatchery procedures to control bacteria.

Although this research did not identify the specific effects of the water treatment processes in place, which may have improved larval health, it did show that the processes used at the time were not resulting in a stable bacterial population in the larval rearing tanks. Since this time we understand that new, more stringent, water treatment processes have been put in place.

The information generated here is also of interest to the wider shellfish and marine aquaculture industry. Detailed descriptions of the microbial ecology of hatcheries using DNA-based methods have not been widely used or published and our results will contribute to improved understanding of the interactions between larvae, bacteria and the environment.

1.6 Further development

At the time this research was carried out (September 2009 – June 2012) we suggested that monitoring of the quality of the intake seawater to the hatchery was important. As microbiologists we were unable to analyse the water for toxic chemicals of either natural or anthropogenic origin. As there was no clear correlation with the bacterial community structure and the occurrence of disease, it is possible that another factor is causing the larvae to become susceptible to attack from opportunistic pathogens such as *Vibrio* spp. whilst being able to co-exist with these bacteria at other times.

At the time this project began, very little work had been carried out using DNA-based technologies to explore the relationship between the entire microbial community of oyster (or other shellfish) hatcheries and the health of the larvae. However as these methods have become more accessible, several international research groups are exploring the relationship between

larval health, genetics, their immune system and the larval "microbiome" – all the microbes associated with larvae (for example Trabal et al. 2013). In similar ways in which links are being discovered between human health and our microbiome, links are also being found between aquatic animal health and the microbiome (Jaafer et al. 2013). Although it will take some time before these discoveries translate into management practices for the prevention and cure of disease, it is important that this knowledge is developed specifically for the Australian industry.

1.7 Planned outcomes

The new knowledge and understanding of the oyster hatchery environment that was produced by this research has benefitted our industry partner Shellfish Culture Ltd by providing evidence on which to base management decisions.

This project contributes to the Australian Seafood CRC output 1.3: Removal or reduction of key production constraints in selected aquaculture systems. The production of healthy spat is one of the largest constraints on the oyster industry worldwide. By improving the understanding of the environment in which the oyster larvae are being produced and by finding no evidence to link disease occurrence to a particular bacterial community, despite a thorough attempt, this project has allowed the hatchery to focus on other possible causes of disease.

1.8 Conclusions

Each of the objectives of this project was successfully met: new methods were developed and optimised for the analysis of microbial communities in shellfish hatcheries and significant variability in the communities on several scales within and between the hatcheries was described. We could not detect a microbial community, nor a specific group of microbes consistently associated either with mass mortality events or with healthy spat other than a group of cyanobacteria that were more abundant when mass mortality epizootics occurred.

The lack of a consistent microbial community associated with disease indicates that differences in the community, as a whole, are not responsible for the mass mortality events. We have been unable to identify a microbial community structure associated consistently with either the production of healthy larvae or the occurrence of mass mortality. This means that subtle changes in the microbial community, such as the appearance or disappearance of one or a few strains or a change in the activity of particular strains, may be responsible for the different outcomes in larvae health.

Alternatively, other physic-chemical factors such as changes in water temperature or the presence of micro-algae or chemicals in the water may be affecting the health of the larvae making them more susceptible to opportunistic pathogens.

The most important findings of this project are listed below.

- There is a high degree of variability in the microbial communities associated with an oyster hatchery that is not clearly correlated with production outcomes. This variation occurred on both a daily and a seasonal basis.
- There was no clear correlation between the bacterial community structure in the water or with algae feed and with successful larval production, although the bacterial communities in the algal feed were significantly affected by the level of nutrients supplied to them. However this analysis was hampered by the fact that only one successful batch of larvae were raised during our sampling.
- The two hatcheries operated by Shellfish Culture contained significantly different bacterial communities, and within each hatchery, the bacteria found in each component of the hatchery (larvae, tank biofilm, clean tank water, algal feed and tank water) were distinct from each other.
- The water treatment processes in place did not produce a more stable community and affected the bacteria present in the water less than the natural variation in the intake seawater.
- High-throughput pyrosequencing of seawater samples showed that there was no clear correlation between the bacteria present and whether mass mortality events occurred, although some groups of cyanobacteria were more numerous at these times.
- Algal cultures that were assumed to be axenic (not to contain bacteria) on the basis of plate counts did in fact contain bacterial DNA.

2. Spoilage communities in Australian produced modified atmosphere packaged Atlantic salmon

2.1 Introduction and Background

The spoilage process in fish is well-documented and consists of autolytic degradation by fish enzymes and the production of unpleasant odours and flavours as a result of microbial action (Gram & Huss, 1996). Typically in the chilled seafood supply chain, microbial-mediated changes dominate the spoilage process (Huss, 1995; Gram & Huss, 1996; Emborg et al., 2002). The bacteria responsible for spoilage in marine fish varies according to the harvest environment, the degree of cross-contamination and the preservation methods applied post-harvest. The primary spoilage bacteria in aerobically packed fish are from the genera *Pseudomonas* and *Shewanella* while in modified atmospheres, *Photobacterium* as well as lactic acid bacteria (LAB) such as *Lactobacillus* and *Carnobacterium* are responsible for spoilage (Gram & Huss, 1996; Dalgaard, Gram & Huss, 1993; Emborg et al., 2002).

The spoilage of Atlantic salmon by microbial action is generally thought to be the same species as those responsible for the spoilage of other temperate marine fish. *Shewanella putrefaciens*, *Shewanella baltica*, *Pseudomonas* spp., *Carnobacterium maltaromaticum* and *Photobacterium phosphoreum* have all been implicated in the spoilage of Atlantic salmon (Emborg et al., 2002; Gram and Huss 1996; Schirmer et al., 2009). Generally, *P. phosphoreum* is accepted as the specific spoilage organism (SSO) for modified atmosphere packaged (MAP) Atlantic salmon, although recent work also suggests that *Hafnia alvei* might also be a specific spoilage organism for this product (Mace et al., 2013). Mace et al. (2012) also found that LAB, predominantly *Lactococcus piscium*, were present in high numbers in spoilt MAP salmon although *P. phosphoreum* was also present. Other authors have acknowledged that the role of LAB in the spoilage of MAP fish may be underestimated due to the difficulty of culturing LAB from fish (Leroi 2010), which has been attributed to acetate found in many LAB-selective media such as de Man, Rogosa Sharpe (MRS) agar.

Modified atmosphere packaging with different ratios of oxygen, nitrogen and carbon dioxide has been used extensively over the last 80 years to increase the shelf-life of chilled protein products such as meat and fish (Valley & Rettiger, 1997). The role of carbon dioxide in particular has been the subject of many studies with several reports on its effect on different groups of bacteria (Dalgaard et al., 1997; Boskou & Debevere, 1998; Devlieghere & Debevere, 2000). Carbon dioxide is regarded as a bacteriostatic rather than a bacteriocidal agent (e.g. Eyles, Moir & Davey, 1993), although the

mechanisms by which it works are not well understood (Sivertsvik et al., 2002). Sivertsvik et al. (2002) summarised the four mechanisms responsible for the effect of CO_2 on bacteria as being: alteration of the cell membrane function (including effects on nutrient absorption); direct inhibition of enzymes or decreases in the rate of enzyme reactions; penetration of the bacterial membranes leading to intracellular pH changes and; direct changes in the physico – chemical properties of proteins. Devlieghere and Debevere (2000) found a relationship between the concentration of dissolved carbon dioxide and both the lag phase and growth rates of several gram-negative and gram-positive bacteria. The carbon dioxide had less of an effect on gram-positive organisms, a trend also observed in work by Kimura, Murakami & Fujii (1997) and Koutsomanis et al. (2000). High levels of carbon dioxide potentially select for gram-positive LAB as they are tolerant of carbon dioxide and prefer low levels of oxygen (Dixon & Kell 1989). It has also been observed that although some bacteria can grow in high CO_2 atmospheres, fewer amines (which are responsible for many of the off odours and off flavours in fish products) are produced under these conditions (Boskou & Debevere, 1998). The concentration of dissolved CO_2 in the flesh is influenced by the gas:product volume ratio (*g/p*) and storage temperature (Devlieghere, Debevere & Van Impe, 1998a). Gas:product ratios generally need to be greater than 2:1 to have a significant effect on microbial growth (Devlieghere & Debevere 2000). Lower temperatures are more effective at slowing microbial growth because of both the direct effect of lower temperatures on microbes and because carbon dioxide is more soluble at lower temperature (Devlieghere Debevere & Van Impe, 1998b).

However, MAP does not confer the same benefits on all seafood products, with shelf-life under 100% CO_2 ranging from 6 d at 3 °C for snapper fillets to 18 d at 2 °C for Atlantic salmon fillets (Sivertsvik, Jeksrud & Rosnes, 2002). The ability of some bacterial species found in seafood products, particularly *P. phosphoreum*, to tolerate carbon dioxide, are responsible for this variability in the effect of MAP (Dalgaard et al., 1997; Sivertsvik, Rosnes & Kleiberg, 2003). The role of *Photobacterium* spp. as the SSO in Atlantic salmon stored under MAP was only discovered by the use of NaCl-containing Long & Hammer agar, as the genus has an absolute requirement for salt and does not grow on standard plate count agar (Emborg et al. 2002). This highlights the potential for culture-based methods to fail to detect particular groups of bacteria, especially if they are not expected, a point investigated in detail by Broekaert et al. (2011).

The use of culture-independent methods based on the 16S rRNA gene circumvents this issue and is becoming more common in studies of spoilage communities (Juste et al., 2008); only a few such studies have been carried out on fish. These methods, such as metagenomic sequencing via clone

library construction, have the advantage of being able to detect organisms that are not readily cultured. In addition, the community fingerprinting suite of methods (e.g. tRFLP, denaturing gradient gel electrophoresis) are easily applied to a large number of samples, making them suitable for studies examining changes in entire communities over time rather than changes in particular species.

Rudi et al. (2004) compared microbial communities found on Atlantic salmon using both culture-based and culture-independent (clone library) methods. They found that although the culture-based methods indicated that the communities were dominated by Enterobacteriacaea, sequencing of clones showed that the diverse communities were dominated by *Carnobacterium* spp. and *Brochothrix* spp. In a study of MAP Halibut, Hovda et al. (2007) also used both culture-based and DNA-based methods, reporting that *Photobacterium spp.* and *Brochothrix sp.* dominated the spoilage communities detected by DNA-based methods and that *Pseudomonas spp.* and other Enterobacteriacaea were detected using culture-based methods. Similarly, Reynisson et al. (2009) also found that culture-based methods indicated that spoilage communities on North-Atlantic cod were dominated by *Pseudomonas spp*. whereas DNA-based methods showed that the communities were dominated by *P. phosphoreum*.

2.1.1 Need

The Atlantic salmon industry in Australia is based in Tasmania where the water is cold enough to grow salmon. Some of the product is sold to retailers as fresh, chilled (on ice) whole fish or fillets, but a large proportion is MAP either as fillets to be sold to retailers or as portions to be sold to consumers.

Most of the published studies on spoilage of Atlantic salmon have been carried out in the northern hemisphere; very little information is available for salmon grown in the southern hemisphere. Anecdotal evidence suggests that *P. phosphoreum* is not commonly observed in Australian Atlantic salmon but whether this is due to the methods used or whether it is a real phenomenon is not clear.

Knowledge of the spoilage communities that develop on Australian Atlantic salmon, and the variability in these communities, is required to understand the shelf-life of this product and to determine which available predictive models and tools are relevant for determining product shelf-life.

2.1.2 Objectives

The overall objective of this project was to define SSOs that impact the quality of processed fresh fish, define processing conditions and intervention

strategies that influence their presence, and identify microbial species that can competitively exclude spoilage organisms.
Specifically the objectives of this project were to:

- characterise the spoilage communities and variability in spoilage communities of modified atmosphere packaged Atlantic salmon;
- determine the spoilage communities that develop under optimised conditions; and
- develop a temperature and carbon dioxide model for the growth of spoilage organisms.

