Developing targeted strategies for improving product quality through selected low value seafood supply chains

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NON TECHNICAL SUMMARY

2006/209	**Developing targeted strategies for improving product quality through selected low value seafood supply chains**

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OBJECTIVES:

1. To complete microbiological and temperature analyses of six seafood supply chains.
2. To determine the impact of specific spoilage organisms/products on product quality through the test supply chains.
3. To identify and validate supply chain intervention and/or value-adding protocols which improve shelf-life or enable the development of new products.

NON TECHNICAL SUMMARY:

The project outputs have contributed to or will lead to the following outcomes:

1. The effectiveness of sanitiser application on a variety of finfish, and other seafood such as crabs and octopus has been completed. The use of sanitisers proved effective for some seafood species (e.g. Sea Mullet), but not for others (e.g. octopus and crabs).
2. The correlation between spoilage and bacterial numbers has been elucidated for all six seafood species investigated. For some there was a good correlation (e.g. sea mullet), for others there was no correlation (e.g. octopus), and for others the bacterial levels in the environment it is stored in had as large an impact on the perception of spoilage as the bacterial levels in the flesh (e.g. mussels)
3. Optimisation of sanitising and storage regimes to decrease numbers of spoilage bacteria and enhance shelf-life in selected finfish species was completed and where appropriate changes are suggested. As one approach was not suitable for all sectors of the industry, different recommendations are made for different sectors.
4. For Blue Mussels the float test was found to have a good correlation with days stored, and microbiological counts.
5. Refrigeration of mussels in freely draining conditions while washing frequently reduced overall bacterial levels
6. Treatment of chilled whole crabs with sanitisers did not impact the growth of microorganisms or rate of spoilage.
7. Treatment of whole cooked Blue Swimmer Crabs with sanitisers did not significantly lower the rate of bacterial growth.
8. A fully validated Quality Index Method for Australian Salmon was developed.
9. Publication (websites, SQMI pamphlet) of information on the effective use of sanitisers in the fishing industry has been made.
10. Communication with producers has been carried out during the course of this project by participation with a number of seminars for the seafood industry. Further communication will be carried out in the future to make the industry aware of the final results.
11. This project has resulted in a culture collection of over 5,000 organisms many of which have been identified. Many different techniques for the identification of bacteria have been developed including biochemical, cell wall fatty acid, and sequencing based identification which have already been used by other researchers.
12. Short documents for each selected seafood line with results and recommendations to improve product quality and shelf-life and/or develop new products. These documents will be produced at the conclusion of the writing of the final report.
13. A report to be written for an upcoming issue of Seafood Directions.

Recently we have seen the emergence of the discerning seafood consumer, who is in the market for high quality, and yet affordable, easy to prepare seafood products. To enhance profitability whilst meeting changes in consumer expectations, seafood sectors, particularly those with lower value products, must be constantly engaged in whole-of-chain strategies to improve quality, increase shelf-life and develop innovative products.

Strategies which maintain quality and improve shelf-life, and result in the development of innovative products, are usually associated with a reduction in microbiological spoilage. It is surprising therefore that, apart from specific monitoring for pathogens (e.g. *E. coli*; *Listeria* sp.), there has been very little work on microbiological analyses of Australian seafood supply chains, and in particular the relationship between microbiology and spoilage, Rather, quality versus spoilage has generally been measured by qualitative indices such as the quality index.

The spoilage of six different seafood species was investigated and means to overcome spoilage investigated. Each species had unique microbial populations and the impact of microorganisms on spoilage varied greatly between species. The use of sanitisers had a significant impact on the rate of spoilage of some species, and in some cases they had no impact. A one size fits all approach to controlling spoilage on seafood does not appear to be applicable based on the findings of this study. Variation was not only found between major groups (e.g. finfish, crustaceans, molluscs, and cephalopods), but also within groups.

Spoilage of Sea Mullet occurred rapidly, with low bacterial levels increasing rapidly between 5 and 20 days of storage on ice. Changes to the way the Sea Mullet was held, and the use of sanitisers in ice slurries slowed the rate of spoilage of Sea Mullet. The use of sanitisers in the ice slurry has a significant impact on both the bacterial counts and quality index of sea mullet. There was no significant difference in the rate of spoilage or in the microbiological populations on fish caught in different geographical locations. The treatment of Sea Mullet with sanitisers results in an overall reduction in all bacterial levels and did not appear to target particular bacterial groups or species.

The rate of growth of bacteria and the rate of spoilage (as determined by the Quality Index Score) was slower for Goldband Snapper than for Sea Mullet. The microbial population on the skin of fresh Goldband Snapper appears to be more diverse than that found on Sea Mullet and using sanitisers gave a marginal improvement in quality index score and bacterial levels. This marginal increase would possibly not be financially viable for Goldband Snapper, but may be of use for other tropical snappers with a shorter shelf-life. A PhD project funded by the Australian Seafood CRC was commenced following preliminary results of this project. A thesis based on this work by Rachel Tonkin (Curtin University) has been submitted.

The spoilage of refrigerated octopus does not appear to be related to microbiological growth. Storage of octopus in drained iced under refrigeration resulted in the dehydration of the tips of tentacles and the formation of gelatinous blebs. The storage of octopus in an ice slurry stopped the dehydration of the tentacle tips, but worsened the rate of bleb formation. The formation of blebs was found not to be due to the presence of high levels of bacteria.

Investigations into the spoilage of Blue Mussels found bacterial growth was higher in the liquor expressed by the mussels than in the mussel meat, and the bacterial growth in the

liquor appeared to be the cause of a large proportion of the off odours detected during spoilage. Refrigeration of mussels in freely draining conditions and washing mussels frequently reduced overall bacterial levels. Unspoilt Blue Mussels had a diverse micro-flora. A test to assess the freshness of mussels was developed. The float test was found to have a good correlation with days stored, and microbiological counts.

The spoilage of Blue Swimmer Crabs was found to be significantly different depending on whether they were cooked or uncooked. Aerobic psychrotrophic bacteria showed the greatest growth in uncooked refrigerated Blue Swimmer Crabs. Bacterial levels were not affected by the sex of the crab with only changes in different species of bacteria impacting on spoilage. The storage of Blue Swimmer Crabs in freely draining ice or in a refrigerator with a damp cloth coverage produced slightly less bacterial growth than when crabs were held in non-drained ice. Treatment of crabs with sanitisers had no impact on the rate of spoilage of either cooked and uncooked crabs.

A Quality Index Method (QIM) for Australian Salmon was produced. The QIM scores correlated well with the days of storage, plus the bacterial counts. When investigating the bacterial counts on Australian Salmon there was no significant difference between the standard plate count and modified Long & Hammer count. The presence of *Serratia liquefaciens* on both the unspoilt and spoilt Australian Salmon is of great significance as this species has previously been found to produce biogenic amines (including histamine).

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BACKGROUND

In response to market access issues and also to emerging legislative requirements under the Food Standards Australia New Zealand (FSANZ) Food Standards Code, various sectors of the seafood industry have focused efforts on their respective supply chains to improve product safety and quality. This has resulted in the development of various Codes of Practices for handling lobster, tuna, shark, mussel, swordfish, and other species, as well as whole of chain strategies such as the AQL-1 project and traceability initiatives. Western Australian seafood supply chains in particular have benefited from this work as supply chains are generally extended and subject to extreme temperature fluctuations.

A recent trend has seen the emergence of the discerning seafood consumer. Such a consumer is in the market for a high quality, and yet affordable, easy to prepare seafood product. He/she may also demand more information on the product, including the history of the product from harvest to purchase. To enhance profitability whilst meeting changes in consumer expectations, various seafood sectors, particularly those with lower value products, must be constantly engaged in whole of chain strategies to improve quality, increase shelf-life and develop innovative products.

Strategies which maintain quality, improve shelf-life and result in the development of innovative products are usually associated with a reduction in microbiological spoilage. It is surprising therefore that, apart from specific monitoring for pathogens (e.g. *E. coli*; *Listeria* spp.), there has been very little work on microbiological analyses of Australian seafood supply chains, and in particular the relationship between microbiology and spoilage. Rather, quality versus spoilage has generally been measured by qualitative indices such as the quality index. Such indices are extremely useful in supply chain studies and analysis but they do have a degree of subjectivity. Indeed, a preliminary study has indicated that the QI may not be related to the total viable count of bacteria. With a greater understanding of the mechanisms of microbiological spoilage within a specific supply chain, and the development of supply chain specific strategies to mitigate such spoilage, improvements in product

development/value may be possible.

Factors affecting Microbiological Growth on Foods

A number of factors affect the ability of microorganisms to grow on foods. This can be grouped into a series of factors (Adams & Moss, 2008) viz:

- Intrinsic Factors: Factors that are specific to the physico/chemical composition of the food matrix in (or on) which a microorganism is growing.
- Extrinsic Factors: The environmental conditions under which a food is stored.
- Implicit Factors: The properties and interactions of the microorganisms present.
- Processing Factors: The means by which a food has been manufactured.

The intrinsic factors include:

- Nutrient Content (the types of nutrients available)
- pH
- Redox Potential (E_h)
- Antimicrobial Barriers & Constituents
- Water Activity (a_w)

The extrinsic factors include:

- Temperature (the temperature at which the food is handled and stored)
- Gaseous Atmosphere (the atmosphere in which the foods are held e.g. In air, vacuum packaging, Modified Atmosphere Packaging - MAP)
- Relative Humidity

Implicit factors are the properties of the organisms themselves, including how they respond to their environment and interact with one another (Adams & Moss, 2008). An organism's specific growth rate is an example of an implicit factor. Organisms with the highest specific growth rate are likely to dominate over time For example, moulds grow well on fresh meat, however they do not grow at the same rate as bacteria and so are commonly out competed. Other implicit factors include mutualism, where the growth of one organism stimulates the growth of another and antagonism, when a micro-organism produces inhibitory compounds or sequesters essential nutrients to limit the growth of surrounding micro-organisms. Other examples include heat shock proteins, which protect the cell from heat damage and sigma factor RpoS, which is a general stress response regulator (Adams & Moss, 2008).

The optimum temperatures under which microorganisms will grow is often used to group them. Table 1 shows the temperatures under which different groups of microorganisms are grouped. Most organisms which cause spoilage on seafood belong to the psychrotrophic and psychrophilic groups as these are the groups which are most likely to grow at a faster rate under refrigeration (Adams & Moss, 2008).

Table 1 Cardinal temperatures for bacterial growth.

	Temperature (˚C)		
Group	Minimum	Optimum	Maximum
Thermophiles	40-45	55-75	60-90
Mesophiles	5-15	30-40	40-47
Psychrophiles	-5 to +5	12-15	15-20
Psychrotrophs	-5 to +5	25-30	30-35

Food spoilage and shelf life

Food spoilage is considered as any change which renders a product unacceptable for human consumption. Australian Food Safety Standards state that "a food product is not suitable for consumption if the food or a substance within the food is damaged, deteriorated or perished to an extent that affects its intended use" (F.S.A.N.Z., 2007). Defining what is unacceptable is often subjective and varies depending on personal tastes, socioeconomic and cultural background etc. All foods which have foodborne pathogens or toxins are viewed as unacceptable. Other causes of food unacceptability include insect damage, loss or change in flavour, odour or taste, physical damage, or microbial growth (e.g. slime production, growth of moulds) (Steele, 2004).

The shelf life of a product is defined as the time during which products will remain safe, retain desired sensory, chemical, physical and microbiological characteristics, and also to comply with any label declaration of nutritional data (Kilcast & Subramanian, 2000). Extending the shelf life of seafood would be advantageous for the industry in WA due to the vast distances which must be covered for fresh fish to reach the majority of consumers. A large proportion of Australia's domestic seafood markets are supplied with fresh and frozen fish from the domestic catch (BRS & FRDC, 2008). Most Australian commercial fishing enterprises are not close to major capital cities where much of the catch is sold. Extended travel distances are common, especially in Western Australia with fish travelling for 2-3 days

in a refrigerated truck before being distributed to retailers. Fish with an extended shelf life are able to be stored for longer periods and retain their quality and freshness reducing wastage and allowing increased opportunity for sale. An additional benefit is that consumers then have the opportunity to experience quality fresh food products not locally available.

Microbiological spoilage and detection

It has been shown that the shelf life of fresh fish is greatly influenced by the growth of microbial populations and autolysis (Jeyasekaran, Maheswari, Ganesan, Jeya Shakila, & Sukumar, 2005). Fish are a good substrate for microbial growth due to their high moisture content, neutral to low pH and high nutritional value (Huis in't Veld, 1996). The flesh (muscle) of healthy, newly caught fish is sterile; however, the skin, gills and intestines may carry considerable bacterial loads. Bacterial loads on the skin surface may range from 10^2 – 10^7 cfu/cm^2 whilst the gills and intestines contain between 10^3 and 10^9 cfu/g (Huss, 1995).