2.2 Methods

2.2.1 Spoilage communities on commercially produced MAP salmon

Experimental design and sampling
The structure and variability of the microbial communities present on Atlantic salmon were measured at the beginning and end of shelf-life. In Study-1, six packs of MAP (55% CO_2 in N_2) Atlantic salmon from three different batches (use-by dates) were purchased (Table 2.1) from a local supplier and stored in the laboratory at 4 °C until three days after the use-by date (i.e. 15 d after commercial packaging). Each pack contained one or two pieces of salmon, each of which was sampled separately. Sterile swabs were used to sample approximately 10 cm^2 of the surface of the salmon pieces in triplicate. Swabs were frozen at -20 °C until DNA extraction and microbial community analysis.

In Study-2, two MAP packs of salmon from three batches were purchased and opened three days after commercial packaging. One piece from each pack was sampled in triplicate and analysed immediately and the other piece repacked in MAP (30% CO_2 in N_2), and stored at 4 °C for a further 12 d (i.e. 15 d after the initial packaging). Viable bacteria were enumerated and the surface of the fish swabbed in triplicate for DNA extraction and microbial community analyses.

Table 2.1: Sampling schedule for packs of MAP salmon.

Date					
3/8/09	Pack 0				
	Piece 1				
30/8/09	Pack 1		Pack 2		
	Piece 2	Piece 3	Piece 4	Piece 5	
16/11/09	Pack 3		Pack 4		Pack 5
	Piece 6	Piece 7	Piece 8	Piece 9	Piece 10

DNA extraction
DNA was extracted from frozen swabs using the UltraClean Microbial DNA kit (MoBio) with the following modifications. Three hundred microlitres of the first solution from the kit (Microbead solution) was added to the swab which was then vortexed for 2 min. This solution was transferred to the microbead tube and the manufacturer's directions followed thereafter.

Microbial community analysis
The overall microbial community structure was examined by tRFLP analysis of the 16S rRNA gene (see Appendix 1). Clone libraries were constructed from a small number of samples (chosen on the basis of the tRFLP results) as described in Appendix 1.

Microbial enumeration
Plate counts were carried out on samples to determine the total number of culturable heterotrophic and LAB present. Samples of approximately 30 grams were stomached for 1 min in diluent (0.1% peptone, 0.85% NaCl). Triplicate serial dilutions were plated onto nutrient agar (Oxoid), MRS (de Man, Rogosa and Sharpe agar(Oxoid)) or Long & Hammer agar (per litre: 20g proteose peptone, 10g NaCl, 40g gelatine, 1g K_2HPO_4, 0.25 g ammonium ferric citrate). Plates were incubated aerobically for 24 h at 25°C (MRS) or at 10°C for 4 d (long and hammer and nutrient agar) prior to enumeration.

Isolate identification
A selection of isolates with different colony morphology from the plates of the highest dilutions were obtained and tentatively identified by partial sequencing of the 16S rRNA gene. PCR was carried out as described for the tRFLP, except that unlabelled primers 519F and 1492R were used. PCR products were sent to Macrogen Inc (South Korea) for sequencing.

2.2.2 Development of spoilage communities with optimised packaging
Harvest and packaging of fish
The aim of this experiment was to determine the spoilage community that developed when maximum possible shelf-life was reached. To do this, cross-contamination was minimised during processing and maximum dissolved carbon dioxide in the fish flesh used in combination with low temperature storage.

Atlantic salmon (between 3 and 4.5 kg each) were commercially harvested from Van Dieman Aquaculture, Tamar River Tasmania and immediately packed in plastic lined polystyrene boxes containing ice for transport to the Australian Maritime College processing facility. On arrival, fish were cooled for a further 2 h on ice. Seven individual fish with a total weight of 32.3 kg were gutted, skinned, filleted and cut into portions of approximately 20 g. Each

portion was individually packaged into 2 layer co-extrusion barrier (NY/TIE/LLDPE) bags with an oxygen transmission rate of 50-55 cc/m^2 per 24 h ATM at 23 °C-75% RH using a model Compaq vacuum packer (Kramer + Grebe, Biedenkopf, DE) with a Witt gas mixer with 100% CO_2 and a gas product ratio of greater than 5:1. Fish processing and portion preparation was completed within 4 h of harvest. All surfaces and equipment were sanitised prior to processing with quaternary ammonium compound disinfectants (FS Formula 7000, Zep USA under licence by Calman Aust) and acidified sodium chlorite (Vibrex Food Care at 500 ppm). During processing, water was used to rinse the cutting board between fish to minimise cross-contamination. Packed MAP bags were stored on ice in a cool room for the duration of the experiment (31 d). Temperature loggers were placed in similar bags in the ice-box alongside the fish.

Triplicate samples were measured approximately every five days. At this time one piece was also swabbed for later DNA extraction and swabs were frozen at -18 °C until required.

Microbial community analysis

DNA was extracted from swabs as described above and clone libraries constructed from day 0, 15 and 30 samples as described in Appendix 1.

Microbial growth

The total number of bacteria present was determined at each time point using plate counts on Long & Hammer agar and molten standard plate count agar (CM0463) with 1% NaCl, as described above.

2.2.3 Development of growth model

Packaging of fish

Packs of commercially harvested and MAP packaged Atlantic salmon were obtained from processing facilities in Tasmania on the day they were MAP packed. The packs were transported to the University of Tasmania on ice where they were aseptically opened and individual pieces removed. Pieces were sub-sampled into similarly sized portions of approximately 30 g and repacked into bags (Orved 95GR smooth bags 2332025) in modified atmospheres (carbon dioxide content from 30 to 100%, remainder nitrogen) with a Technovac vacuum packer. Samples were stored at a range of temperatures between 0 ° and 10 °C. Data loggers (Thermochron ibuttons) were used to monitor the actual storage temperature.

Sensory analysis

In a pilot study, sensory analysis of the Atlantic salmon was also included. A sensory test was developed based on similar tests used in the Tasmanian industry with an overall acceptability score rated from 1 (extremely

acceptable) to 5 (extremely unacceptable) based on texture, aroma and appearance. Details of the sensory attributes are given in Table 2.2.

Table 2.2: Sensory attributes used to score salmon portions.

	1	3	5
Appearance	Glossy flesh No discolouration No to little drip Clean mucus (if present)	Dull / opaque Slight discolouration Moderate drip	Sticky secretions Slimy appearance Creamy mucus Obvious drip
Aroma	Sea / seaweed / fresh Light Sweet	Sourish stale	Very sour Very stale Putrid or rancid Ammoniac sulfur
Texture	Firm Elastic Smooth surface	Less firm Slightly tacky	Soft consistency Sticky / slimy surface

Microbial enumeration

At each sampling time, two random bags were chosen. Each piece was given a sensory score and then stomached in saline peptone diluent (0.85% NaCl and 0.1% bacteriological peptone) and serially diluted. Total viable counts were obtained by pour-plating with Standard Plate Count (APHA) agar (Oxoid CM0463). Plates were incubated at 25 °C for 48 h prior to enumeration.

Model development

DMFit (web version available from ComBase: http://www.combase.cc) was used to fit the data of the number of colony forming units as a function of time to a growth model (Baranyi and Roberts 1994) to obtain the maximum specific growth rates (μ_{max}) hereafter referred to as growth rate. The maximum specific growth rates were then used to derive a secondary growth model (Ratkowsky et al. 1982). Following the work of Koutsoumanis et al. (2000), a Beldrahek-type model was fitted to the growth data:

$$\sqrt{\mu max} = a(T-T_{min})\sqrt{(CO_{2max} - CO_2)} \quad (1)$$

where a is a constant, T is temperature (K), CO_2 is the percentage carbon dioxide in the pack, T_{min} is the theoretical minimum temperature (K) and CO_{2max} (%) is the theoretical maximum carbon dioxide level at which the bacteria will grow. The square root transformation serves to stabilise the variance.

2.3 Results

2.3.1 Variability in spoilage communities on MAP Atlantic salmon

Spoilage communities after 15 days in MAP study-1

A clone library was constructed from one sample which generated 65 good quality sequences from 10 different genera. The percentage composition of the library is shown in Figure 2.1 (pack 0). The most common were *Carnobacterium* and *Iodobacter* but there were also several representatives of the Gammaproteobacteria (*Morganella*, *Yersinia*, *Serratia* and *Rahnella*) and a single Alphaproteobacterial genus, *Roseobacter*. The Carnobacteria were split into two main groups related to *C. divergens* and *C. maltaromaticum*.

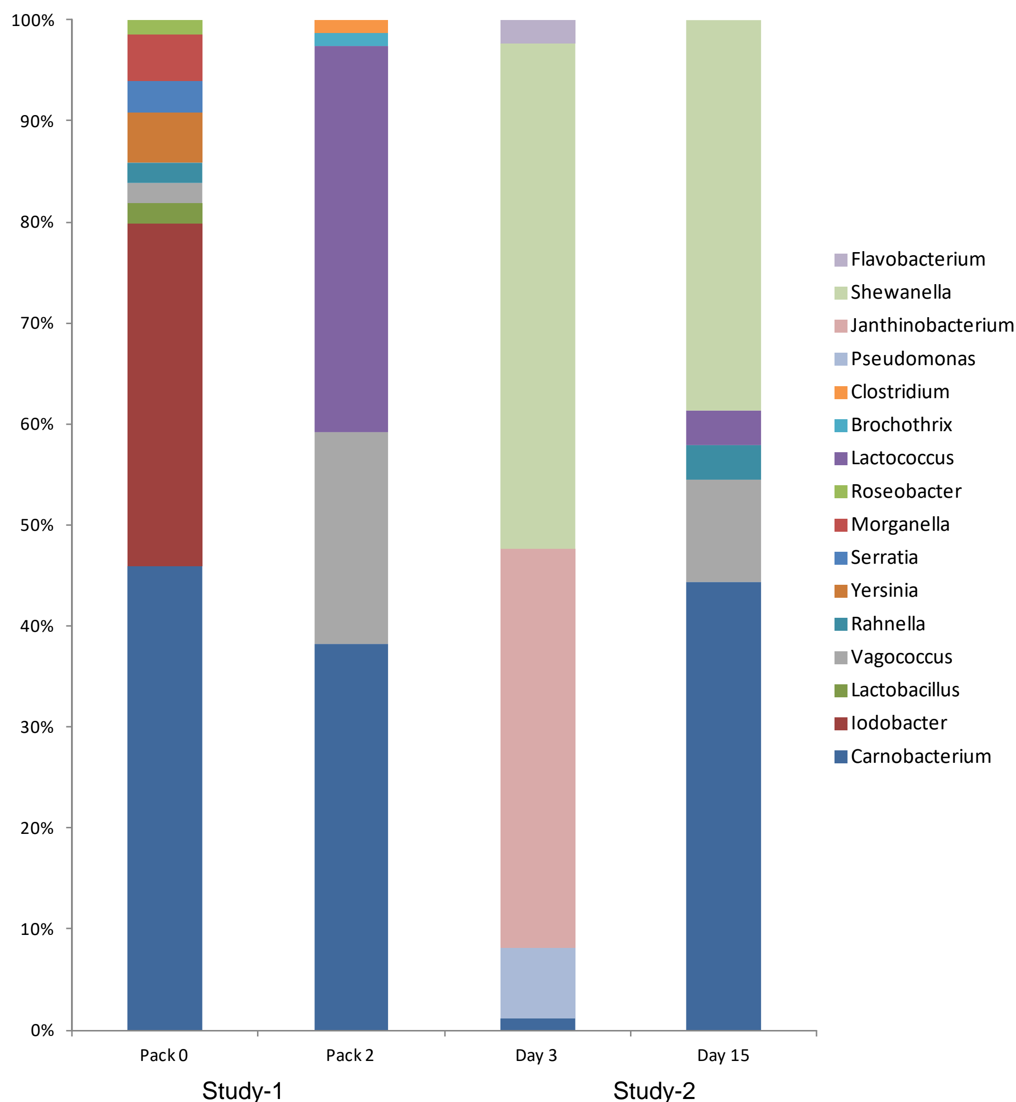


Figure 2.1 Percentage composition of clone libraries of from MAP Atlantic salmon. Study-1 samples were stored for 15 d in 55% CO_2 in N_2, Study 2 samples were taken after 3 and 15 d initially in 55% CO_2 in N_2 then repacked into 30% CO_2 in N_2.

The microbial community structure on different pieces of salmon from different packs with three different use-by dates (and hence three different packaging dates) was examined using tRFLP. Multivariate statistics were used to examine whether there were significant differences in the microbial communities found either on different pieces of salmon in different packs or from pieces packaged on different days. The similarity between communities is shown in a MDS plot (Figure 2.2) and the strength and significance of the similarity / dissimilarity between groups of samples tested using ANOSIM (Table 2.3). The lowest possible *p*-value generated by this permutation test for differences between pieces was 0.1, as there were only three samples from each piece. This is a practical limitation as dividing the piece of salmon into smaller sections to swab (to create more samples from each piece) would reduce the size of the microbial community sampled.

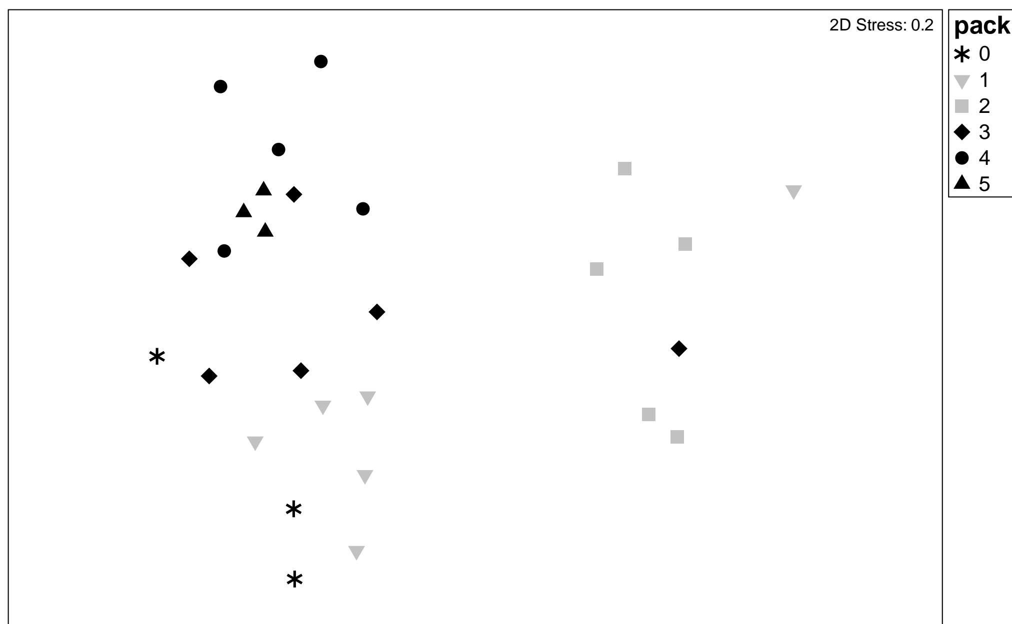


Figure 2.2 : MDS comparing microbial communities found in different packs of MAP salmon by tRFLP. Three samples were taken from each of two pieces in each pack. Pack-2 was packed on the same day as pack-1.

It is quite clear that pack-2 is significantly different to the other packs. In Table 2.3, the ANOSIM test is significantly different ($p < 0.05$) for all the comparisons between pack-2 and other packs. Packs with different use-by dates also tended to be different to each other whilst of the four comparisons that could be made between packs sampled on the same day, two were significantly different.

A second clone library was generated from the atypical pack. This library was much less diverse containing sequences form only five genera, all of which were from the phylum Firmicutes (Figure 2.1). Sequences from the genus *Carnobacterium* were again present along with *Lactococcus*, *Vagococcus*, *Brochothrix* and a single *Clostridium* sequence.

Table 2.3: ANOSIM values generated by testing for differences between packs of salmon. Significant differences are in bold.

	Packs	**ANOSIM R value**	***p***
Same use-by date	**1,2**	**0.525**	**0.015**
	3,4	0.149	0.123
	3,5	-0.136	0.738
	4,5	-0.087	0.696
Different use-by dates	0,1	0.299	0.119
	0,2	**0.938**	**0.018**
	0,3	0.123	0.274
	0,4	**0.682**	**0.018**
	0,5	**0.667**	**0.100**
	1,3	**0.230**	**0.017**
	1,4	**0.509**	**0.006**
	1,5	**0.481**	**0.024**
	2,3	**0.609**	**0.011**
	2,4	**0.904**	**0.008**
	2,5	**0.908**	**0.018**

Comparison of communities in early and late shelf-life

Plate counts for total bacteria (on Nutrient and Long & Hammer agars) and LAB (MRS agar) were carried out between two and three days into the shelf-life of MAP salmon, and three days after the use-by date (day 15) (Figure 2.3). The counts on Nutrient and Long & Hammer agars were not significantly different, although the numbers on Long & Hammer agar were usually somewhat (less than 5%) higher. At day-3, LAB comprised between 8 and 100% of the total culturable bacteria. However, at day-15, they were between 0.05 and 0.95% of the total culturable bacteria.

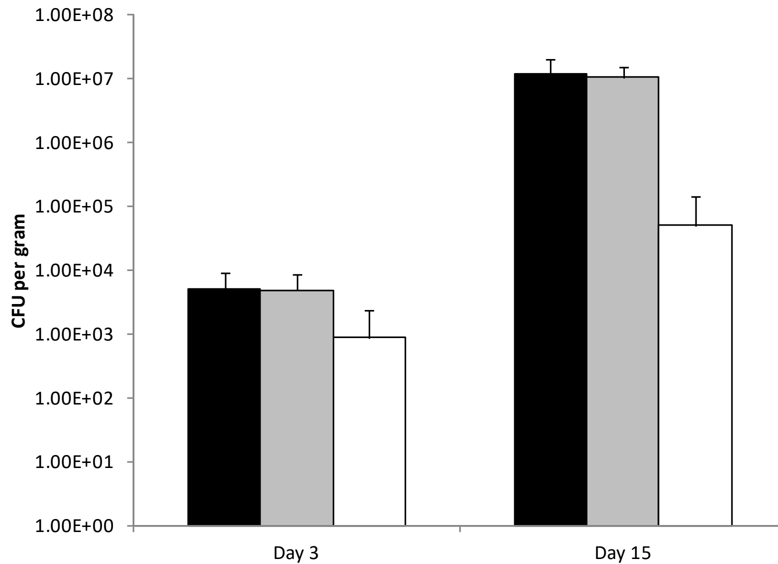


Figure 2.3: Numbers of total aerobic plate counts on Long & Hammer (black), nutrient (grey) and MRS (white) agars from Atlantic salmon after 3 and 15 d storage at 4°C in MAP (initially 55% CO_2 in N_2, then 30% CO_2 in N_2). The error bars indicate the standard deviation of six pieces of salmon each measured in triplicate.

Differences in the overall microbial community structure were measured using tRFLP (Figure 2.4). The communities from samples taken on day-3 clustered together very tightly and were significantly different to the communities from day-15. The communities from day-15 showed more spread, with pack-5 significantly different to the others (although pack-5 at day-3 was not significantly different to the other day-3 packs). Pack-5 also had the highest proportion of LAB at day-15.

Two clone libraries were constructed, one from a day-3 sample and one from the corresponding day-15 sample (Fig 2.1). The genus *Carnobacterium* dominated (44%) the day-15 clone library as it did for the day-15 clone libraries generated in study-1. However, the next most numerous sequences belonged to the genus *Shewanella* (38%). *Shewanella* also dominated the day-3 clone library (50%) where only one *Carnobacterium* sequence was detected. *Janthinobacterium* (39%) and *Pseudomonas* (7%) were the other genera represented by more than one sequence in the day-3 clone library. The main changes between the day-3 and -15 bacterial communities are that although *Shewanella* remains a large proportion of the community, it decreases in percentage; the proportion of LAB, especially *Carnobacterium,* increased and *Janthinobacterium* were not observed at all on day-15.

Sequences from these clone libraries have been deposited in GenBank under accession numbers JF719342 – JF719542.

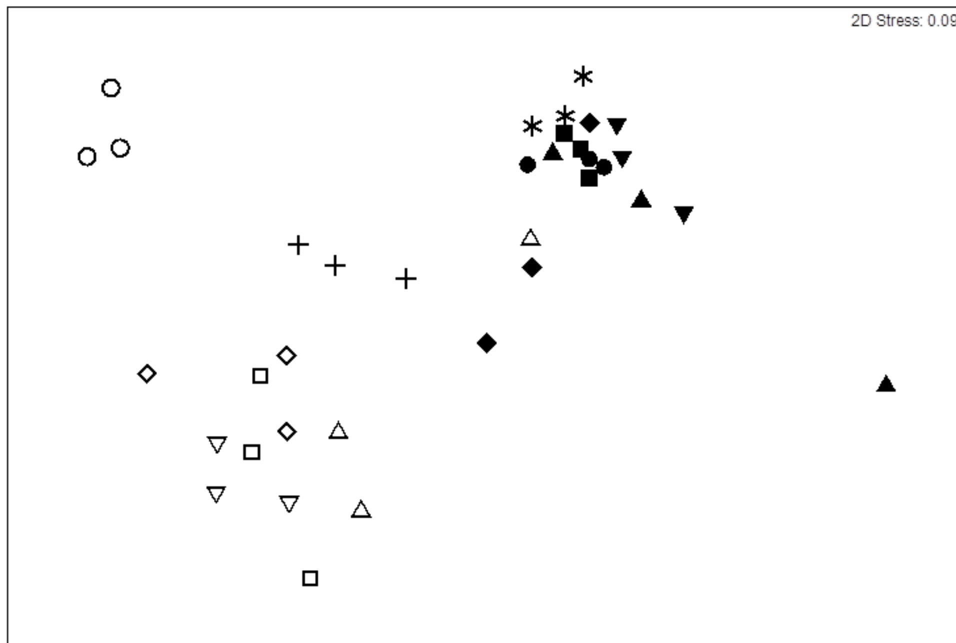


Figure 2.4: MDS comparing the similarity of the overall microbial community structure, as measured by tRFLP, in samples taken from day-3 (dark symbols and star) or day-15 (open symbols and cross). The different symbol shapes are from different packs.

2.3.2 Validation of a method for producing MAP salmon with limited microbial growth

Visual inspection of the salmon at each sampling time (approximately every 5 d) revealed few signs of spoilage (eg slime, odour, liquid) until day-25. At day-31, there was no particular odour and very little slime present. Over time the gas was absorbed by the fish and the bags contracted. At day-5, a mean of 92% CO_2 remained in the headspace and by day-11 insufficient gas was in the headspace to conduct a reading.

Three individually packaged pieces of salmon were analysed at each time point to generate the growth curve shown in Figure 2.5. Two different plate counts are shown: using plate count agar incubated at 25 °C for 2 d (following the Australian standard method) and using long and hammer agar incubated at 10 °C for 4 d (modified method after Emborg et al., 2002). Samples were also plated onto Lyngby Iron agar to detect sulphide producing bacteria and onto MRS agar to detect LAB, but these counts were always zero. The two different plate counts showed a similar pattern of growth although the numbers obtained by the modified method were always higher than those obtained using the standard method. There was an extended lag time of 10 – 15 d before exponential growth began. The bacterial numbers reached 10^6 CFU g^{-1} after 20 – 25 d (depending on enumeration method) and then a plateau is reached after 31 d at 10^8 CFU g^{-1}. Counts were not continued past 38 d.