A high bacterial load, calculated from the total plate count, is used to indicate that a fish has reached the end of its useable shelf-life, however, the number of bacteria present on a fish does not always relate to fish spoilage. For example, fish harvested from warmer waters have a higher bacterial load than those from colder regions (Chattopadhyay, 1999; Shewan, 1977) yet it is well documented that fish caught from warmer waters have a longer shelf life than those caught from temperate waters (Surendran et al., 1989; Surti et al., 2002). The difference in shelf life is thought to be associated with the initial low number of specific spoilage organisms carried by tropical fish in their natural habitat (Surti et al., 2002). Therefore, high bacterial numbers are not always the cause of deterioration and spoilage.

Specific spoilage organisms (SSOs) are usually present on fresh or lightly preserved seafood in very low concentrations, making up a small percentage of the total microflora. Under certain conditions, these spoilage organisms are able to proliferate at an increased rate, producing off flavours, and are eventually responsible for sensory rejection of the fish (Dalgaard, 2003). The SSOs differ for individual species of seafood and in some instances it is only a single bacterial species responsible for spoilage (Gram & Dalgaard, 2002)(Gram & Dalgaard, 2002). The well-known spoilage organism for iced marine fish is *Shewanella putrefaciens,* while *Pseudomonas* spp. are known spoilage organisms for iced fresh water fish (Gram & Dalgaard, 2002). The environment from which the fish are caught; temperate,

tropical and sub-tropical waters, does not appear to effect the type of SSOs, with all marine fish experiencing the same SSOs when stored in chilled aerobic conditions (Gram, 1996).

Techniques for bacterial enumeration

The isolation of spoilage flora and specific spoilage organisms from marine fish requires specialised media. The media selected needs to enumerate and isolate the spoilage bacteria commonly found on fish at both low and high temperatures, hydrogen sulphide producers and microbial contaminates.

Standard Plate Count

The standard plate count is a technique used to determine the total number of aerobic mesophilic organisms present on a food sample. Visible colonies are counted after food is inoculated onto Plate Count Agar using standard procedure and conditions (Huss, 1988). Along with a thorough knowledge of the processing system, for example holding temperatures, handling of the fish and packing, a standard plate count allows the investigator to measure microbial contamination experienced by the fish (Huss, 1988). It is important to note that standard plate counts measure the entire microbial population and therefore has no correlation to the eating quality or shelf life of the fish (Huss, 1988). As well as determining the number of mesophilic organisms contributing to the spoilage flora on spoiling fish, a standard plate count is useful for determining the overall microbial contamination experienced throughout the supply chain.

Iron Agar Count

Iron agar is a media used to enumerate hydrogen sulphide producing SSO, such as *Shewanella* species. *Shewanella* spp. degrade sulphur containing amino acids and produce volatile sulphides including hydrogen sulphide (Vogel et al., 2005), and it is these compounds that contribute to the off odours experienced in spoiling fish (Lapin & Koburger, 1974). Originally lead acetate agar was used to enumerate hydrogen sulphide producers, however as Tittsler & Sandholzer (1937) demonstrated Iron agar allowed results to be determined after a shorter incubation time and with more clarity. A positive result for hydrogen-sulphide producing colonies will result in the colony turning black in comparison to the lead acetate agar which resulted in only a black-brownish colour almost so faint the result was often uncertain (Tittsler & Sandholzer, 1937). Further studies have concluded that adding L-cysteine will enhance and stabilise the blackening of the colonies (Gram et al., 1987).

Long and Hammer Count

Long and Hammer agar was used to enumerate the total aerobic counts in fresh and lightly preserved seafoods. Due to its low incubation temperature (15°C) and high salt content this media allows for the detection of psychrotolerant bacteria commonly found on seafood. Long and Hammer agar can also help differentiate between certain species of bacteria from phenotypic characteristics. An investigation into the spoilage bacteria on shrimp phenotypically characterized some common spoilage bacteria grown on Long and Hammer agar (van Spreekens, 1977). *Shewanella putrefaciens* colonies produced a salmon pink endopigment when grown on Long and Hammer agar and *Psychrobacter* colonies were grey/white with some having a wrinkle appearance (van Spreekens, 1977).

NEED

Surveys have identified a market absence and yet consumer demand for well-presented, well-priced, top quality fresh and frozen WA seafood products. The market value of WA seafood may be further enhanced by the development of high quality value-added convenience products. These products may be developed using innovative processing technologies that ensure that taste and texture quality equivalent to a fresh product are achieved. Documented attention to food safety and environmental issues should also be addressed as these have been identified in consumer surveys as of importance to the discerning purchaser. Such value-adding, resulting in an extended shelf-life, may also result in export opportunities not currently available to WA suppliers.

To modify current industry practices, and develop new processing protocols to meet these emerging markets, there is a need for species specific, whole of supply chain microbiological and biochemical research, aligned with product quality assessment. Low value seafood with generally short shelf-lives will be chosen as the target sectors to maximize the potential increase in profit by extending shelf-life or by developing new product lines.

There have been no investigations to identify specific spoilage organisms that are found on seafood along the WA coast. Virtually nothing is known about any of the micro-flora of WA marine species. In terms of finfish in other parts of Australia, cool water fish are generally spoiled by *Shewanella putrefaciens* and similar pseudomonads, but the situation in warm water species is less clear with *Pseudomonas fragi* having been reported. The occurrence of

these on WA species is unknown, but without this knowledge it will not be possible to design appropriate preservation, storage and packaging strategies to deal with spoilage organisms.

OBJECTIVES

1. To complete microbiological and temperature analyses of six seafood supply chains
2. To determine the impact of specific spoilage organisms/products on product quality through the test supply chains
3. To identify and validate supply chain intervention and/or value-adding protocols which improve shelf-life or enable the development of new products.

MATERIALS

Product	Manufacturer	Catalogue Number
Peptone (0.1%) Salt Solution (9ml)	PathWest Media	T7187
Brain Heart Infusion Broth (BHIB)	PathWest Media	T4730
Triple Sugar Iron Agar (TSIA) slopes	PathWest Media	T1207
Trypticase Soy Broth Agar (TSB agar)	PathWest Media	P1331
alpha Cyano-4-hydroxycinnamic acid (Vitek MS CHCA)	bioMérieux - Australia Pty. Ltd.	411071
API 20NE	bioMérieux - Australia Pty. Ltd.	20050
API 20E	bioMérieux - Australia Pty. Ltd.	20100
ID 32 Staph	bioMérieux - Australia Pty. Ltd.	32500
Rapid ID 32 Strep	bioMérieux - Australia Pty. Ltd.	32600
50CHB/E Medium	bioMérieux - Australia Pty. Ltd.	50430

Culture Media Preparation

Maximum Recovery Diluent (MRD)

Maximum recovery diluent was prepared as described in Australian standard AS 5013.11.1 (Anon 2004). MRD was prepared by dissolving 1 g of enzymatic digest of casein and 8.5 g of sodium chloride in 1 L of distilled water. All ingredients were dissolved by heating and the pH adjusted to 7.0 ± 0.2 at 25 ˚C and autoclaved at 121˚C for 15 minutes.

Plate Count Agar (PCA)

Plate count agar was used in a standard aerobic plate count to measure the total number of organisms that form visible colonies in food, water and waste water samples. Plate count agar

was prepared by adding 5 g of tryptone, 2.5 g of yeast extract, 1 g of D – glucose and 15 g of agar to 1L of distilled water. The mixture was dissolved by heating and the final pH adjusted 7.0 ± 0.2. The mixture is then autoclaved at 21˚C for 15 minutes (Buchbinder, Boris et al. 1953; Anon. 1994). Approximately 15mL of PCA was added to 90mm petri dishes. Poured PCA plates were held at 4˚C for up to 2 months.

Modified Long and Hammer Agar (mL&H)

20g of proteose peptone No.2 (Becton Dickenson), 40g of gelatin (Sigma), 10g of Sodium chloride (BDH AnalaR), 15g of bacteriological agar (Oxoid) and 1g of potassium phosphate (Sigma) mixed into 1L of distilled water. The ingredients were dissolved by heating and the pH adjusted to a final pH of 7.0 ± 0.2 at a temperature of 20-25˚C using 1 M sodium hydroxide,. The media was autoclaved at 121˚C for 15 minutes, and stored in refrigerated conditions for up to 3 months. On the day of use the media was melted in a boiling water bath and 2 ml 10% ammonium ferric citrate solution was added per 1L of media (van Spreekens 1974; Nordic Committee on Food Analysis 2006). Approximately 15mL of PCA was added to 90mm petri dishes. Poured PCA plates were held at 4˚C for up to1 month.

Iron Agar (IA)

20g of proteose peptone No.2 (Becton Dickenson), 6g of Lab Lemco powder (Oxoid), 6g of yeast extract (Oxoid), 0.6g of ferric citrate (Sigma), 0.6g of sodium thiosulphate (Sigma), 10g of sodium chloride (BDH AnalaR) and 24g of bacteriological agar (Oxoid) was mixed into 1L of distilled water and dissolved by heating. pH was adjusted to a final pH of 7.4 ± 0.2, using 1 M sodium hydroxideand autoclaved at 121˚C for 15 minutes. The media was stored in at 4.0˚C for up to 3 months. On the day of use 0.04% L-cysteine solution is added to the melted agar just before use (Gram, Trolle et al. 1987; Nordic Committee on Food Analysis 2006). Approximately 15mL of IA was added to 90mm petri dishes. Poured PCA plates were held at 4˚C for up to 1 month

Modified Long and Hammer broth (mL&Hb)

Long and Hammer broth was prepared dissolving 20g of proteose peptone No.2 (Becton Dickenson), 40g of gelatin (Sigma), 10g of Sodium chloride (AnalaR), and 1g of potassium phosphate (Sigma) in 1L of distilled water.

The ingredients were dissolved and adjusted to a final pH of 7.0 ± 0.2, using 1 M sodium hydroxide. The media was autoclaved at 121˚C for 15 minutes (van Spreekens 1974; Nordic Committee on Food Analysis 2006).

RODAC (Replicate Organism Detection and Counting) plate (Agar contact)

The RODAC plates used for bacterial enumeration were made from a disposable plastic 65x15mm plates (BBL, Maryland USA). The plates were filled with Plate Count Agar, Iron Agar and Long and Hammer Agar. Between 15.5 and 16.5 ml. of molten agar was pipetted into each plate, with the meniscus of the agar seen to form a convex surface (Sveum, Moberg et al. 1992).

METHODS

Bacterial enumeration

RODAC plates

To sample the bacterial load of finfish and octopus the RODAC plates were placed against the surface of each fish, just below the dorsal fin. The three sample points were taken at different points along the dorsal fin. Three RODAC plates, containing either PCA, IA or mL&H agar), were used per fish. Each plate was then incubated according to the growth media requirements (see below).

Flesh excision

$2cm^2$ square of flesh and skin was excised from the fish. and placed in a stomacher bag and weighed on a pre-tared A & D EW 300B digital scale. A 1 in 10 dilution was prepared using maximum recovery diluent (MRD) and the sample was homogenised using a stomacher for 1 minute. Subsequent 1 in 10 dilutions were then prepared using peptone water (0.1%) + 0.85% Saline. Dilutions ranged from 10^{-1} through to 10^{-7}, and two dilutions were plated every sample point to ensure accurate counts were obtained. All dilutions were plated onto Plate Count Agar, Iron Agar and Long and Hammer Agar using the spiral plate method.

Swabbing Method

Where it was not feasible to use the flesh extraction method a swab sampling method was introduced. Samples were collected by rubbing a sterile swab in a back and forth motion while turning the swab over a measured area of $2cm^2$ on the fish above the dorsal fin. Once the area has been sampled the swab is placed in 10ml of peptone water (0.01%) +0.85% Saline and the solution was mixed suing a vortex mixer for 1 minute. Further serial dilutions were performed ranging from 10^{-1} through to 10^{-7}, and two dilutions were plated every sample point based on a probable count for each sample point.

Preparation of Initial Dilution of Mussel Meat

Mussels were prepared for testing and homogenised as described in Australian Standard AS 5013.11.3 - 2006 Food Microbiology - Preparation of test samples, initial suspension and decimal dilution for microbiological examination - Specific rules for the preparation of fish and fishery products.

Spiral Plater

The Whitley Automated Spiral Plater (WASP) was operated in accordance with manufacturers instruction manual (Don Whitely Scientific 1995). The spiral plater works by dispensing a liquid sample (or homogenized dilution of a sample) from a stylus onto a rotating agar plate in an Archimedes spiral. The volume of liquid dispensed in a defined area becomes less as the stylus moves towards the outer edge of the plate. After incubation, the number of organisms can be determined using a dark-field colony counter and a counting template. 50 µl of dilution was pipette onto the PCA, Iron agar and Long and Hammer agar plates and incubated as described below.

Spread Plate

Serial 1/10 dilutions were prepared by transferring 1mL of diluted sample into 9mL of MRD. 100µL of each dilution was transferred onto duplicate plates and spread evenly over the plates using sterile hockey sticks. Inoculated media was incubated as detailed below.