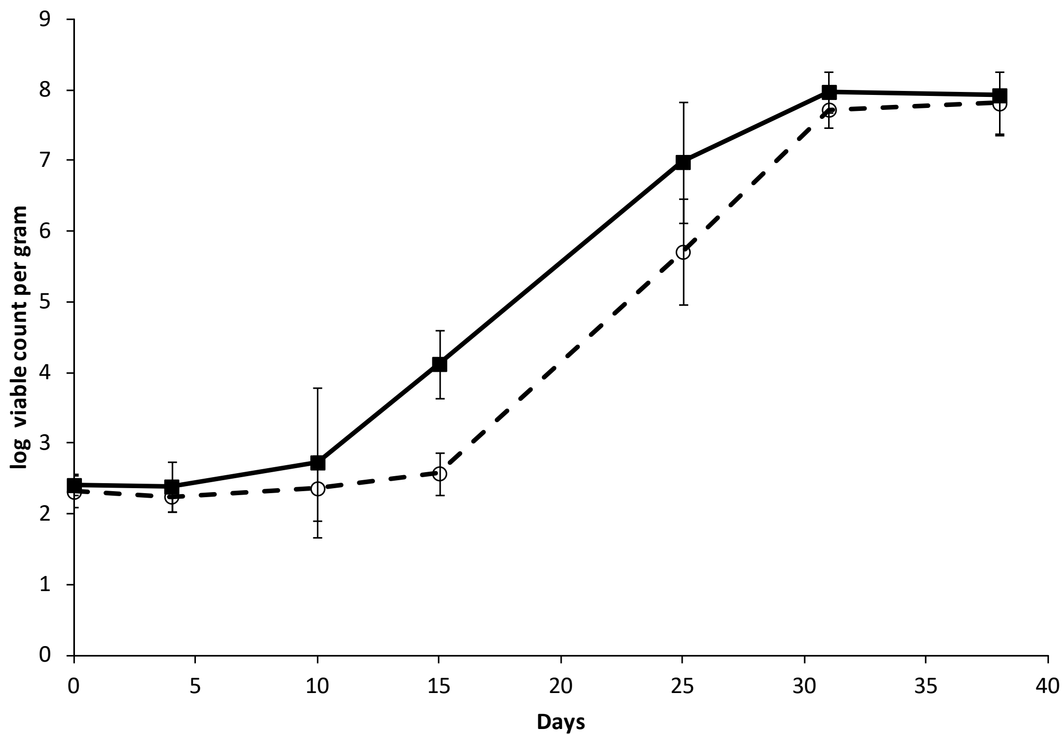


Figure 2.5: Growth curve of total bacteria on Atlantic salmon fillets packed in 100% CO_2. Solid line represents counts carried out using pour plates of plate count agar incubated at 25 °C and the dashed line represents counts carried out using Long & Hammer agar incubated at 10 °C.

Colonies growing on both the standard plate count and Long & Hammer agars were isolated, purified and identified to genus level by partial sequencing of the 16S rRNA gene. Table 2.4 shows the identity and origin of isolates. *Staphylococcus* and *Pseudomonas* were isolated from day-0 but only *Pseudomonas* were isolated from day-21 and -31 plate counts. The complete 16S rRNA gene sequence was determined for four representative isolates. Comparison to the 16S rRNA gene of type strains from the Pseuodomonas genus showed that these isolates belonged with the *P. fluorescens* group of psychrotolerant, fluorescent pseuodmonas or the *P. fragi* group of psychrotolerant, non-fluorescent pseuodmonas.

Clone libraries were constructed from DNA extracted from day-0, day-15 and day 31 salmon fillets. Very limited diversity was observed in any of the clone libraries. Sequences from the genera *Gramella*, *Micrococcus*, *Acidovorax* and *Achromobacter* were retrieved more than once from the day-0 clone library but again, only *Pseudomonas* was detected in the day 15 and 30 libraries.

The storage experiment was repeated to determine whether *Pseudomonas* would again be the dominant microorganism present. After 11 d, there were an average of 840 CFU g^{-1} total aerobes present and 300 *Pseudomonas* CFU g^{-1}. Both sets of plate counts remained less than 1×10^3 CFU g^{-1} until day-33 when total aerobic plate counts ranged from 1×10^3 to 1×10^5 CFU g^{-1} and *Pseudomonas* plate counts also ranged from 2×10^3 to over 1×10^5 CFU g^{-1}.

2.3.3 Effect of temperature and carbon dioxide on growth of spoilage bacteria

Sensory analysis of the portions of MAP salmon was carried out alongside the bacterial enumeration for the first set of samples in this part of the project (Figure 2.6). There were a strong positive correlations between the two: as the numbers of bacteria increased, the acceptability decreased. The correlations were 0.93 (3.5 °C), 0.90 (9 °C) and 0.96 (10 °C). Such a high correlations suggests that TVC counts are a good indicator of sensory shelf-life.

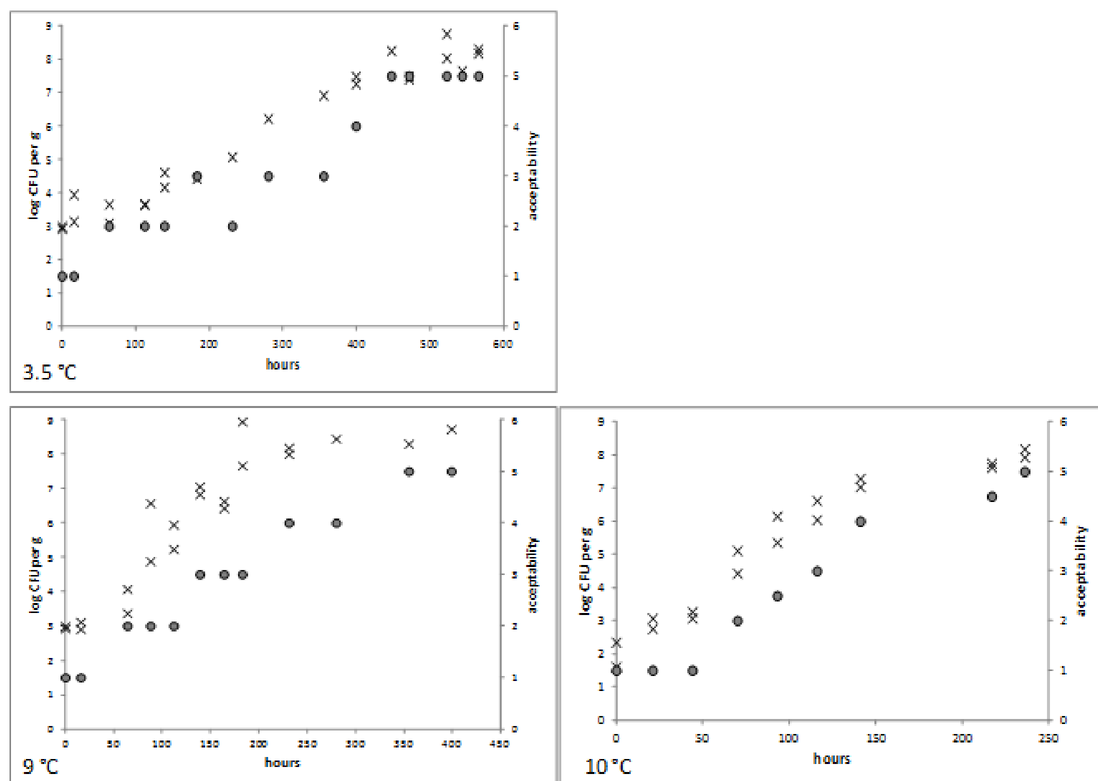


Figure 2.6: Correlation between sensory scores and total viable counts. The circles represent the sensory scores and the crosses the total viable count.

Growth curves were obtained for total viable bacteria on Atlantic salmon in three different modified atmospheres (30, 55 and 98% CO_2) stored at several temperatures between 0 and 10 °C. Examples of these curves at 98% CO_2 are presented in Figure 2.7. The growth curves obtained here are different to those obtained in the previous study (section 2.2) with the most striking difference being the absence of the long lag phase of approximately 15 d that was observed previously. The growth rates determined in this study were compared to those predicted by the Seafood Spoilage and Safety Predictor version 2.0 (Dalgaard et al. 1997) for *Photobacterium phosphoreum* on MAP salmon (Table 2.4). Although the growth rates are similar at low temperatures (around 0 °C), at higher storage temperatures (10 °C) the rates obtained in the current study are significantly lower.

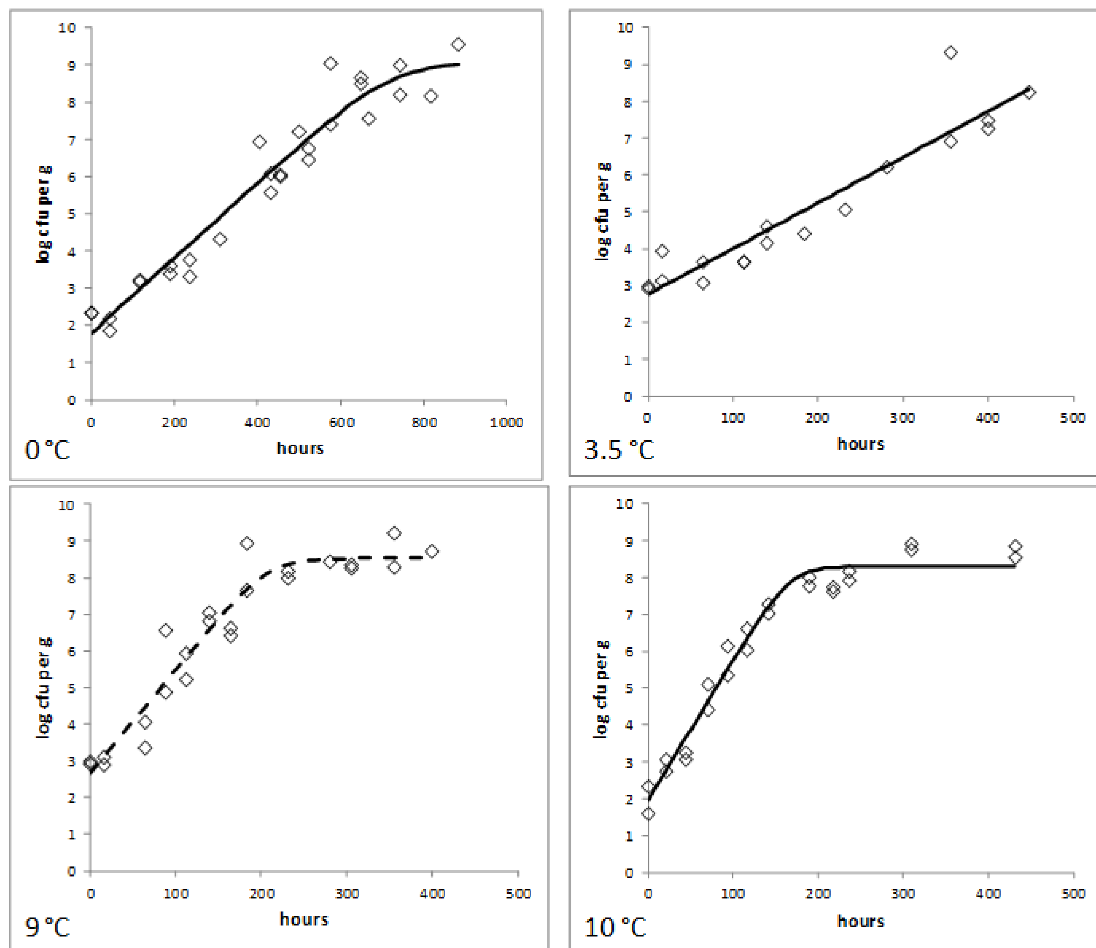


Figure 2.7: Growth rates for total bacteria on skin-on portions of Atlantic salmon at temperatures between 0 and 10 °C in MAP (98% carbon dioxide). The diamonds are actual data points, the solid line is the fitted model.

Table 2.4: Comparison of growth rates obtained in this study with growth rates used in the Seafood Spoilage and Safety Predictor (SSSP).