Incubation of media

Following inoculation of the medium the plates were incubated as detailed below:

Medium	Test	Incubation Temp	Incubation Time
Plate Count Agar	Standard Plate Count	30±1˚C	48±2h
Iron Agar	Specific Spoilage Organisms	25±1˚C	48±2h
Modified Long & Hammer	Psychrotrophic bacteria	15±1˚C	120±2h

Following incubation all colonies on Plate Count Agar and modified Long & Hammer agar are counted. Only colonies with black centres are counted on Iron Agar. Modified Long & Hammer agar plated were also checked for the presence of bioluminescent bacteria,

specifically to check for the presence of *Photobacterium phosphoreum* (Dalgaard, Mejlholm et al. 1997). This was performed by photographing the modified Long and Hammer agar plate in a darkened room using a Canon 5D SLR camera set at 800 ISO, aperture setting of 4.0,and shutter speed of 13 seconds with a 24-105mm F4.0 lens. Figure 1 below is an example of a bioluminescent on modified Long and Hammer agar.

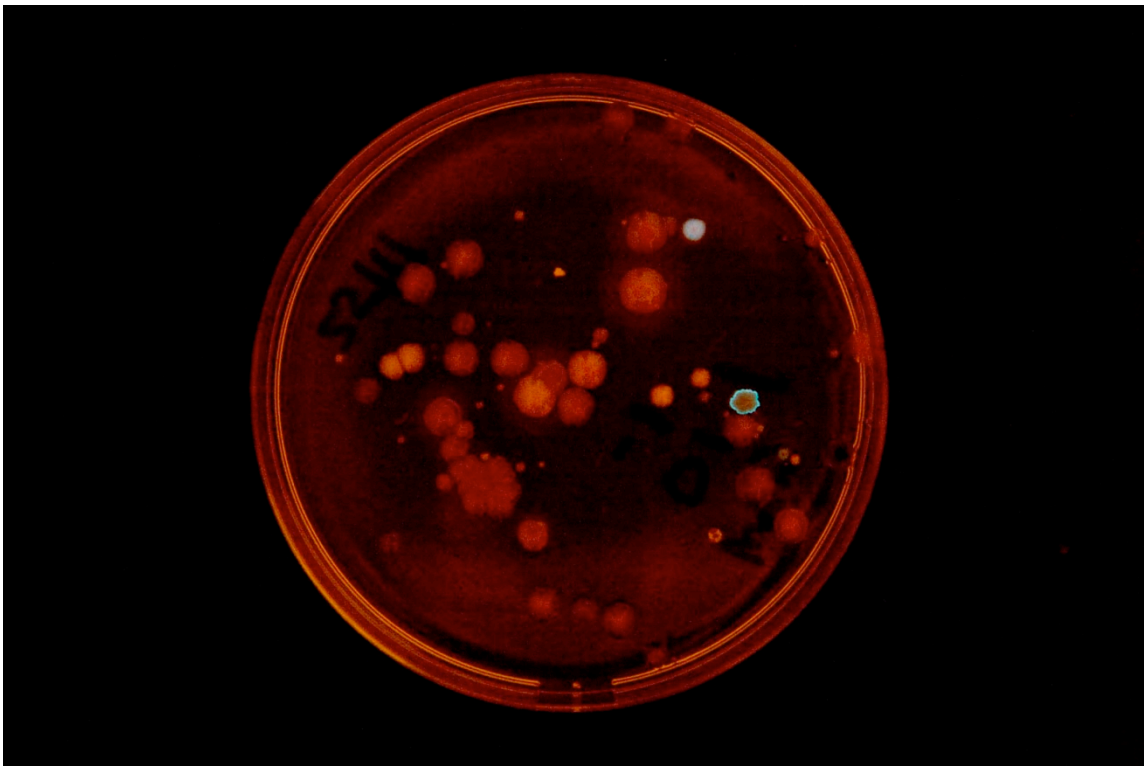


Figure 1 Bioluminescent bacteria growing on modified Long & Hammer agar.

Bacterial Identification

Storage

Bacterial isolates were purified and stored at -20˚C in brain heart infusion broth (BHIB) containing 15% glycerol.

Primary identification

Primary bacterial identification was performed using the following techniques:

- Gram stain
- Colony morphology
- Colony pigment
- Motility
- Catalase
- Oxidase

- Optimum growth temperature
- Oxidative/ Fermentative metabolism

Further Identification Procedures

Preliminary identification of all isolates allowed them to be sorted into groups, based on their phenotypic characteristics. Further identification was performed specific identification tests including biochemical reactions, fatty acid analysis and DNA analysis to identify the organisms to a species level. The methods used varied from isolate to isolate depending on the complexity of species identification.

Biochemical Analysis

Biochemical analysis utilised a series of commercial identification kits including:

- API 20NE
- API 20E
- API 50CH
- API ID 32 STAPH
- API Rapid ID 32 STREP
- BIOLOG GN2 plates

The biochemical identification of *Shewanella* species was performed using a combination of results from the API 20NE strip and the BIOLOG GN2 plate. Appendix 1 summarises the expected biochemical results compiled from a large number of papers.

Fatty Acid analysis- MIDI system

Microbial fatty acid profiles were determined by the MIDI Sherlock system using gas chromatography to analyse extracted microbial fatty acid methyl esters (FAMEs) (Kunitsky, Osterhot et al. 2006).

The fatty acid analysis was performed in compliance with the manufacturers recommended procedures (Sigurgisladottir, Hafsteinsson et al. 1999). Samples were prepared by inoculating the organism onto Trypticase Soy Broth Agar and incubating at 28°C for 24 hours. It is necessary to test bacteria closest to its log stage of growth and to ensure there is an appropriate amount of bacterial cells for analysis. The bacteria was harvested followed by saponification, methylation, extraction and a base wash were performed on the bacterial cells.

Brief centrifugation was performed to clarify the layers before two thirds of the top layer was pipetted into a GC vial for analysis (Sasser 1990).

Fatty acid determination was performed using a microbial identification system (Hewlett-Packard). The gas chromatograph used was a HP Agilent GC series 6890, with a split injector system and a flame ionisation detector. An Agilent Ultra 2 capillary column (25.0m, 200µ) was used with Hydrogen as the carrier gas. The column temperature program rose from 288˚C to 310˚C in 1.25 min, with the initial oven temperature starting at 170˚C (Muller, Schmid et al. 1998).

The Sherlock MIS Software was the data processing software used for identifying the organisms (Pathwest, 2011; Sasser 1990).

16S rRNA gene sequencing

DNA sequencing of the 16S rRNA gene is an alternative method for rapidly and accurately identifying bacteria. The DNA sequencing was performed by the Molecular Typing Department at PathWest Laboratories. The method used for DNA sequencing was followed from the Molecular Diagnostic Methods Manual at PathWest based on published procedures (Sanchez-Alonso, Barroso et al. 2009), based on a universal PCR and DNA sequencing method of the 16S rRNA gene (Ozogul 2009). An extract was prepared for sequencing by heating suspensions of bacterial cultures in ultrapure water (Fisher Biotech). The suspensions were centrifuged and the supernatant was used for further analysis. Eight micro litres of the supernatant was added to duplicate PCR mix tubes These inoculated tubes were transferred to a thermocycler (Applied Biosystems 2720) and put on a cycle using a Non-hot start program with an AT of 55°C.

The PCR products were then transferred for ethidium bromide gel electrophoresis (2.5% agarose) and photographed. If a satisfactory band was produced the product was sequenced using the Applied Biosystems xl 3130 Sequencer.

RESULTS & DISCUSSION

1. SPOILAGE OF SEA MULLET

1.1. Bacterial Growth on Retail Sea Mullet.

Fresh whole sea mullet was obtained from a retail shop. The fish were transported to the laboratory in ice. On arrival the fish were stored on ice in a refrigerator. The container of iced fish was set up with a drain to ensure the fish did not stand in water. Samples were taken at days 1, 5, 9, and 14. Total mesophilic bacterial numbers (as assessed using the Standard Plate Count method) started at a relatively low level of around 1000 CFU/cm^2, rising steadily to a count of just under 1x10^{8}CFU/g at day 14. Total psychrotrophic bacterial numbers were assessed using the Long & Hammer agar method and started at around 10000 CFU/g rising to a maximum count of around 3.3x10^7 CFU/g. The total number of Specific Spoilage Organisms (SSOs) (enumerated using Iron Agar) started at levels of around 1000 CFU/cm^2 rising to approximately 1x10^8 CFU/g at day 14 (see Figure 1 below). The SSOs were predominantly *Shewanella* species and these have been identified as being a major cause of spoilage of refrigerated fin fish (Gram and Huss 1996).

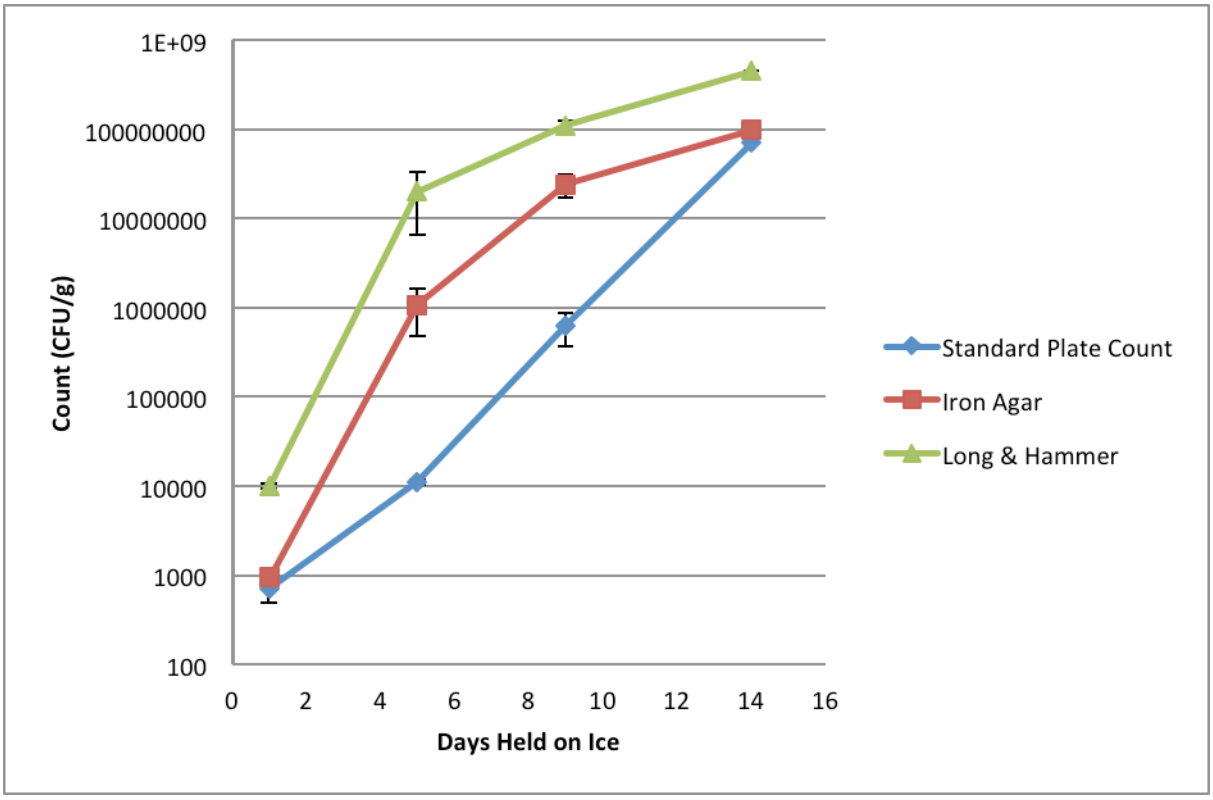


Figure 1.1 Bacterial growth on Sea Mullet as assessed by the Standard Plate Count, Iron Agar, and Long & Hammer agar methods (error bars indicate standard error of the mean).

1.2. Effect of Geographic Location on Spoilage of Sea Mullet

The effect of place of capture was investigated on the spoilage of Sea Mullet. Figures 1.2.1 and 1.2.2 below show the results for Sea Mullet caught at Rockingham and Green Head.

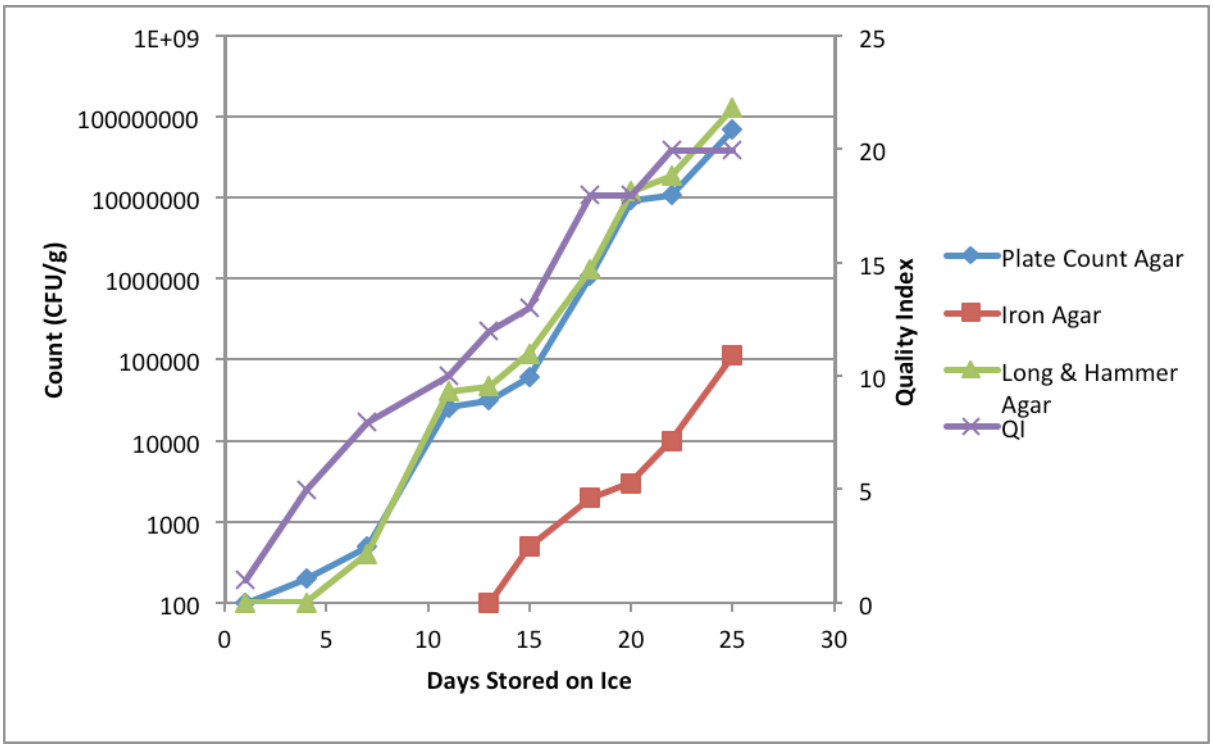


Figure 1.2.1 Quality index and bacterial counts of chilled Sea Mullet caught at Rockingham.

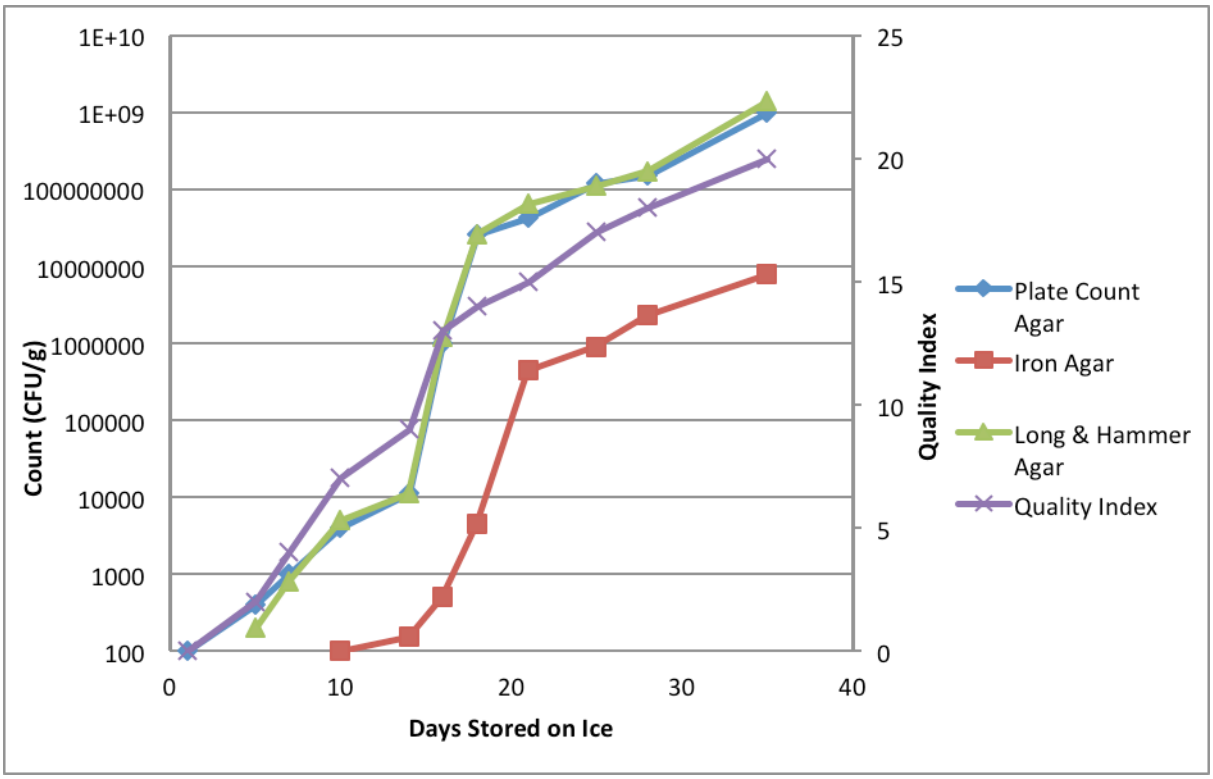


Figure 1.2.2 Quality index and bacterial counts of chilled Sea Mullet caught at Green Head.

The rate of spoilage of Sea Mullet was not found to be significantly different between mullet caught at Rockingham and mullet caught at Green Head. Following netting, fish from both locations were immediately sampled and then placed into a slurry of ice and sea water. The fish were then transported to the laboratory and held overnight in the ice slurry under refrigeration. The fish were then transferred to a plastic container with a drain point and iced. These were held under refrigeration prior to testing.

It was observed in the initial trials at Rockingham that there was a significant background of non-hydrogen sulphide producing bacteria growing on the Iron Agar plates. This background micro-flora growing on these plates are likely to cause a reduction in the counts of SSOs on Iron Agar. Attempts were made to adjust incubation temperatures to overcome this problem summarised in Figure 1.2.3 below.

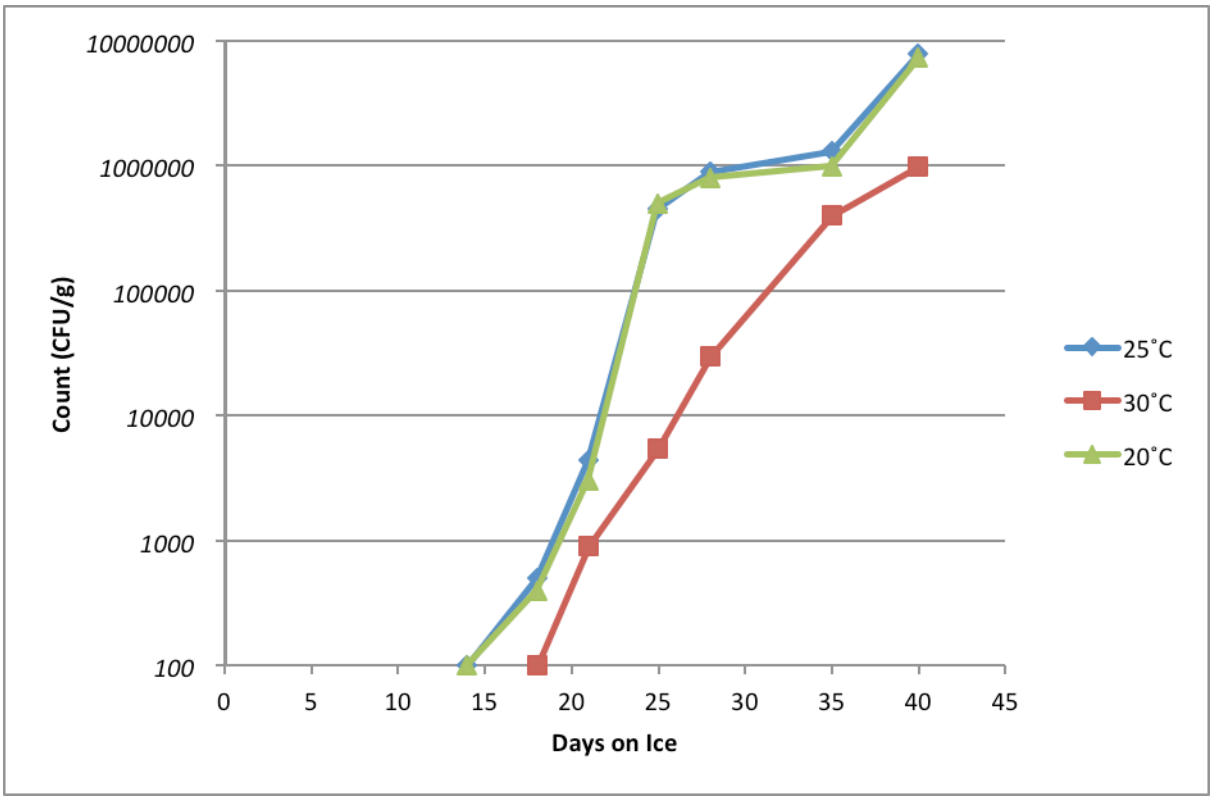


Figure 1.2.3 The effect of incubation temperature on the Specific Spoilage Organism count of Iron Agar.

Adjustment of the incubation temperature did not improve the count of SSOs on Iron Agar. At 30˚C the background micro-flora on the iron agar plates was markedly reduced. Unfortunately, there was also a reduction in the number of SSOs (hydrogen sulphide producing bacteria). Whilst there was a slight reduction in the size of the background micro-flora colonies at 20˚C there was also a reduction in the size of the colonies of SSOs and the background micro-flora was so numerous as to still cause likely interference with the SSO count.

1.3. Cold Chain Trials of Sea Mullet

A series of temperature trials were performed to assess the cold chain of sea mullet. Figure 1.3.1 is an example of the typical temperature profile of the mullet which has been netted and placed into an ice slurry. The ambient temperature indicated the temperature within the ice slurry/ refrigerated ice. The internal temperature was measured within the muscle of the mullet. The tested mullet had been placed into an ice slurry for 3 to 5 minutes prior to dispatching and insertion of the temperature probe. No significant issues were identified.

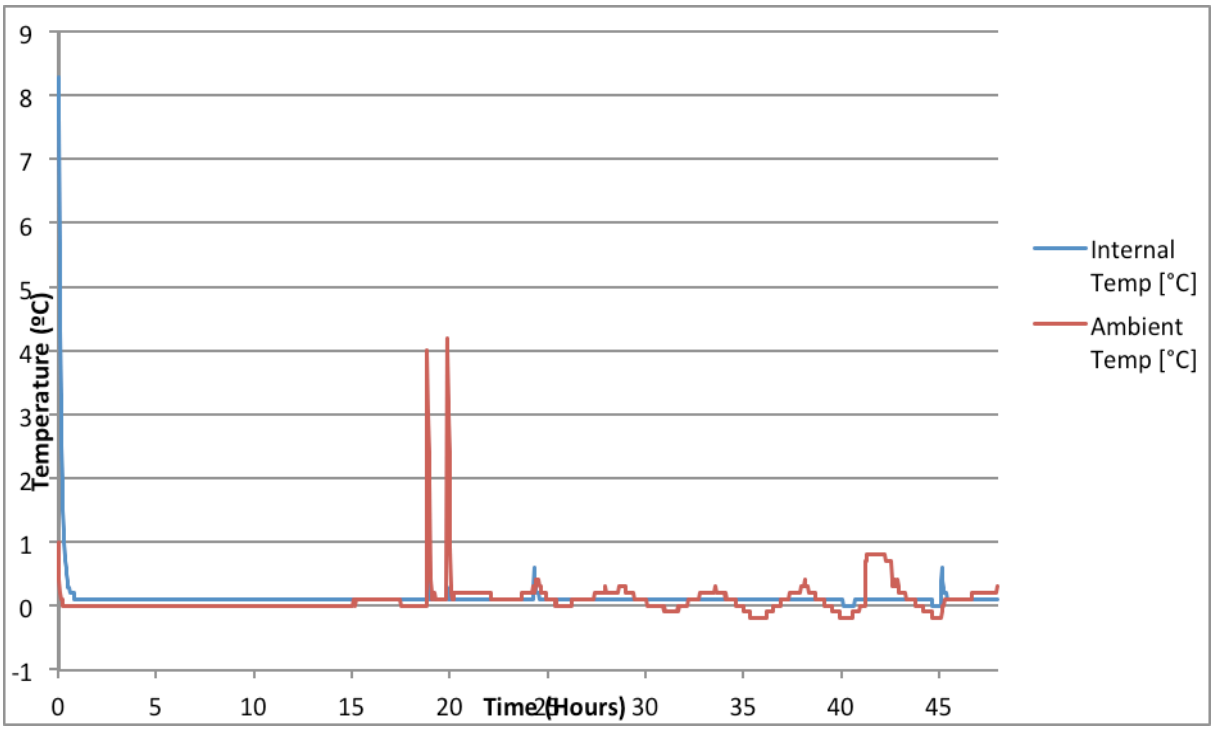


Figure 1.3.1 temperature profile of netted sea mullet placed into ice slurry prior to transport.

1.4. Use of Sanitisers to Extend the Shelf-life of Whole Chilled Sea Mullet

The addition of sanitisers to the initial ice slurry used when Sea Mullet is caught was investigated, two types of sanitisers were investigated. The first was a hydrogen peroxide based sanitiser. The sanitiser was added to the ice slurry at the concentration recommended by the supplier. On capture the fish were transferred to the ice slurry and allowed to sit in the slurry overnight prior to repacking for transport to wholesalers. The Sea Mullet was then transferred into ice stored in a freely draining container held in a refrigerator. Figure 1.4.1 details the effect of the use of the sanitiser on the quality index (QI) of Sea Mullet.