Temperature (°C)	**Atmosphere (% CO_2, balance N_2)**	**Specific growth rate (this study)**	**Specific growth rate (SSSP)**
0	98% CO_2	0.023	0.029
10	98% CO_2	0.087	0.132
0	55% CO_2	0.032	0.042
10	55% CO_2	0.092	0.188
0	30% CO_2	0.034	0.048
10	30% CO_2	0.094	0.215

The square root of the specific growth rate was plotted against temperature for each modified atmosphere tested and produced a linear relationship (Figure 2.8).

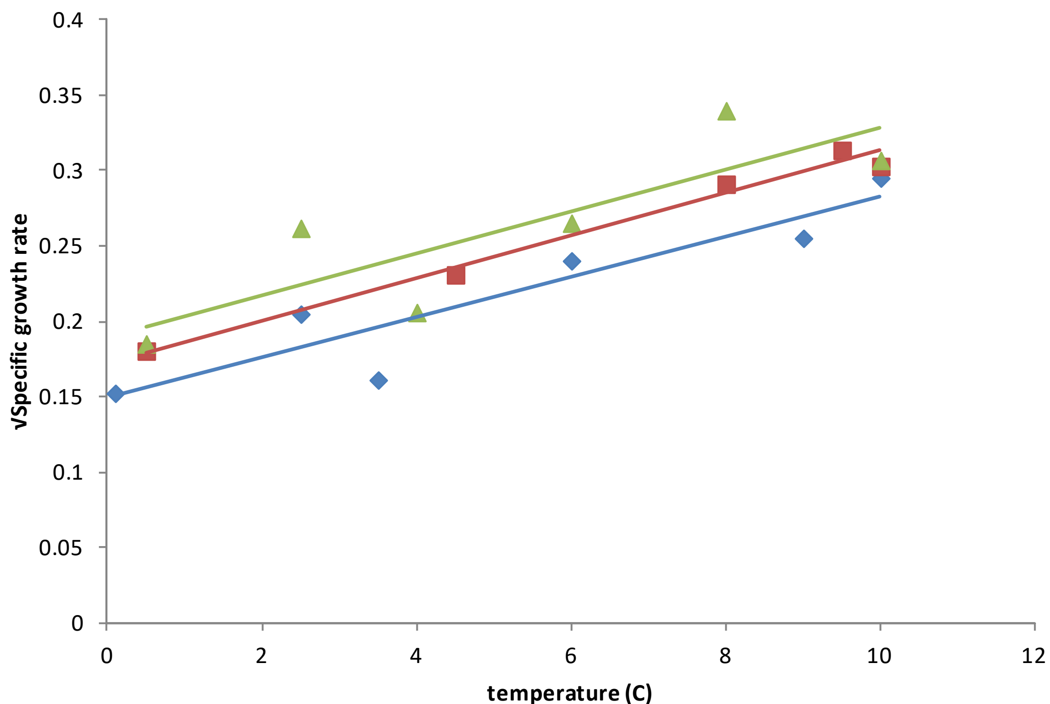


Figure 2.8: Comparison of relationship between maximum specific growth rate and temperature for three different modified atmospheres (green = 30% CO_2, brown = 55% CO_2 and blue = 98% CO_2).

The Bělehrádek-type model described in Koutsoumanis et al (2000) was fitted to our combined temperature and CO_2 data (Figure 2.9). The root mean squared error of this model was 0.0216 for the square root of the specific growth rate. The values obtained for *a*, T_{min} and CO_{2max} are shown in Table 2.5 with a comparison to the values obtained by Koutsoumanis et al. (2000) for different species of bacteria on red mullet (*Mullus barbatus*). These models are compared to our data in Figure 2.10.

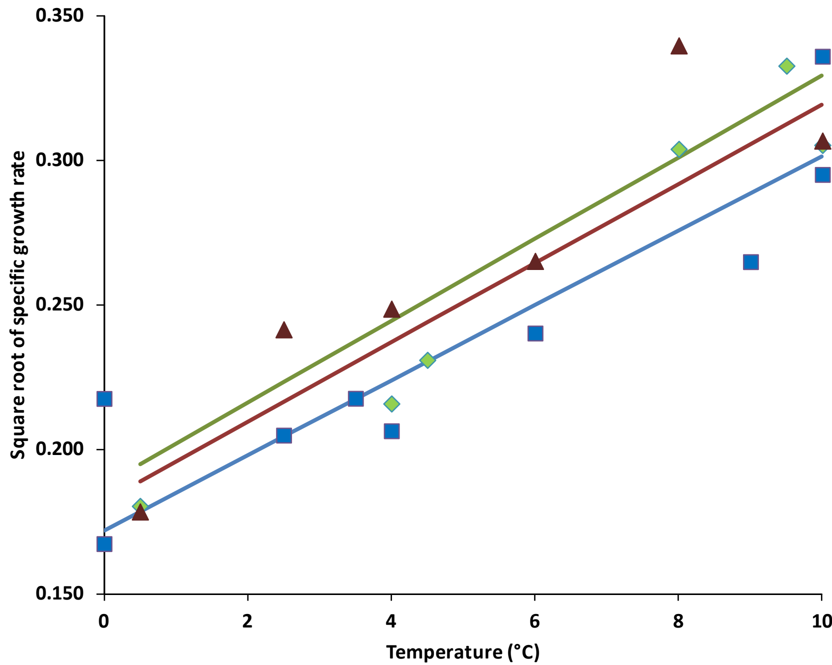
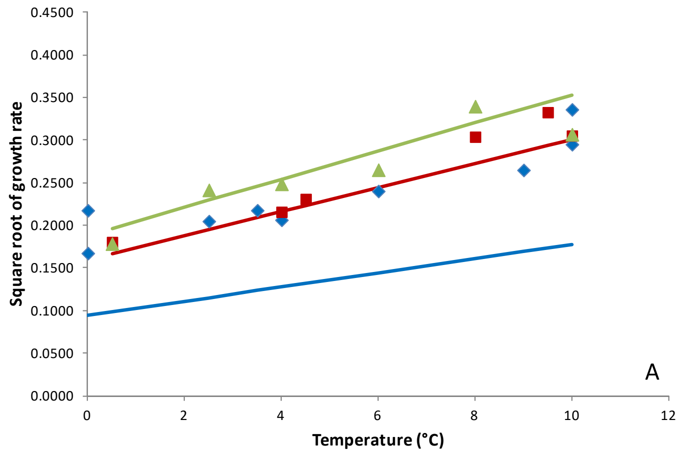
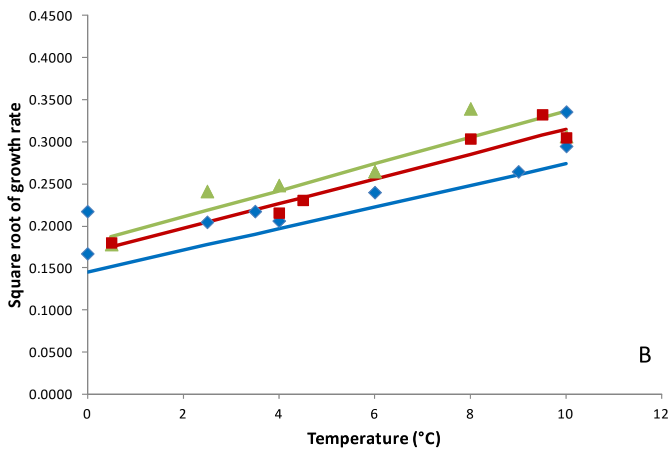


Figure 2.9: Fitted secondary model describing the effect of temperature and carbon dioxide on the maximum specific growth rate of the total viable bacteria on Atlantic salmon packed in CO_2 / N_2 mixes. The symbols are actual data points and the lines are the model predictions (green = 30% CO_2, brown = 55% CO_2 and blue = 98% CO_2).

Table 2.5: Comparison of the model from this study to those in Koutsoumanis et al. (2000).

	This study	**Koutsoumanis et al. (2000)**			
	Total viable	*Pseudomonas spp.*	*Shewanella putrefaciens*	*Brochothrix thermosphacta*	Lactic acid bacteria
A	6.9×10^{-4}	1.73×10^{-3}	1.85×10^{-3}	1.46×10^{-3}	1.11×10^{-3}
$\mathbf{T_{min}}$ (K)	259.8	262	263	262	262
$\mathbf{CO_{2max}}$ **(%)**	452	121	156	187	232





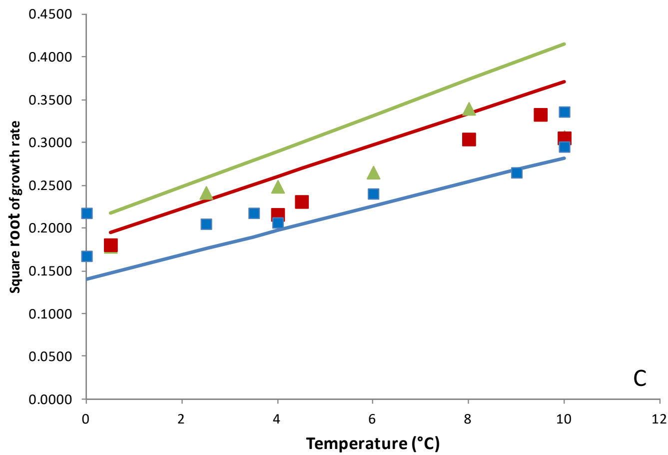


Figure 2.10: Comparison of the models for *Pseudomonas* (A), LAB (B) and *Shewanella* (C) from Koutsoumanis et al (2000) (straight lines) to our data (symbols) in modified atmospheres of 30% CO_2 (green), 55% CO_2 (brown) and 98% CO_2 (blue).

2.4 Discussion

2.4.1 Spoilage communities on Australian produced MAP Atlantic salmon

After 15 d storage under MAP, DNA-based analysis of the spoilage communities on commercially produced Atlantic salmon showed that they were dominated by *Carnobacterium* spp. (Figure 2.1). Plate count data indicated that LAB were only a small part of the total population, however it is known that Carnobacteria do not grow well on MRS agar, which is commonly used to enumerate LAB. Other media were also trialled in this study (see Elliker et al. 1956 and Davidson and Cronin 1973) however microscopic observation of colonies that grew on these agars showed the presence of gram-negative rods indicating that these agars were not selective enough to be used for enumeration. Carnobacteria can have both beneficial and detrimental effects in seafood and so their presence requires greater investigation to understand their role in the spoilage of this product. Recent work by Mace et al. (2013) has revealed that greater variety of bacteria may be responsible for the spoilage of Atlantic salmon than previously thought.

The spoilage communities observed by the sequencing of clone libraries also revealed the presence of a range of gammaproteobacteria including *Morganella*, *Rahnella*, *Serratia* and *Yersinia*. Other studies (for example Dalgaard et al. 1997, Emborg et al., 2002; Hovda et al., 2007; Rudi et al., 2004) have also reported that gammaproteobacteria are minor members of the spoilage community.

Photobacterium spp. were not detected using either culture-based or DNA-based methods. The culture-based methods used (Long & Mammer agar incubated at 10 °C) were those recommended by the group that originally identified *P. phosphoreum* as the SSO of MAP Atlantic salmon, therefore we are confident, had they been present, that we would have detected *P. phosphoreum*. Anecdotal reports from industry partners also suggested that within the salmon processing industry, Photobacteria are not considered an important SSO in Australia.

Variability in the structure of the spoilage communities was observed for the first time. Previous studies have focussed on isolation of the SSOs that are responsible for the production of off-odours and flavours. However, the minor members of the community are also important because they can influence the growth and metabolism of the SSO and hence influence the process of spoilage. In the first study, we observed that one pack out of six had a significantly different community based on the tRFLP analysis. More detailed clone library analysis showed that the reason for this difference was the presence of *Iodobacter* and various Gammaproteobacteria in this one pack.