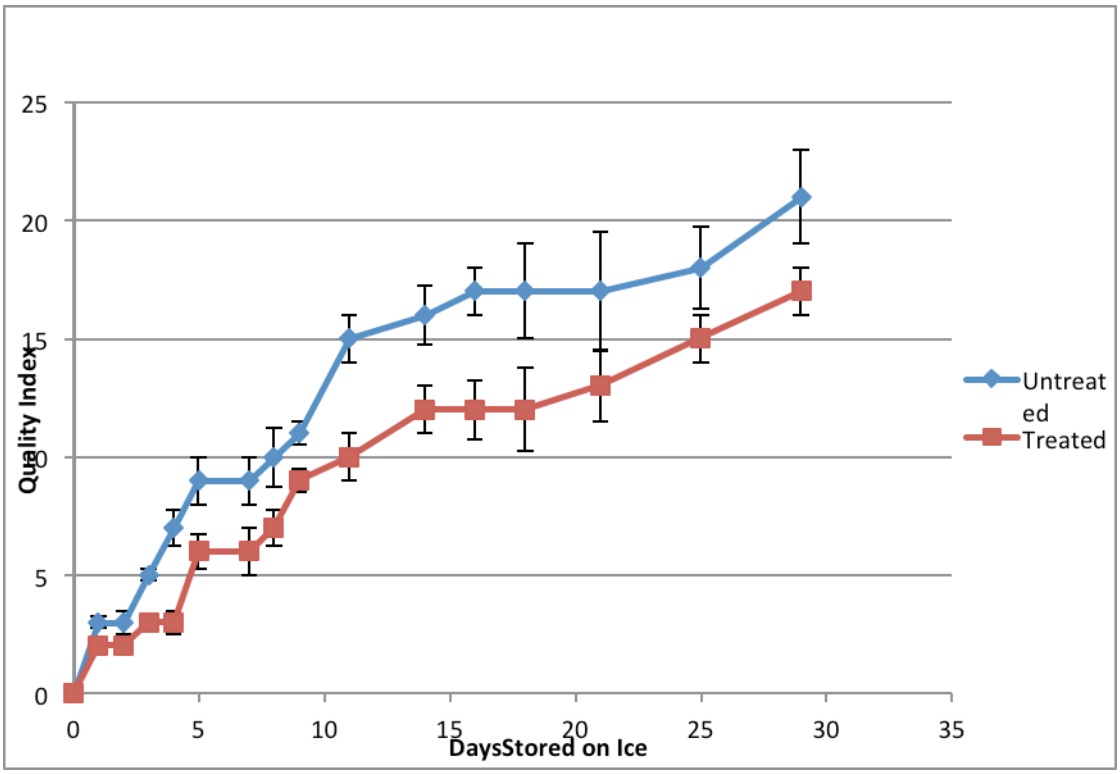


Figure 1.4.1 Effect of peroxide sanitiser treatment on the quality index of Sea Mullet.

The use of sanitisers in ice slurry resulted in a significant reduction in the rate of spoilage of Sea Mullet as determined by the Quality Index Method. At day 1 there was an average of a one point difference between the QI score of treated and untreated mullet. Following 7 days of storage on ice there was an average difference of 3 points in the QI scores and by day 14 the average difference was 6 points. A difference in the QI scores of between 3 and 6 points was maintained for up to 29 days of storage. After 29 days the untreated Sea Mullet had an average QI score of 21 whilst the treated Sea Mullet was 17. Figures 1.4.2 to 1.4.4 summarise the effect of the sanitisers on the microbiological counts on treated and untreated Sea Mullet.

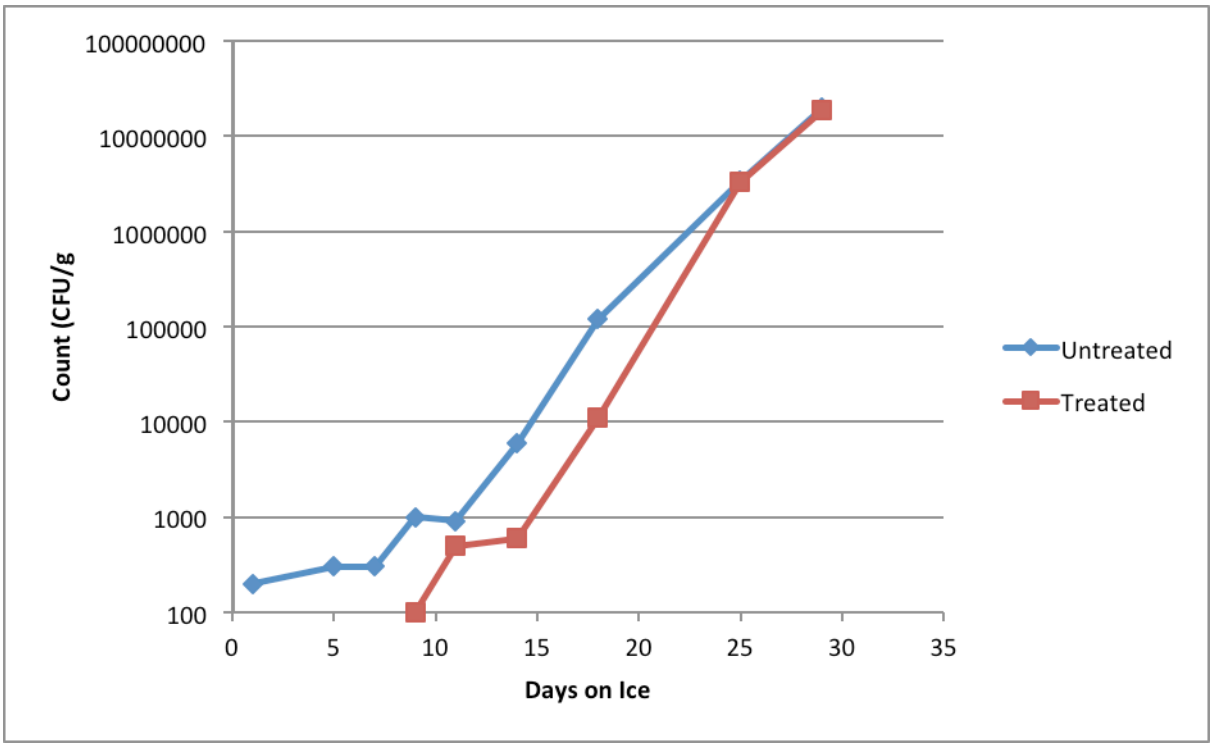


Figure 1.4.2 Effect of the use of hydrogen peroxide sanitiser on the Standard Plate Count of Sea Mullet.

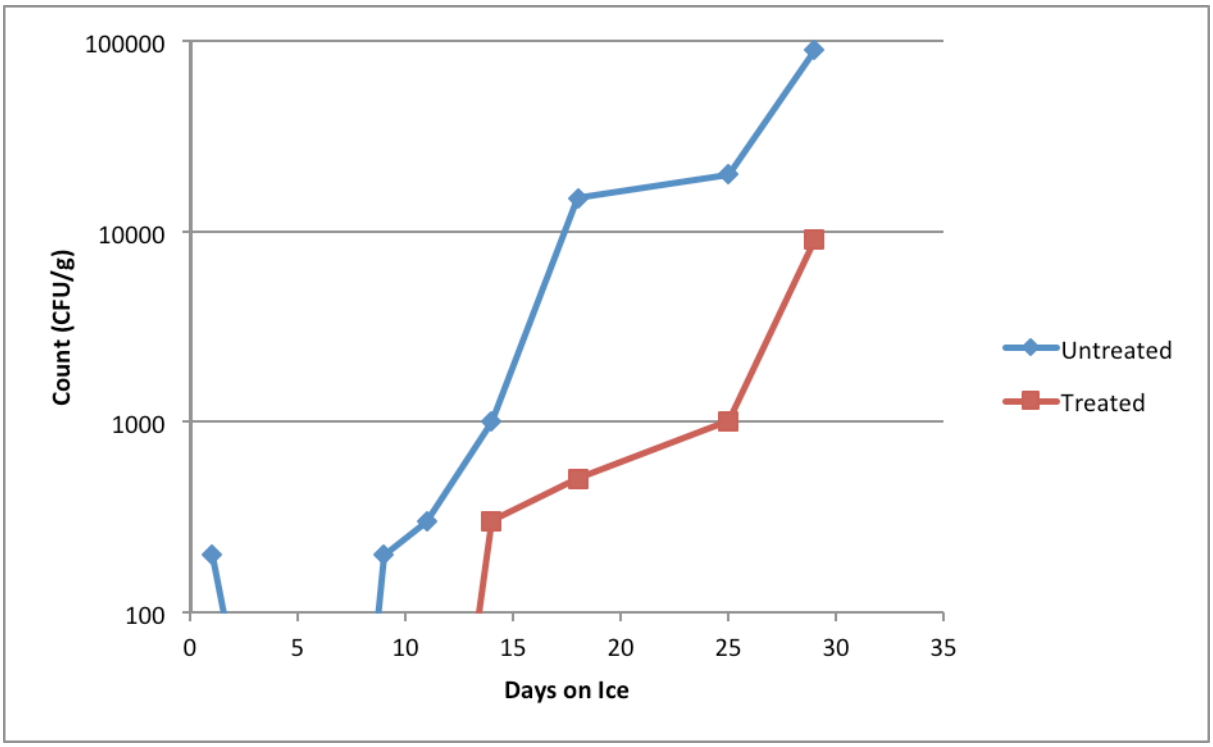


Figure 1.4.3 Effect of the use of hydrogen peroxide sanitiser on the Specific Spoilage Organism (Iron Agar) count of Sea Mullet.

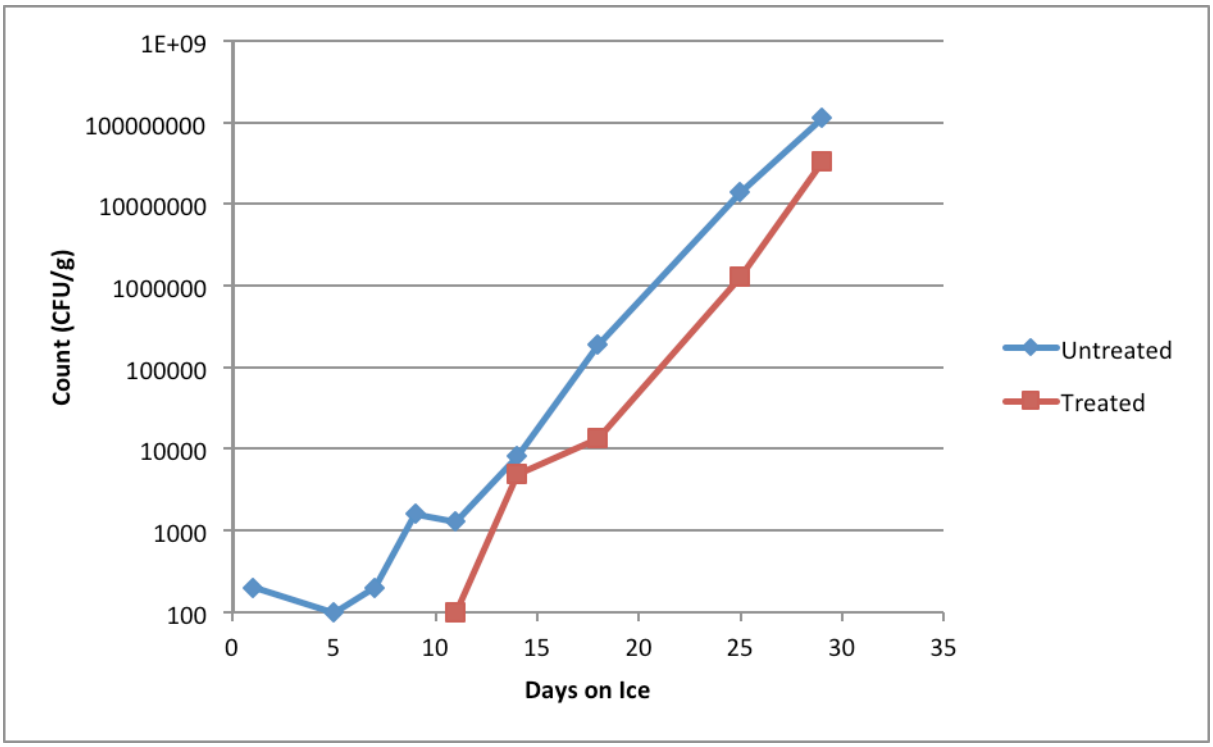


Figure 1.4.4 Effect of the use of hydrogen peroxide sanitiser on the Psychrotrophic Plate count (Modified Long & Hammer) of Sea Mullet.

The use of the sanitiser saw a reduction in the counts for mesophilic bacteria, psychrotrophic bacteria, and specific spoilage organisms. The specific spoilage organism counts (iron agar count) for both the untreated and treated Sea Mullet is likely to be underestimated due to the presence of high background organism counts. For all of the microbiological counts the use of a sanitiser in an ice slurry saw an increase in the time for a countable number of organisms to be detected there was also a decrease in the total numbers of specific spoilage organisms and psychrotrophic bacteria. Whilst there was a delay in the growth of mesophilic bacteria, the total number of mesophilic bacteria on the treated mullet was almost identical to the numbers on untreated mullet. The majority of these organisms were found to be *Pseudomonas fluorescens*, which are able to grow at psychrotrophic temperatures.