Iodobacter is usually associated with freshwater fish and so its presence here may indicate transient cross-contamination or perhaps different storage conditions that allowed this group of organisms to survive.

These findings are important because they have implications for the use of tools to predict shelf-life and spoilage, based on storage temperatures. Different organisms have different growth requirements and growth rates and this will affect the rate of deterioration of quality.

2.4.2 An optimised method for production of MAP portions

In our study using an optimised method to process and package portions of Atlantic salmon, low initial numbers of bacteria (10^2 CFU g^{-1}) combined with several growth hurdles (high levels of carbon dioxide, high gas:product ratio and low storage temperature) resulted in an extended lag time before microbial growth was observed (Fig 2.5). The importance of low bacterial numbers at the time of packaging has been noted previously (Leroi et al. 2001; Slattery, Reeves & Warfield, 1996) and should be emphasised. The storage conditions then prevented the growth of most bacteria that remained on salmon after packaging and in the absence of competitors, and the presence of very small amounts of oxygen, *Pseudomonas* were eventually able to grow.

After 30 days, the organoleptic changes observed were minimal. Spoilage by *Pseudomonas* is usually associated with fruity or sulfhydryl odours and production of hypoxanthine resulting in a more bitter less bland taste. Spoilage due to *Photobacterium* is associated with the production of trimethylamine, a characteristic off-odour. None of these odours were present and very little slime was observed. Recent work by Mace et al (2013) characterised the main odours produced the dominant organisms they had isolated from spoilt salmon as pyrrolidine (*Hafnia alvei*), sour and amine (*Photobacterium phosphoreum*) and sour and cheese feet (*Carnobacterium maltaromaticum*), but we detected none of these.

2.4.3 A model of the effect of temperature and carbon dioxide on growth of spoilage bacteria on Australian MAP Atlantic salmon

In the initial growth curve measurements, basic sensory analysis of Atlantic salmon portions was carried out at the same time as sampling for bacterial enumeration. It is generally accepted that there is a correlation between the numbers of spoilage bacteria and the quality of a food, but in some cases the relationship between the number of total bacteria and food quality is not as clear. Our results in the initial trials showed that there was a strong positive correlation between the numbers of bacteria present and the quality of the salmon (Figure 2.6). A related study on aerobically stored Australian Atlantic

salmon also found a strong relationship between bacterial numbers and quality (Churchill, 2013).

The curves generated (Figure 2.7) by DMFit fitted well (standard errors < 0.6, $R^2 > 0.9$) to the data on bacterial numbers over time and the maximum specific growth rates for 22 different combinations of carbon dioxide and temperature were used to generate the secondary model. Several different types of models were explored including those used by Koutsoumanis et al. (2000) in modelling the effect of carbon dioxide and temperature on the growth of different bacterial species on red mullet and polynomials of the type used by Dalgaard et al. (1997) for the growth of *Photobacterium phosphoreum* on MAP Atlantic salmon. Similar to Koutsoumanis et al. (2000), the Bělehrádek type model fitted our data best. The models developed by Koutsoumanis et al. are not directly comparable to our data as they were developed using datasets that were inherently different to ours. Our study aimed to develop a model for the growth of spoilage bacteria on Atlantic Salmon as measured by the standard method (plate count agar pour plates incubated at 25 °C for 48 h), whereas the Koutsoumanis et al. modelled the growth of different bacteria individually on red mullet. However, it is interesting that the constants in our model were the most similar to those obtained by Koutsoumanis et al. for LAB (Table 2.5) and a comparison of the values predicted by the Koutsoumanis models to our data also shows the highest similarity is with the LAB model (Figure 2.10). Although we did not examine the bacterial community structure on the spoilt salmon in this part of the project, based on our previous work it seems likely that LAB were a large part of the community on the product in this study as well. The standard media and growth conditions we used may have under-estimated the numbers of LAB. The *Pseudomonas* model fits well for atmospheres with lower amounts of CO_2 and the *Shewanella* model fits for atmospheres with high amount of CO_2 but overall the LAB model is a better fit.

We also compared our model to the model for MAP Atlantic salmon fillets provided in the SSSP suite of models (Table 2.4). Again, our study targeted the entire spoilage community rather than a single organism (in this case *P. phosphoreum*). At low temperatures, growth rates were very similar, however at 10 °C the growth rates were significantly different. In practical terms, starting with the same number of bacteria (1×10^3 CFU per gram) at 10 °C and 55% CO^2, the SSSP predicts it would take 1.7 d for bacterial numbers to reach 1×10^6 CFU per gram whereas our growth rates suggest it would take 3 d.

2.5 Benefits and adoption

The research carried out in this project will benefit all Australian companies involved in the production of MAP Atlantic salmon. The identification of the spoilage community is very important to the industry because different species of bacteria have different growth rates, and hence will affect product quality and shelf-life differently, under the same handling conditions. Expectations and predictions of shelf-life based on the assumption of different organisms being present will not be accurate. Local companies now have rigorous scientific evidence to substantiate their anecdotal evidence and observations regarding spoilage of Australian Atlantic salmon.

We have also provided evidence that it is possible to extend the shelf-life of MAP Atlantic salmon if small improvements are made to processing, packaging and storage conditions. This could result in small but significant improvements in quality and shelf-life. The use of the new model to predict bacterial numbers and shelf-life also offers producers a way to develop their cold transport chains and reduce wastage resulting from discarding of product that is not actually spoilt.

2.6 Further development

The role of LAB in MAP fish is not well understood. Previously they were generally considered important in smoked rather than fresh fish. In the last two to three years, as DNA-based methods have become more widely used in food microbiology, other research groups have found that LAB are present in high numbers on MAP seafoods. These bacteria, particularly *Carnobacterium* spp., are known to have both beneficial effects on food (for example by inhibiting the growth of other spoilage organisms) but also can produce off-odours and -flavours (Mace et al. 2013a). Some work in this area is being carried out in other countries, including the development of new methods to accurately quantify these organisms (Mamlouk et al., 2012; Mace et al., 2013a). As *Photobacterium* is not the SSO for Australian product it is important that we understand the role of LAB in Australian produced MAP products.

The work on the optimised process for MAP Atlantic salmon showed that microbial growth could be inhibited to the point where autolytic degradation rather than microbial spoilage determines the product shelf-life. More work is required to test the sensory acceptability of salmon packaged this way and to determine at what point the autolytic processes reduce its quality.

The new growth model needs to be developed into a simple tools for monitoring product quality based on actual storage temperatures. This will be discussed further with the Tasmanian Salmonoid Growers Association who supported this part of the project.

2.7 Planned outcomes

The project contributes to the Australian Seafood CRC outcome 1.7 “Smart processing technologies and practices”.

This project provides evidence that it is possible to extend the shelf-life of MAP Atlantic salmon and improve the product quality and shelf-life with small changes to the production process. An extended lag time during which there was very little bacterial growth was observed when the numbers of bacteria present on the fish at the time of packaging was reduced and multiple growth hurdles incorporated into the storage conditions. The factors important in this were: short time between harvest and packaging; cleanliness of processing surfaces including the use of running water; temperature control during processing, transport and storage; and amount of carbon dioxide in packs (high carbon dioxide levels and high gas:product ratio). Incorporation of all of these into commercial processes is likely to be uneconomical; however, incorporation of some of these into commercial production is feasible and will have the benefit of improving the product’s sensory quality when it reaches the consumer or allowing an extension of the shelf-life as measured by microbial numbers.

The use of the model produced in the last part of this project will allow accurate prediction of bacterial numbers based on knowledge of the initial bacterial numbers and storage time and temperature. This may lead to a reduction in the amount of product discarded because it is assumed that the product is of poor quality or contains high bacterial numbers and will also provide more confidence in the quality of the product as it is sold to consumers.

2.8 Conclusions

All the objectives of this project were achieved. The spoilage communities in commercially produced MAP Atlantic salmon was determined and found to be significantly different to those reported in the same product produced in other countries. Evidence was provided that the growth of bacteria on this product can be reduced using simple, low-cost methods (for example increasing the volume of gas in MAP packs). Finally, because the communities on Australian product are different, a model that more accurately describes the growth of these communities was produced for use by local producers.

The most important findings from this project are:

- Spoilage communities found on MAP Atlantic salmon were dominated by LAB, particularly from the genus *Carnobacterium* although culture-based methods underestimate the occurrence of LAB.
- These communities varied, mostly due to differences in the minor members of the community.
- *Photobacterium* spp (the usual specific spoilage organism of MAP Atlantic salmon) are not detected on Australian salmon.
- In an optimised MAP system there was an extended lag time of up to 15 days before bacterial growth began and over 20 days before the numbers of bacteria reached 1×10^{6} CFU g^{-1}; these numbers are often reached after 12 – 15 days in commercial systems.
- A model, specific for Australian produced Atlantic salmon, was developed that allows the prediction of bacterial numbers based on the carbon dioxide content of the atmosphere and the actual temperatures experienced during storage.

3. Microbial communities on prawn supply chains

3.1 Introduction and Background

Several species of prawns are caught in all Australian waters coastal waters. The temperature ranges from the temperate Spencer Gulf in the south (annual range 12 – 24 °C) to the tropical northern prawn fishery which has an annual temperature range of 27 – 30 °C (Naval METOC: http://www.metoc.gov.au/products/data/aussst.php). It is well established that seafood from tropical waters tends to develop spoilage communities that are dominated by gram-positive organisms (such as *Bacillus* or LAB) whereas seafood from temperate waters are usually dominated by gram-negative bacteria such as *Pseudomonas* or *Shewanella* (Gram and Huss 1996). Given the wide range of water temperatures from which prawns are harvested in Australia, it is likely that the initial flora on the prawns will be different for each fishery. The initial microflora present on seafood is one factor that determines which SSO grows and hence influences the spoilage process. Harvesting, processing and storage conditions also affect the development of the spoilage community as well as affecting autolytic spoilage. Different initial communities may result in the growth of different SSO and hence a different shelf-life for similar products from different regions.

Only one study has investigated variation in the initial spoilage flora from prawns caught in different waters within Australia. Chinivasagam et al. (1996) compared prawns caught in shallow, warmer waters with prawns caught in deeper, colder waters in Queensland. As might be expected, they found that the initial bacterial flora depended on the environment of capture with more gram-positive bacteria on the prawns from the warmer water and mainly *Pseudomonas* spp. on the prawns from the deeper, colder water. However, the spoilage community that developed was influenced by the storage conditions, with *P. fragi* dominating prawns stored on ice and *Shewanella* dominating prawns stored in an ice slurry. The authors concluded that the initial flora influenced the shelf,-life as longer shelf-life was obtained when the spoilage organisms had to out-compete the dominating initial flora.

There are few reports of the bacteria found on freshly caught prawns. A study conducted in India found that the flora on Indian white shrimp (*Penaeus indicusm* also known as *Fenneropenaeus indicus* or Banana prawn) comprised *Aeromonas, Pseuodmonas, Vibrio, Flavobacteria* and *Serriatia* (Jeyasekaran et al. 2006). One study of seafood in Greece (Papadopoulou et al. 2007) examined different seafoods purchased from local retailers and stored for approximately 24 h. They also found a large number of samples contained *Aeromonas*. However, they did not detect any *Pseudomonas* or

Vibrio but instead observed *Proteus*, *Enterobacter*, *Hafnia* and *Staphylococcus*. Most reports focus only on total numbers, however, spoilage communities appear to contain a significant proportion of psychrotrophic bacteria (Che Rohani et al. 2008; Jeyasekaran et al. 2006), LAB and *Pseudomonas* (Martinex-Alvarez et al. 2005; Lopez et al. 2006). Storage conditions are an important factor with prawns stored under vaucum or in MAP with carbon dioxide less likely to contain *Pseudomonas* and more likely to contain LAB or facultatively anaerobic bacteria (Baug et al. 2009; Jeyaskearan et al. 2006).

3.1.1 Need

There is anecdotal evidence that the quality of Australian prawns deteriorates through the supply chain such that consumers have poor perception of the quality of prawns at the retail outlet. Project 2011/748 “Time-temperature management to maximise returns through the prawn supply chain” aimed to produce predictive models for prawns that could be used alongside temperature monitors to determine at which point in the supply chain the greatest loss of quality occurred. The use of these models with a particular product assumes that the organisms causing the spoilage of that product are the same as the ones used to develop the model. Knowledge of the micro-organisms responsible for the spoilage of prawns from different Australian fisheries is therefore required.

3.1.2 Objectives

The objectives of this project were to:

- describe the microbial communities on prawns from different sources;
- describe the microbial communities on prawns after frozen storage; and
- determine whether this was related to differences in the quality and shelf-life of these products

3.2 Methods

3.2.1 Prawn collection

The prawns used in this study were sub-samples of prawns used for project 2011/748 “Time-temperature management to maximise returns through the prawn supply chain”. Prawns from four separate fisheries were harvested commercially and processed and stored on the boats as described in Table 1. On receipt at the laboratory in Perth (Western Australia) they were either sampled immediately (for 0-month and day-0 samples) or stored for a further period at -10 or -26 °C. Prawns were thawed in water overnight prior to taking day-0 sub-samples. The remaining prawns were stored at 0 or 10 °C for a further 8 d.

3.2.2 Microbial community analysis

The microbial community composition was determined using next-generation, high-throughput pyrosequencing of the microbial 16S rRNA gene. Samples were analysed in duplicate. The prawn homogenate was thawed and 2 ml centrifuged at 10 000 x g for 10 min. The supernatant was discarded and the pellet transferred to the bead tube from the PowerSoil DNA extraction kit (MoBio) and the manufacturer's standard protocol followed. The 16S rRNA gene was amplified in 50 µl reactions using 0.1 µM each of the primers 341F (CCT ACGGGAGGCAGCAG) and 1492R (TACGGYTACCTTGTTACGACTT), 1 ng bovine serum albumin and MyTaq mastermix (Bioline Australia). The thermal cycling conditions were 95 °C for 1 min followed by 30 cycles of 94 °C for 15 s, 55 °C for 15 s and 72 °C for 20 s with a final extension at 72 °C for 3 min. The PCR product was precipitated using ethanol and dried pellets were sent to MR DNA (Shallowater, Texas, USA) for sequencing. PCR products were re-amplified using HotStar Taq Plus mastermix (Qiagen) and the primers 515F and 806R (Caporaso et al. 2011). The thermal cycling program consisted of 95 °C for 3 min followed by 28 cycles of 94 °C for 30 s, 53 °C for 40 s and 72 °C for 1 min with a final extension at 72 °C for 5 min. Sequencing was carried out on an Ion Torrent PGM following the manufacturer's standard protocols. Data was processed using the pipeline developed by MR DNA. Briefly, sequences were trimmed of barcodes and primers then poor quality sequences (length less than 150bp or containing homopolymers of 6 or more or containing ambiguous base calls or chimeric sequence) were removed. Sequences were clustered into operational taxonomic units (OTU) at the 3% dissimilarity level and a representative from each OTU was taxonomically classified using the Classifier tool on the Ribosomal Database Project website (http://rdp.cme.msu.edu/). Finally, a table was constructed showing how many times each OTU appeared in each sample. This data was analysed using multivariate methods in the Primer6 package (PrimerE Ltd).

Table 3.1: Description of products used in study.

Common name	Banana prawn	Bay prawn	King prawn	Endeavour prawn
Scientific name	*Fenneropenaeus merguiensis* or *F. indicus*	*Metapenaeus bennettae*	*Melicertus latisculcatus*	*Metapenaeus ensis* or *M. endeavouri*
Fishery	Northern Prawn Fishery	Moreton Bay	Spencer Gulf	Exmouth
Average water temperatures (°C)	26 – 30	20 – 26	18 – 21	24 – 28
Processing	Green	Green	Cooked	Cooked
Storage	Frozen	Chilled	Frozen	Frozen

3.3 Results

3.3.1 Comparison of four different products

The microbial communities in prawns that were thawed on receipt and then stored at 0 or 10 °C for 8 d were compared to determine whether there were differences between each of the four products and to determine how the communities changed during storage. A table of the percentage composition of the communities, with most OTU identified to genus level, was constructed. Due to the large number of OTU that were present in very small numbers, only those OTU that were present in at least two samples at a level of over 1% of the community in that sample, were included in the multivariate analysis. The similarity between the communities is shown on a multi-dimension scaling plot in which samples that are more similar to each other plot more closely together.

The four products are distinctly different to each other (Figure 3.1). Except for the Banana prawns, the day-0 samples are different to both the 0 and 10 °C day-8 samples. A two-way crossed analysis of similarity (ANOSIM) test revealed that the four different products are statistically significantly different ($R = 0.922$, $P = 0.001$) and that the two time points (0 and 8 d) are also significantly different ($R = 0.728$, $P=0.001$). The only product for which storage temperature appeared to have an effect was the King prawns.

The main bacterial genera observed on these prawns are listed in Table 3.2. Eight days after thawing a mix of gram-positive (*Bacillus* and LAB including *Carnobacterium*) and gram-negative bacteria, mostly *Pseudomonas*, *Serratia* and *Psychrobacter* were present. The day-8 spoilage communities on the Bay and Endeavour prawns were both dominated by a single genus (60% *Psychrobacter* and 55% *Pseudomonas* respectively). The spoilage communities on the King and Banana prawns were an even mix of several genera.

Table 3.2: Main genera found in four different prawn products stored for 8 days at 0 and 10 °C.

	Banana	Bay	King	Endeavour
Day 0	*Bacillus* *Serratia* *Carnobacterium* *Sphingomonodaceae*	*Shewanella* *Psychrobacter* *Pseudoalteromonas* *Sphingomonodaceae* *Caulobacter*	*Bacillus* *Serratia* *Psychrobacter* *Aquimarina*	*Acinetobacter* *Chryseobacterium* *Sphingomonodaceae* *Stenotrophomonas* *Cupravidas* *Pseudomonas*
Day 8	*Bacillus* *Serratia* *Carnobacterium*	*Psychrobacter* *Carnobacterium* *Vagococcus*	*Pseudomonas* *Psychrobacter* *Bacillus* *Serratia* *Sphingomonodaceae*	*Pseudomonas* *Pseudoalteromonas* *Janthinobacterium* *Carnobacterium*

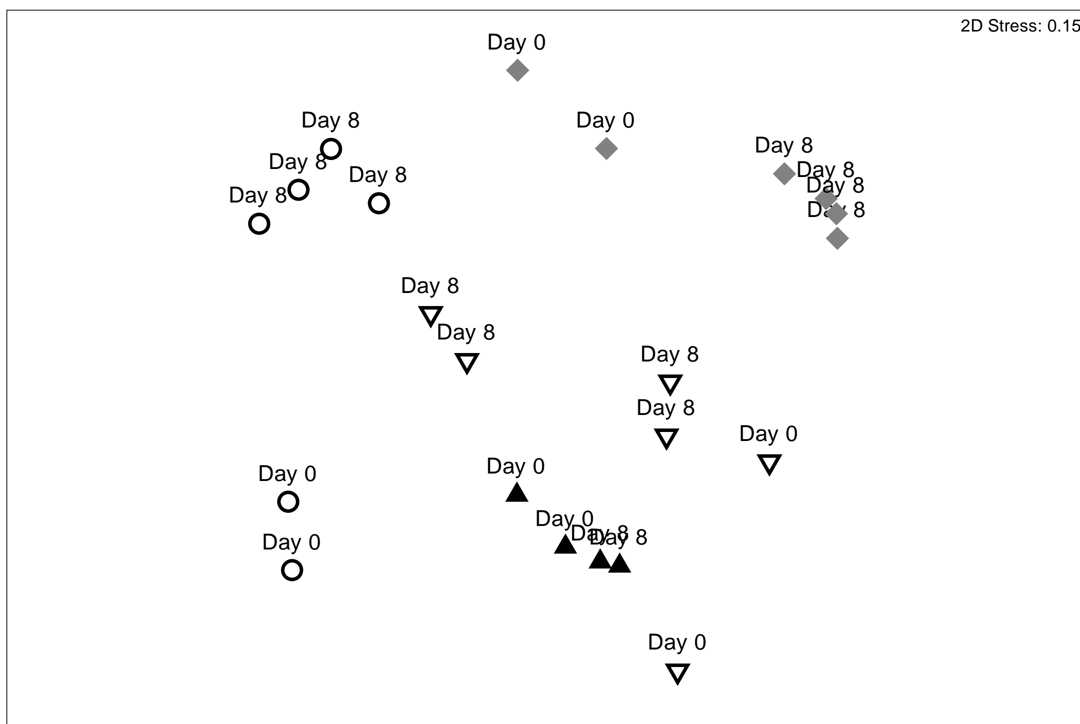


Figure 3.1. MDS plot showing the similarity of the microbial communities found at 0 or after 8 d storage at 0 or 10 °C for four different products: Endeavour (white circles), Banana (black triangles), Bay (grey diamond) or King (white inverted triangle) prawns.

3.3.2 Microbial communities on frozen prawns

Banana and King prawns were stored frozen at -26 °C for up to six months and were sampled immediately (0 months) and after 3 and 6 months. The most common genera detected are listed in Table 3. The Banana prawns were dominated by *Bacillus* and *Serratia* whereas the King prawns were dominated by *Pseudomonas*, *Bacillus*, *Psychrobacter* and an unclassified genus from the Sphingomonodaceae family. At each time point samples were taken on thawing and 8 d later. An MDS plot (Figure 3.2) showed that the spoilage communities on the two species were different (ANOSIM test: R = 0.594, P = 0.001). Interestingly, the communities at 0, 3 and 6 months were significantly different (ANOSIM global R = 0.655 P = 0.001) for the King prawns but not for the Banana prawns (ANOSIM global R= 0.09, P = 0.18).

The microbial communities on Banana prawns stored frozen at -10 °C were also compared to the communities found on Banana prawns stored at -26 °C. The communities present after storage at -10 °C were statistically significantly different (ANOSIM R= 0.365, P=0.002) to those that developed after storage at -26 °C. Although the communities at both temperatures contained *Bacillus* and *Serratia*, *Carnobacterium* were also present after 3 and 6 months at -10 °C, and the genera that were present only in small proportions were different at the two temperatures.

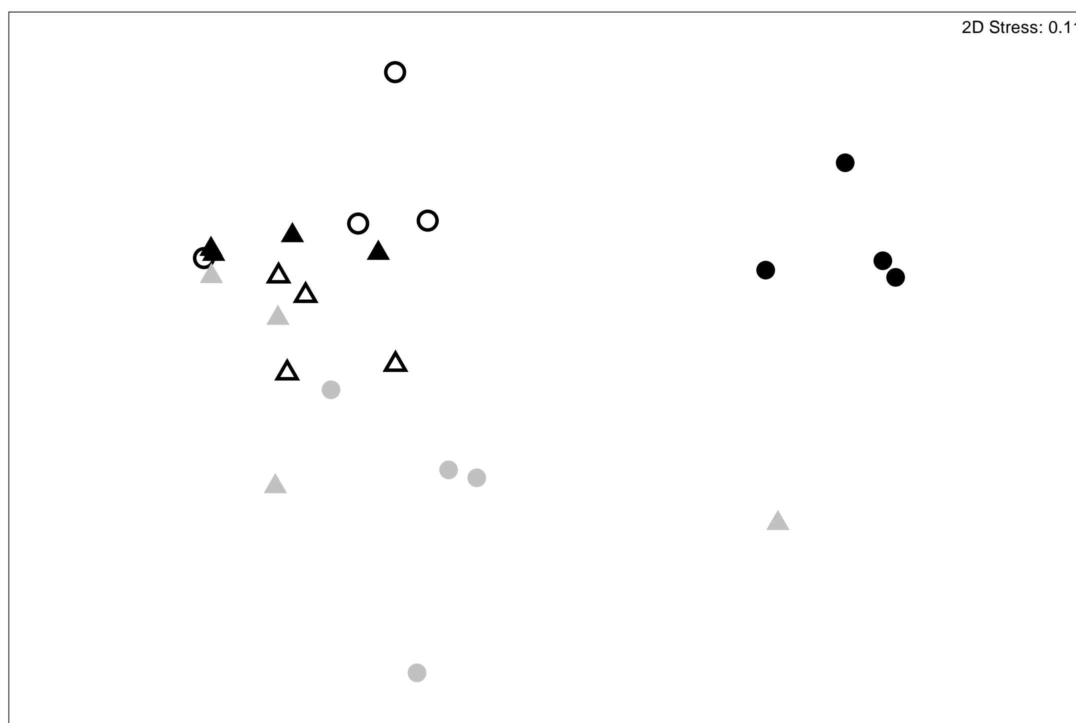


Figure 3.2: MDS plot showing the similarity of the microbial communities found on thawed Banana (triangle) and King (circle) prawns after storage at -26 °C for 0 (white), 3 (grey) or 6 (black) months.