The effect of using chlorine dioxide based sanitisers in ice slurries was also investigated. Un-activated chlorine dioxide was used rather than activated chlorine dioxide. This was to allow the chlorine dioxide to oxidise over a longer period of time, thus releasing smaller concentrations of chlorine over a longer period of time. The experiments were set up as described for the previous sanitiser experiments above. Figures 1.4.5 to 1.4.7 summarise the

effect of chlorine dioxide on the rate of spoilage of mullet as determined using the Quality Index method and the levels of mesophilic bacteria, psychrotrophic bacteria and SSOs. This initial trial indicated that the use of un-activated chlorine dioxide in an ice slurry reduced the rate of spoilage and lowered the levels of spoilage bacteria on sea mullet.

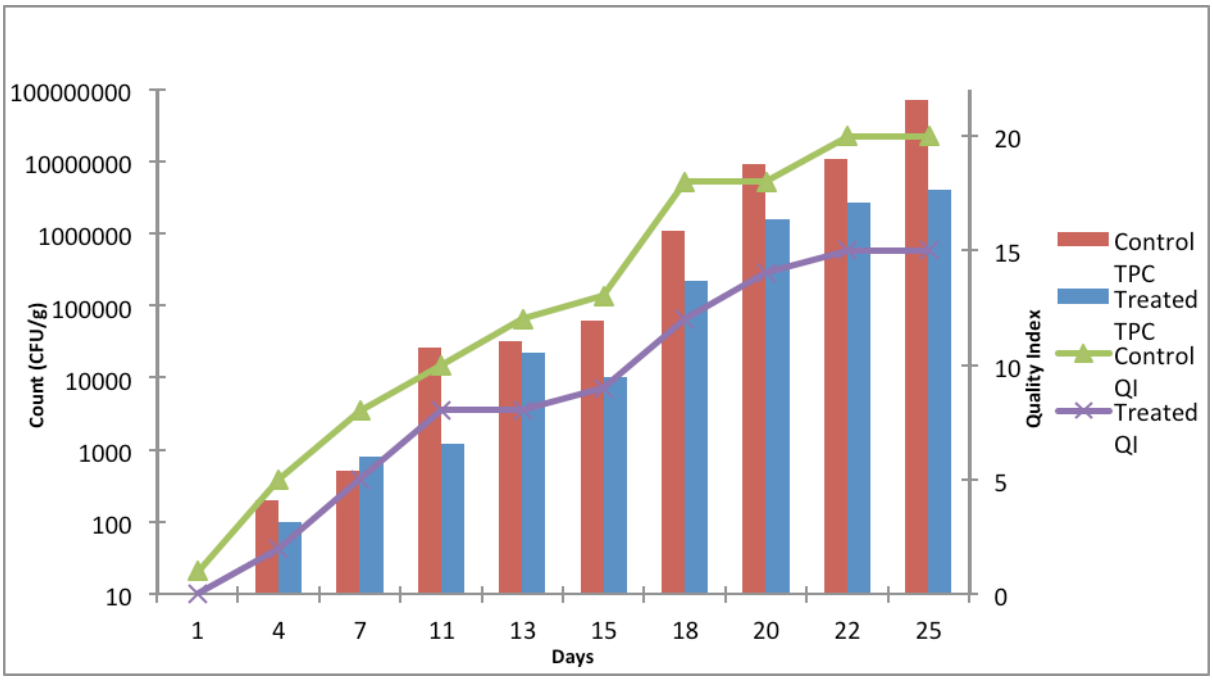


Figure 1.4.5 The effect of chlorine dioxide on the levels of mesophilic bacteria (Standard Plate Count) and quality index of Sea Mullet.

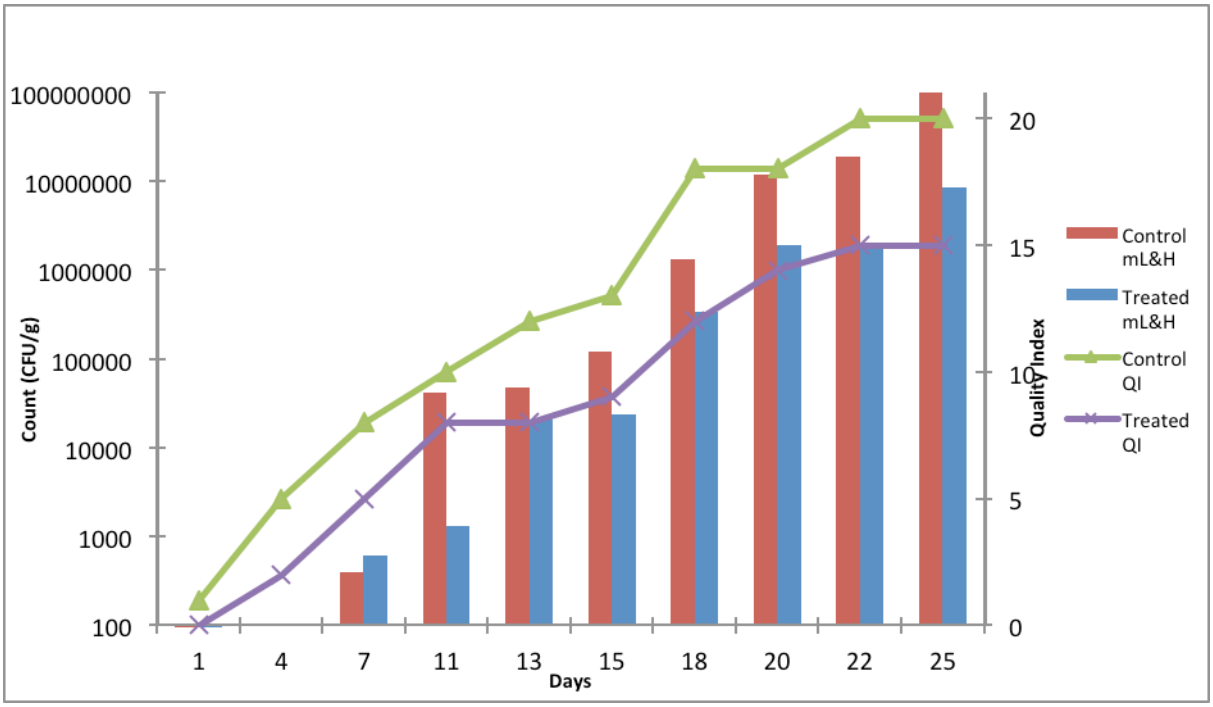


Figure 1.4.6 The effect of chlorine dioxide on psychrotrophic bacteria (modified Long & Hammer Count) and quality index of Sea Mullet.

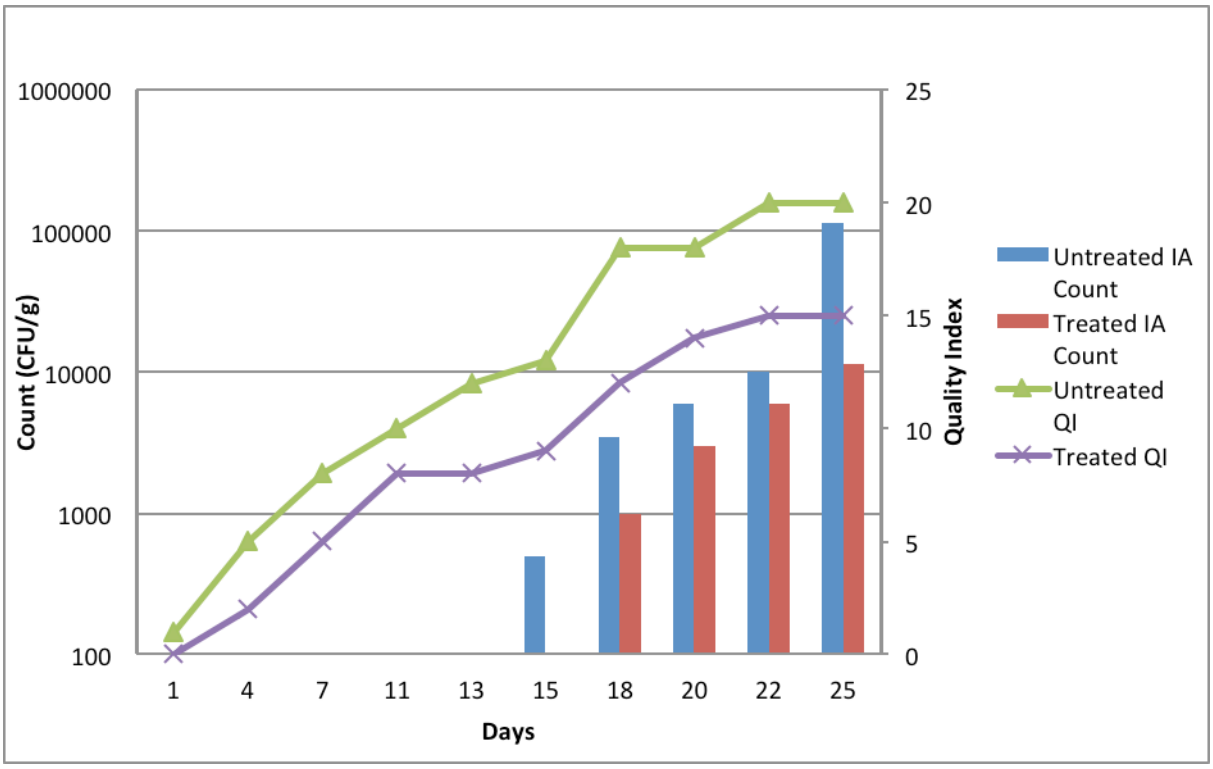


Figure 1.4.7 The effect of chlorine dioxide on Specific Spoilage Organisms (Iron Agar Count) and quality index of Sea Mullet.

As a means of potentially saving on the cost of using a sanitiser in the ice slurry, the effect of halving the concentration of chlorine dioxide on the rate of spoilage of Sea Mullet was also investigated. By halving the manufacturers recommended application rate the fisher would see a significant reduction in costs. Figure 1.4.9 shows the impact of chlorine dioxide concentration on the Quality Index of sea mullet. There was a significant difference in the Quality Index score of the untreated sea mullet compared to both the mullet treated with half strength and full strength un-activated chlorine dioxide with both concentrations of chlorine dioxide treated mullets showing significantly less signs of spoilage than the untreated mullet up to 18 days stored on ice under refrigeration. After this time the average QI scores for the mullet treated with half the recommended concentration of chlorine dioxide had QI score which were lower than the average QI score of the untreated mullet, but were found to be statistically non-significant. The average QI scores for the mullet treated however were

statistically significantly different to the untreated mullet up to and including 35 days storage on ice.

Figures 1.4.10 to 1.4.11 detail the effect of varying the chlorine dioxide concentration on the microbiological quality of Sea mullet. There was little impact on the levels of mesophilic bacteria and psychrotrophic bacteria. However there was a significant difference in the average levels of SSOs on Iron agar. There was a delay in the time taken for SSOs to be detected on Iron Agar. SSOs were detected on untreated sea mullet at day 14, on mullet treated with half strength chlorine dioxide SSOs were detected on day 21, and for the mullet treated with full strength chlorine dioxide SSOs were detected on day 25. As had been previously noted there was significant background interference with background micro-flora on the iron agar plates.