Table 3.3: Dominant genera found on Banana and King prawns 8 days post-thawing after frozen storage at either -10 or -26 °C for up to 6 months.

	Banana prawn		King prawn
	- 10 °C	-26 °C	-26 °C
0 months	*Bacillus* *Serratia* *Carnobacterium*	*Bacillus* *Serratia* *Carnobacterium*	*Pseudomonas* *Psychrobacter* *Bacillus* *Serratia* *Sphingomonodaceae*
3 months	*Bacillus* *Serratia* *Carnobacterium* *Pseudomonas*	*Bacillus* *Serratia* *Sphingomonodaceae*	*Bacillus* *Sphingomonodaceae* *Pseudomonas* *Serratia*
6 months	*Bacillus* *Serratia* *Carnobacterium* *Psychrobacter*	*Bacillus* *Serratia* *Psychrobacter* *Pseudomonas*	*Pseudomonas* *Pseudoalteromonas* *Psychrobacter*

3.4 Discussion

3.4.1 Microbial communities on fresh and stored prawns

In general, the microbial communities present on the prawns thawed and sampled on receipt (that is after 0 d storage) were diverse and in most cases consisted of a mix of marine (e.g. *Pseudoalteromonas, Psychrobacter*) and gut (*Serratia, Carnobacterium*) bacteria. Many more genera were detected than are listed in Table 2. Often these bacteria were present as a small proportion of all the sequences detected. Some genera were detected only in one replicate at one time point whereas others were detected more frequently. This diversity disappeared 8 d after thawing with the communities becoming dominated by a small number (two to four) of different genera. *Bacillus* and *Carnobacterium* were the most common gram-positive organisms. Both are known to have roles in the spoilage of some seafood although some *Carnobacterium* are known to produce bacteriocins which may inhibit the growth of other spoilage bacteria (Leisner, 2007).

Recently, Mace et al. (2014) demonstrated that *Carnobacterium maltaromaticum* produced off odours described as sour, feet/cheese or buttery when inoculated in shrimp (*Penaeus vannamei*) stored under MAP. *Vagococcus*, a genus of LAB related to *Carnobacterium*, has been previously isolated from spoilt cooked shrimp (Jaffres et al. 2009) although its role in spoilage is unclear. *Pseudomonas* are well known as spoilage organisms for fresh seafood (REF). It has been described as the SSO of prawn (or shrimp) in several studies previously (Chinivasagam et al. 1996, Jeyasekaran et al. 2006) where it produced fruity off-odours. Several strains identified as *Psychrobacter* and *Pseudoalteromonas* were isolated from *Crangon crangon* (Brown shrimp) classified as spoilt by sensory methods (Broekaert et al 2013). In a follow up study Broekaert et al. (2013b) explored the off odours produced by these two genera and concluded that *Pseudoalteromonas* produced most of the off-odours although it was likely that *Psychrobacter* were contributing to the breakdown of lipids. A study in cod (*Gadus morhua*) described *Psychrobacter* as lipolytic and producing a strong musty odour (Bjorkevoll et al. 2003).

3.4.2 Difference between species / fishery

Each of the four products tested had distinct microbial communities (Figure 3.1, Table 3.2). These differences most likely arose from a combination of factors: different microbes present initially on the prawns due to harvest from different areas, exposure to different methods of handling and processing and different storage conditions on the boat. It is difficult to attribute the development of the different spoilage communities to any one of these factors in particular; however, it is significant that different spoilage communities develop as this influences the quality and shelf-life of the product.

All the genera identified at spoilage on the different products have been associated with spoilt prawns or shrimp previously. A related study, from which these samples were taken, measured the deterioration in quality of the stored prawns over time using a quality index method (QIM) and found that, although the difference in QIM scores was only two or three points, after 8 d at 10 °C the Bay prawns had the lowest quality and the Endeavour the highest. At this time, *Psychrobacter* and LAB dominated the Bay prawns, whereas *Pseudomonas* dominated the spoilage community on the Endeavour prawns (Table 3.2).

3.4.3 Effect of long-term frozen storage

This is the first study of which we are aware that has investigated the effect of long-term frozen storage on the quality and spoilage of prawns. As observed from the study of prawns thawed on receipt at the laboratory, the microbial community that developed on the Banana and King prawns stored frozen for extended periods of time were different (Figure 3.2). The Banana prawns were dominated by *Bacillus* and *Serratia* at all time points whereas the spoilage communities on the King prawns varied with time and included *Pseudomonas* and *Psychrobacter* as well as *Bacillus* and *Serratia*. The spoilage community of Banana prawns frozen at -10 °C also contained *Bacillus* and *Serratia* but in addition included *Carnobacterium,* which did not appear to survive for long after storage at -26 °C. It may be that *Bacillus* and *Serratia* are particularly well adapted to surviving frozen storage but where *Pseudomonas* have survived the period of storage they are able to recover and grow more quickly once the prawns are thawed.

3.4.4 Correlation with quality measurements

The data on the microbial community structure was compared to the data on prawn quality generated in project 2011/748. The Endeavour prawns from the Exmouth fishery that were cooked and frozen spoilt the least over 8 d once thawed. The spoilage communities on these prawns were dominated by bacteria from the genera *Pseudomonas*, *Pseudoalteromonas* and *Janthinobacterium*. The Bay prawns from the Moreton Bay fishery that were frozen green spoilt the most in the 8 d after thawing. The spoilage communities on these prawns were dominated by members of the *Psychrobacter* genus and LAB including *Carnobacterium* and *Vagococcus*. However, the difference in the QIM scores among all four supply chains were small – only between two and three points after 8 d.

3.5 Further development

It appears that different spoilage communities are found on prawns from different supply chains and it is possible that these communities could be used as a biological marker or fingerprint to establish which supply chain they came from. However, the data obtained in this project should be considered preliminary. Replication is required before general conclusions can be drawn on the micro-organisms responsible for the spoilage of Australian prawns and more work would be needed to establish standard methods for obtaining a microbial community fingerprint before it was used as a marker for identifying supply chains.

3.6 Planned outcomes

This project contributes to the Australian Seafood CRC output 2.3 “Predictive tools to increase value chain efficiency”. Predictive tools are based on knowledge of the micro-organisms that cause spoilage of seafood. This project has provided preliminary information on the spoilage communities found on four different prawn supply chains.

The data obtained in this project contributes to our understanding of the spoilage processes that were observed and modelled in project 2011/748. The results from this study were presented to industry participants at a workshop in Perth in February 2014.

3.7 Conclusion

The data collected in this project is preliminary and needs to be replicated. However, there appear to be significant differences in the spoilage communities and likely specific spoilage organisms found on prawns harvested from different areas. The geographical location might not be the only factor that is responsible for this; processing and storage conditions are also important.

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Appendix I Microbial community analysis methods

Appendix 1A: tRFLP method

The 16S rRNA gene was amplified using Immomix (Bioline) and 0.1 µM of primers 10F (GAGTTTGATCCTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT). Both primers were labelled on the 5' end with either WellRED dye D3 or D4 (SigmaProligo). The thermal cycling program consisted of a 10 min initial denaturation step at 95 °C, followed by 35 cycles of 1 min at 94 °C; 1 min at 55°C and 1 min at 72 °C, with a final step of 10 min at 72 °C. Two separate PCR reactions were pooled before digesting 10 µl of the combined PCR product with 5U of either *HhaI*, *HinfI* or *HaeIII* (New England Biolabs) for 3 h. The digests were purified by ethanol precipitation into a 96-well plate. The purified digests were resuspended in 30 µl of CEQ sample loading solution (Beckman Coulter) with 0.25 µl of GenomeLab size standard 600 (Beckman Coulter). The fragments were separated on a Beckman Coulter CEQ Genetic Analysis system.

The CEQ software (Beckman Coulter) was used to create a matrix of fragment length and peak height for each enzyme–label combination excluding peaks with a height of less than 500 relative fluorescence units. The percentage peak height was calculated for each fragment and fragments that made up less than 1% of the total peak height for a sample were given a value of zero whilst fragments with a peak area over 1% were left as a percentage. The data from the three enzymes were combined into one matrix of percentage peak height for fragment length for all samples. Multivariate methods were used to analyse differences in the microbial communities using the Primer6 package (Primer-E Ltd). Resemblance matrices were generated using either the Bray-Curtis similarity index or a simple matching co-efficient. The Bray-Curtis index is widely used in ecological studies and differs from the simple matching co-efficient as it ignores joint absences in calculating the similarity between two samples. Non-metric multidimensional scaling ordination plots (MDS) were used to explore relationships between groups of samples based on the strength of these calculated similarities/dissimilarities between pairs of samples. In these plots samples that are close together are more similar to each other than samples that are further apart. Tests of the null hypothesis of no difference between sample groups were done using the Analysis of Similarity (ANOSIM) procedure in Primer6 and were based on the previously generated similarity matrices. The data were generally not transformed as the peak area had already been normalised to percentage peak area. However for some analyses a presence/absence transformation was carried out.

Appendix 1B: Clone library construction

The 16S rRNA gene was amplified using Immomix PCR Mastermix (Bioline) with 0.2 uM of each of the primers 10F (GAG TTT GAT CCT GGC TCAG) and 1492R (TAC GGY TAC CTT GTT ACG ACTT). The thermal cycling program consisted of a 10 min initial denaturation step at 95 °C, followed by 35 cycles of 1 min at 94 °C; 1 min at 55 °C and 1 min at 72 °C, with a final step of 10 min at 72 °C and PCR products were cleaned using the UltraClean PCR kit (MoBio). Clean PCR products were cloned using the TOPO-TA cloning kit (Invitrogen) according to the manufacturer's standard protocol. Clones were sub-cultured and placed directly into a PCR reaction with the M13 primers (F: GTA AAA CGA CGG CCAG and R: CAG GAA ACA GCT ATG AC). The thermal cycling conditions were as above except that 30 cycles were carried out and the annealing temperature was 60 °C. Successful amplifications were precipitated using ethanol and the clean, dry PCR product was sent to Macrogen Inc (South Korea) for sequencing. The CLASSIFIER tool on the Ribosomal Database Project (http://rdp.cme.msu.edu/) was used to classify clone sequences to the genus level and any that were below 90% confidence interval were checked for chimeric characteristics using the Pintail program (http://www.bioinformatics-toolkit.org/Web-Pintail/).

Appendix II Access to outputs

All sequence data generated in these projects are publicly available online through the GenBank database at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The sequences can be accessed through the following accession numbers.

Sequences describing the microbial ecology of an oyster hatchery:
Sequences associated with algal cultures: JF707640–JF707766
Sequences associated with water samples: BioProject PRJNA244691 (release date June 30, 2014)

Sequences describing the spoilage communities of Australian produced Atlantic salmon: JF719342–JF719542

Sequences describing the spoilage communities of Australian wild caught prawns: BioProject PRJNA244690 (release date June 30, 2014)