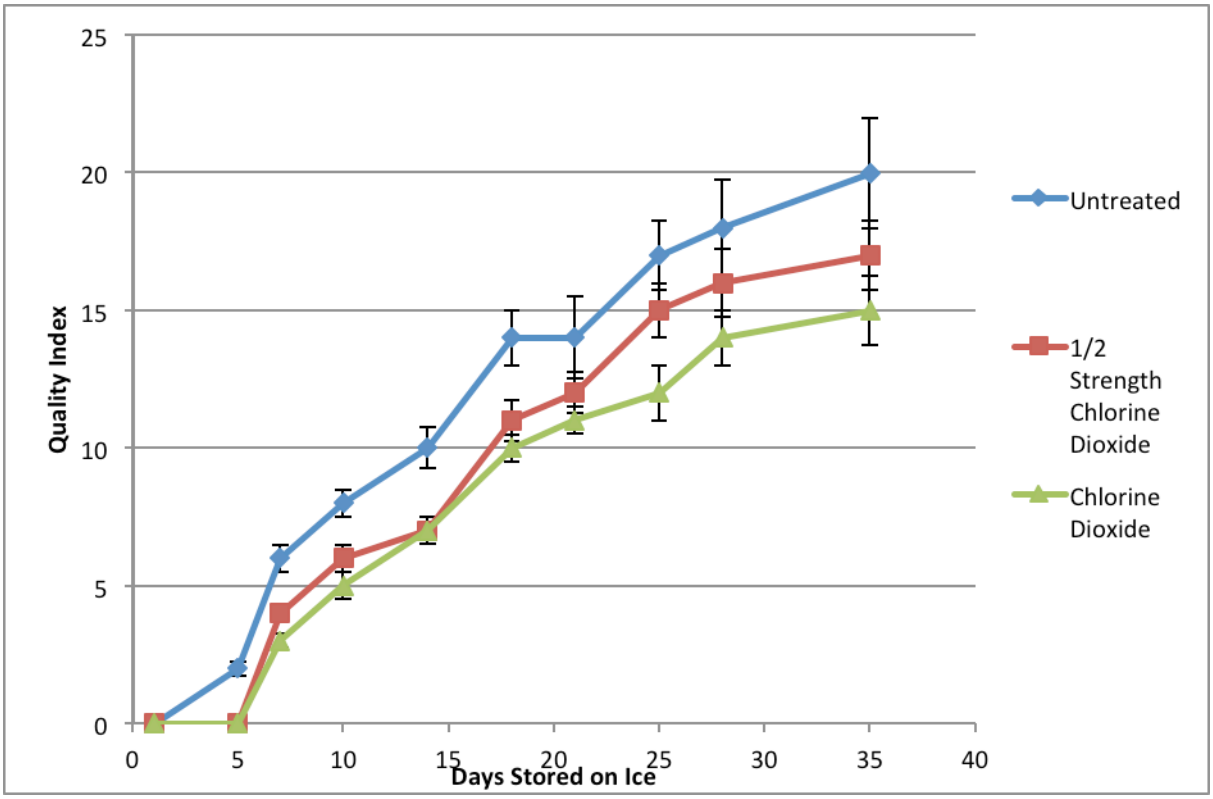


Figure 1.4.9 Effect of chlorine dioxide concentration on the spoilage of Sea Mullet.

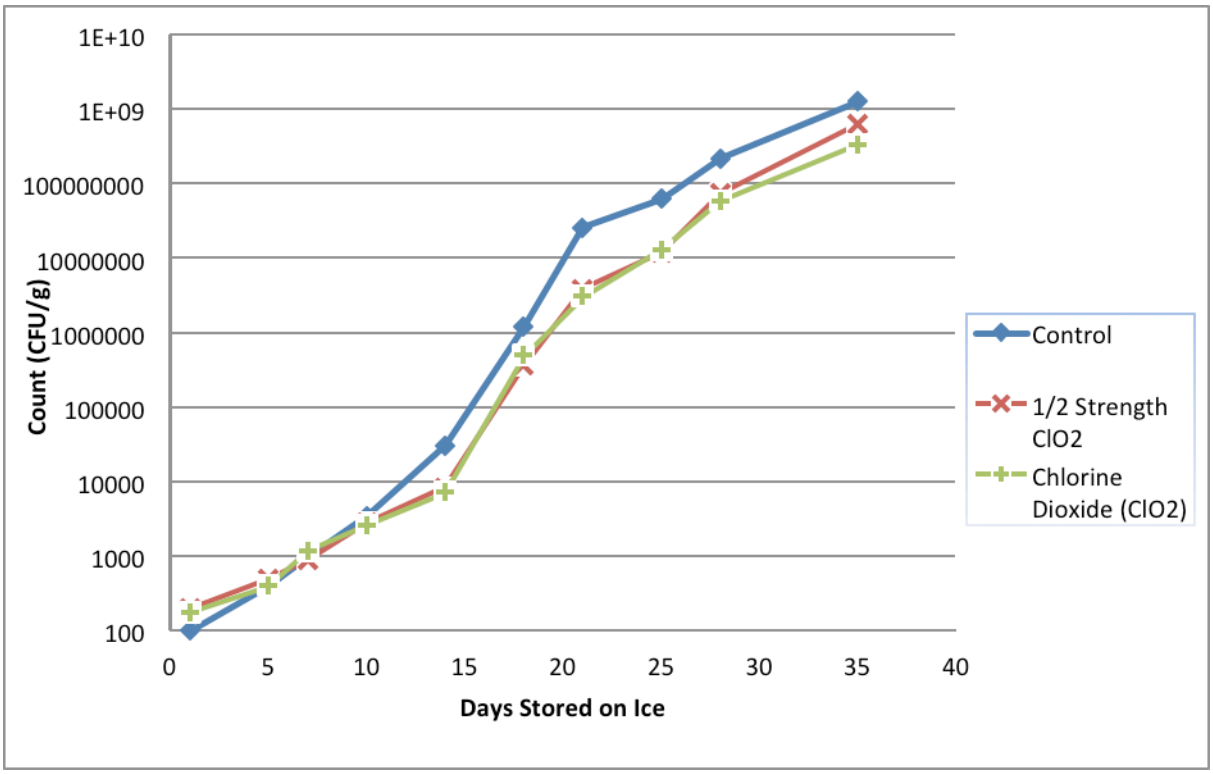


Figure 1.4.10 Effect of chlorine dioxide concentration on the levels of aerobic mesophilic bacteria (Standard Plate Count) on Sea Mullet.

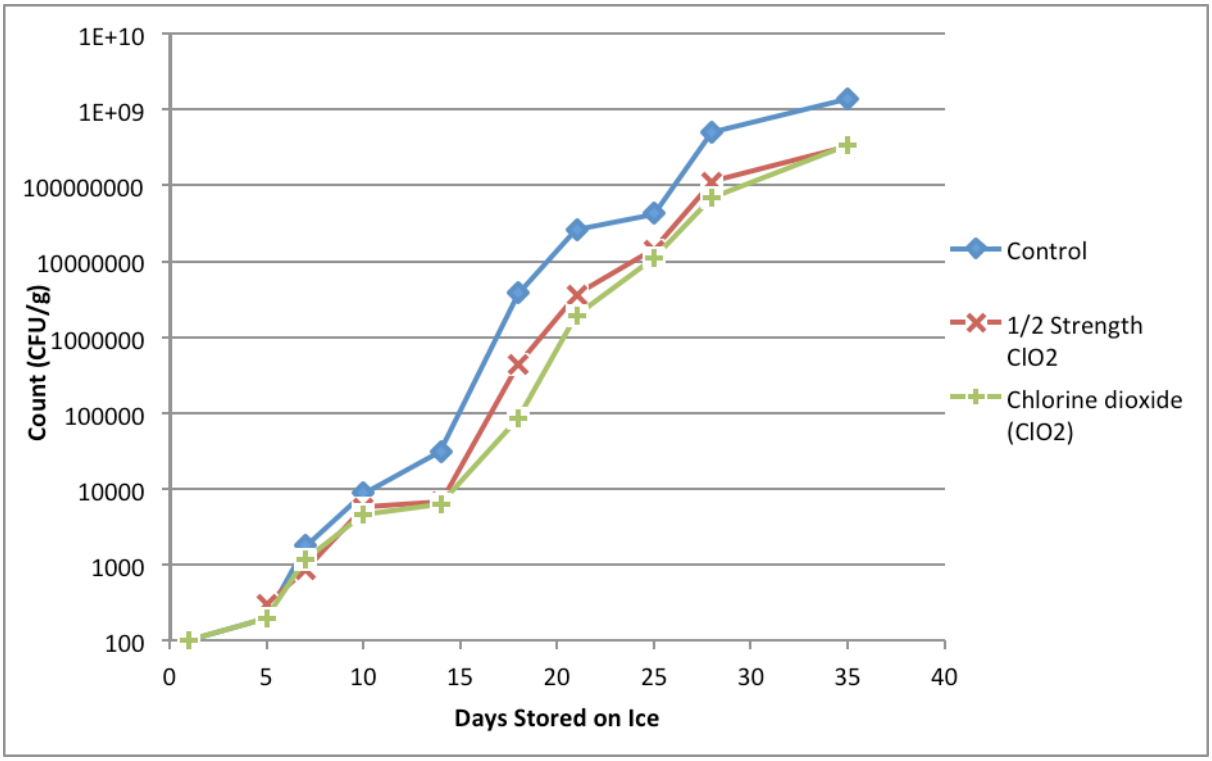


Figure 1.4.11 Effect of chlorine dioxide concentration on the levels of aerobic psychrotrophic bacteria (modified Long & Hammer) on Sea Mullet.

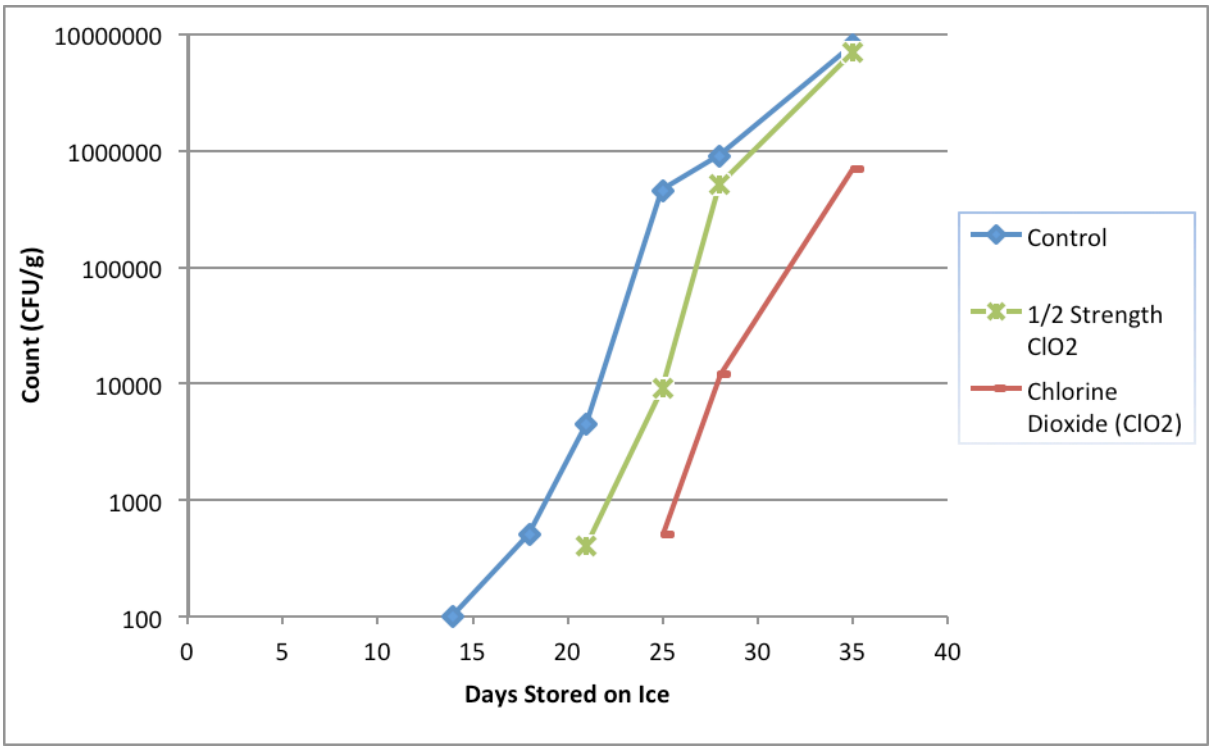


Figure 1.4.12 Effect of chlorine dioxide concentration on the levels of aerobic psychrotrophic bacteria (modified Long & Hammer) on Sea Mullet.

A final series of experiments was performed to confirm the effectiveness of chlorine dioxide. Un-activated chlorine dioxide was added to the ice slurry the mullet were placed into following netting. The fish were held in the slurry overnight and then transferred to ice and refrigerated as previously described. Figure 1.4.13 summarises the effect of chlorine dioxide on the average rate of spoilage of mullet as determined using the Quality Index method. After 5 days held in ice under refrigeration, the treated mullet had an average QI score two points lower than the untreated mullet. Treated fish was on average 3 points lower than the untreated mullet between 7 and 14 days of storage rising to a 5 point difference between 28 and 35 days of storage. After 35 days storage the untreated Sea Mullet had a QI score of 21, whilst the treated Sea Mullet had a QI score of 15. The difference between the average QI score for the chlorine dioxide treated and untreated Sea Mullet was statistically significant.

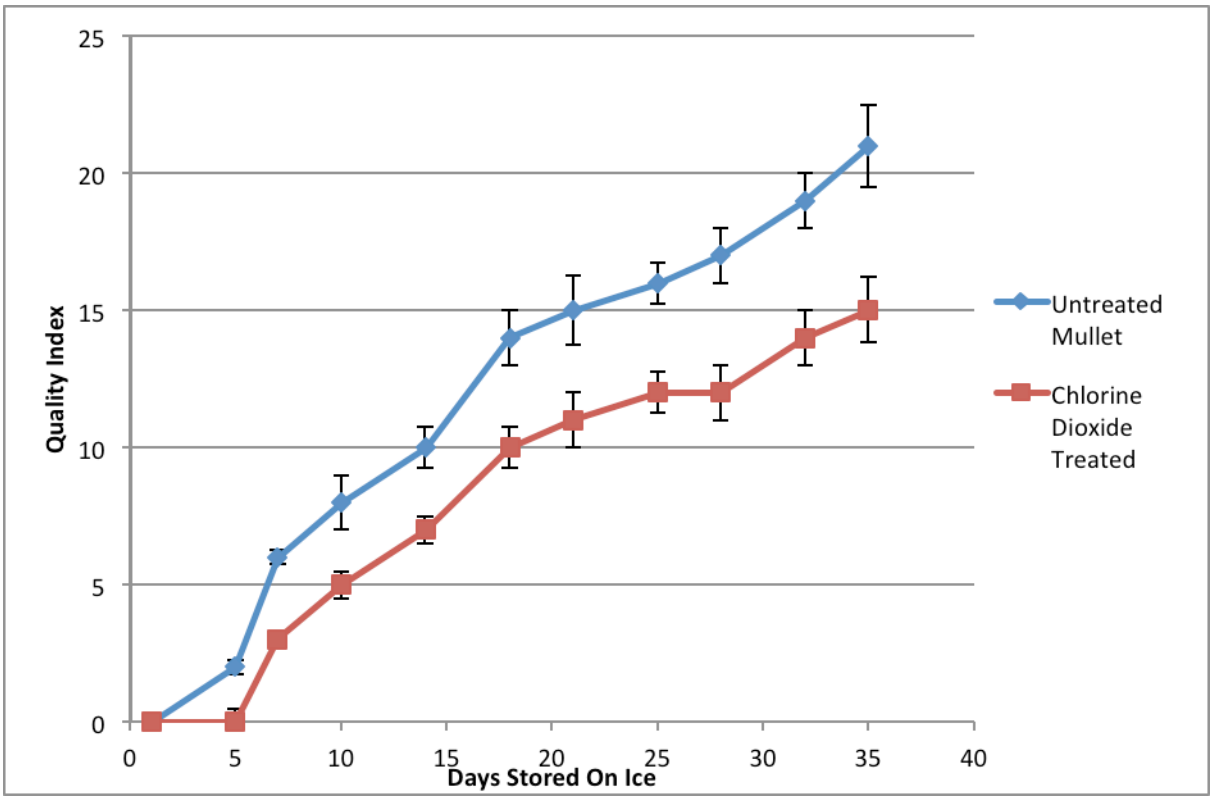


Figure 1.4.13 The effect of chlorine dioxide sanitiser on the quality index of Sea Mullet.

Figures 1.4.14 to 1.4.16 summarise the effect of chlorine dioxide on the average bacterial counts of treated Sea Mullet. Interestingly the reduction in the total number of aerobic mesophilic bacteria as measured by the Standard Plate Count, and the total number of aerobic psychrotrophic plate bacteria as measured by the modified Long and Hammer count was not as high as would have been thought based on the QI scores. The number of mesophilic bacteria was reduced by around 0.3 to 1 log while the number of psychrotrophic bacteria was reduced by between 0.2 to 1.2 log. The use of chlorine dioxide did have a marked effect on the total number of SSOs (see Figure 1.4.15). The SSOs were reduced by between 1 and 3.7 log. There was also a delay in detecting these organisms by 11 days as compared to the untreated mullet. Once again significant numbers of non-SSO bacteria were detected on the Iron Agar plates and therefore the total numbers of SSOs may be significantly underestimated, although the degree of this underestimation is likely to be similar on both the treated and untreated Sea Mullet. The effect of sanitiser treatment on the bacterial populations on Sea Mullet is discussed in section 1.5 below.

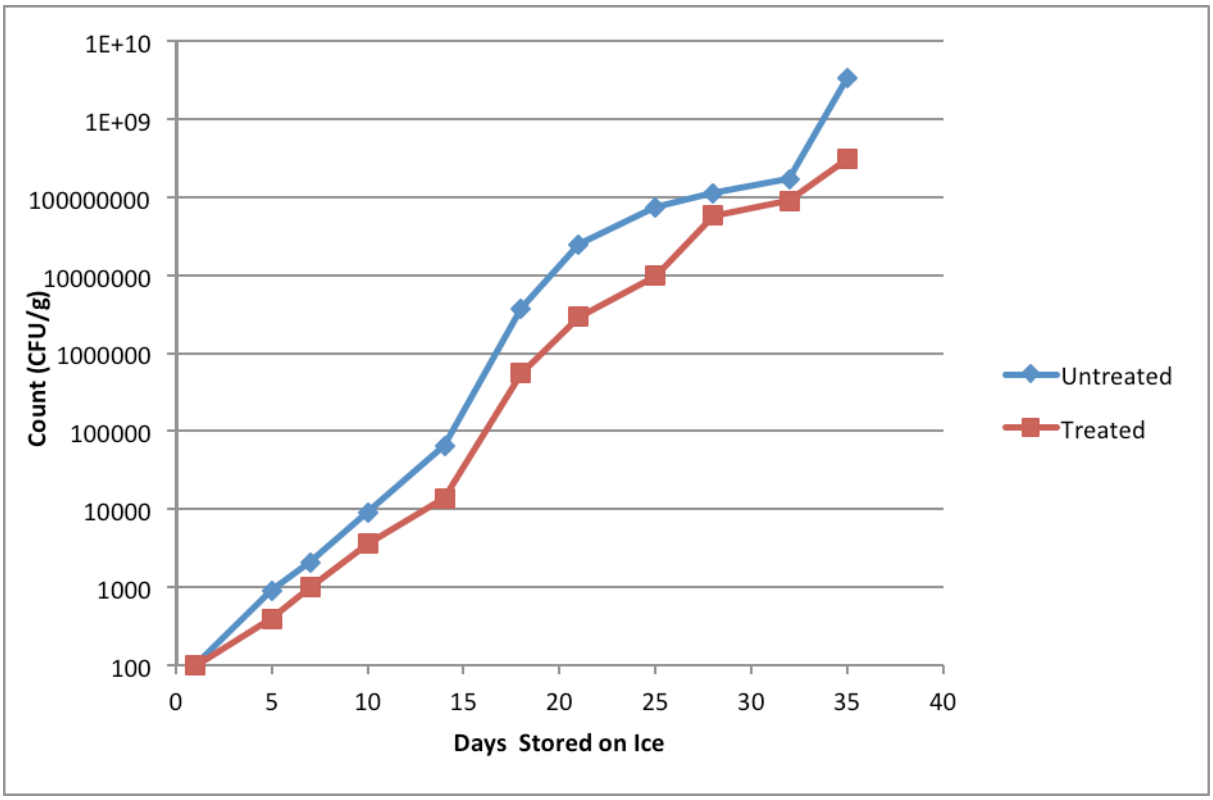


Figure 1.4.14 The effect of chlorine dioxide on the Standard Plate Count of Sea Mullet.

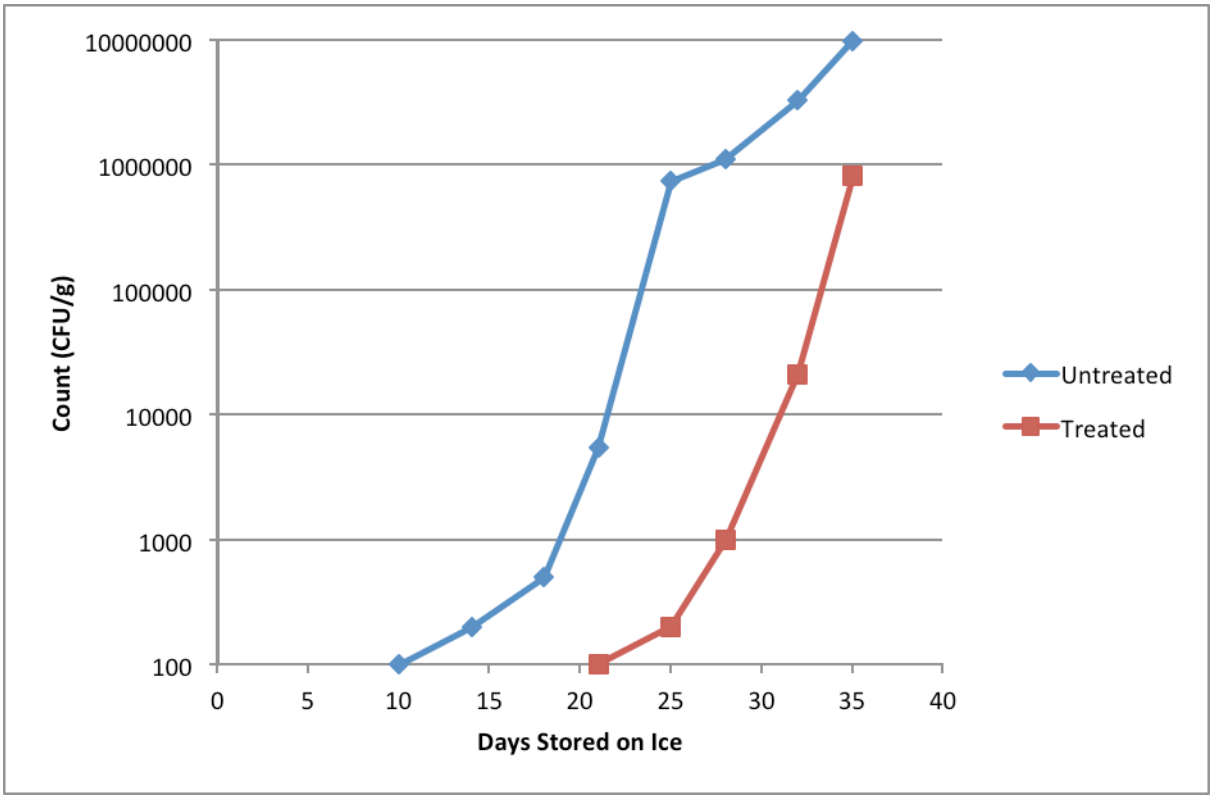


Figure 1.4.15 The effect of chlorine dioxide on the Specific Spoilage Organisms on Sea Mullet.

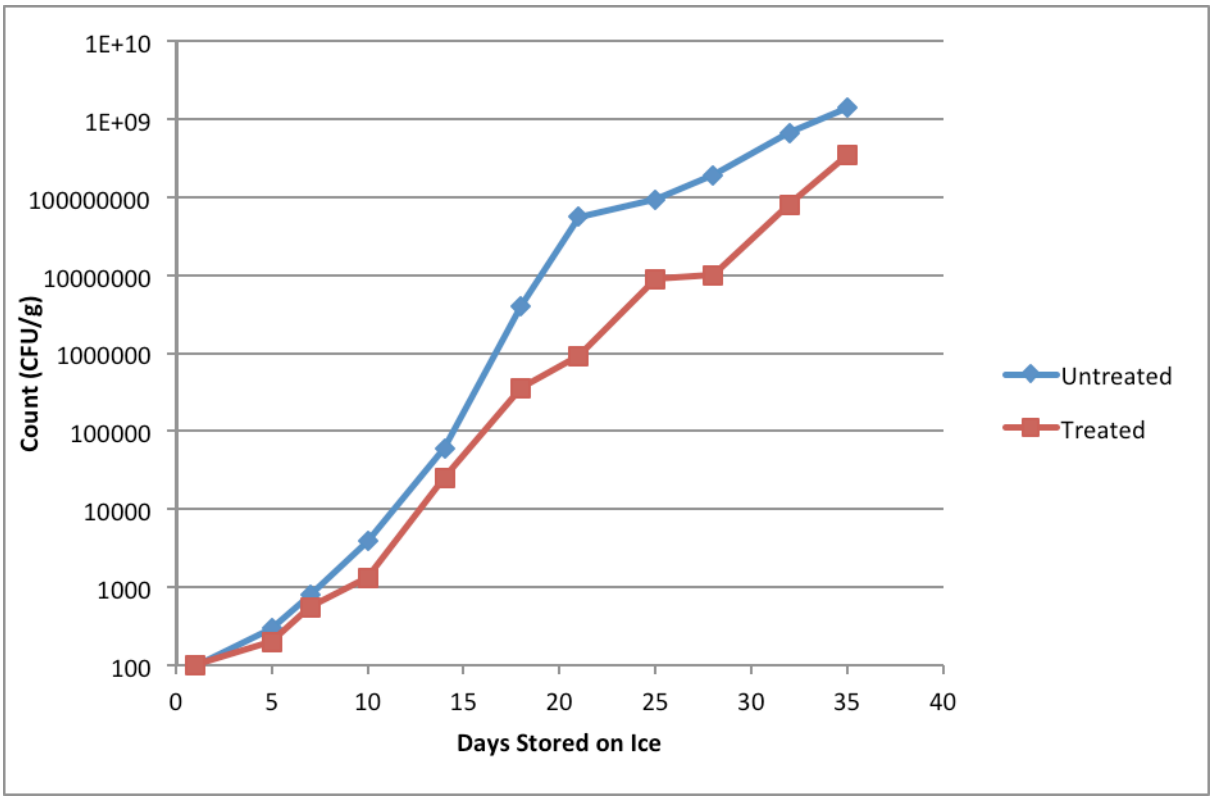


Figure 1.4.16 The effect of chlorine dioxide on the psychrotrophic count of Sea Mullet.

1.5. Bacterial Species Isolated From Sea Mullet.

The major colony types from each agar plate (i.e. Plate Count Agar, Iron Agar & modified Long & Hammer Agar) were selected and tested as described in the Methods section (up to 10 colonies per plate). Figure 1.5.1 shows the proportion of major genera and species present on fresh sea mullet at retail. The figure represents those bacterial species which were present on mullet on at least two of the three experiments.

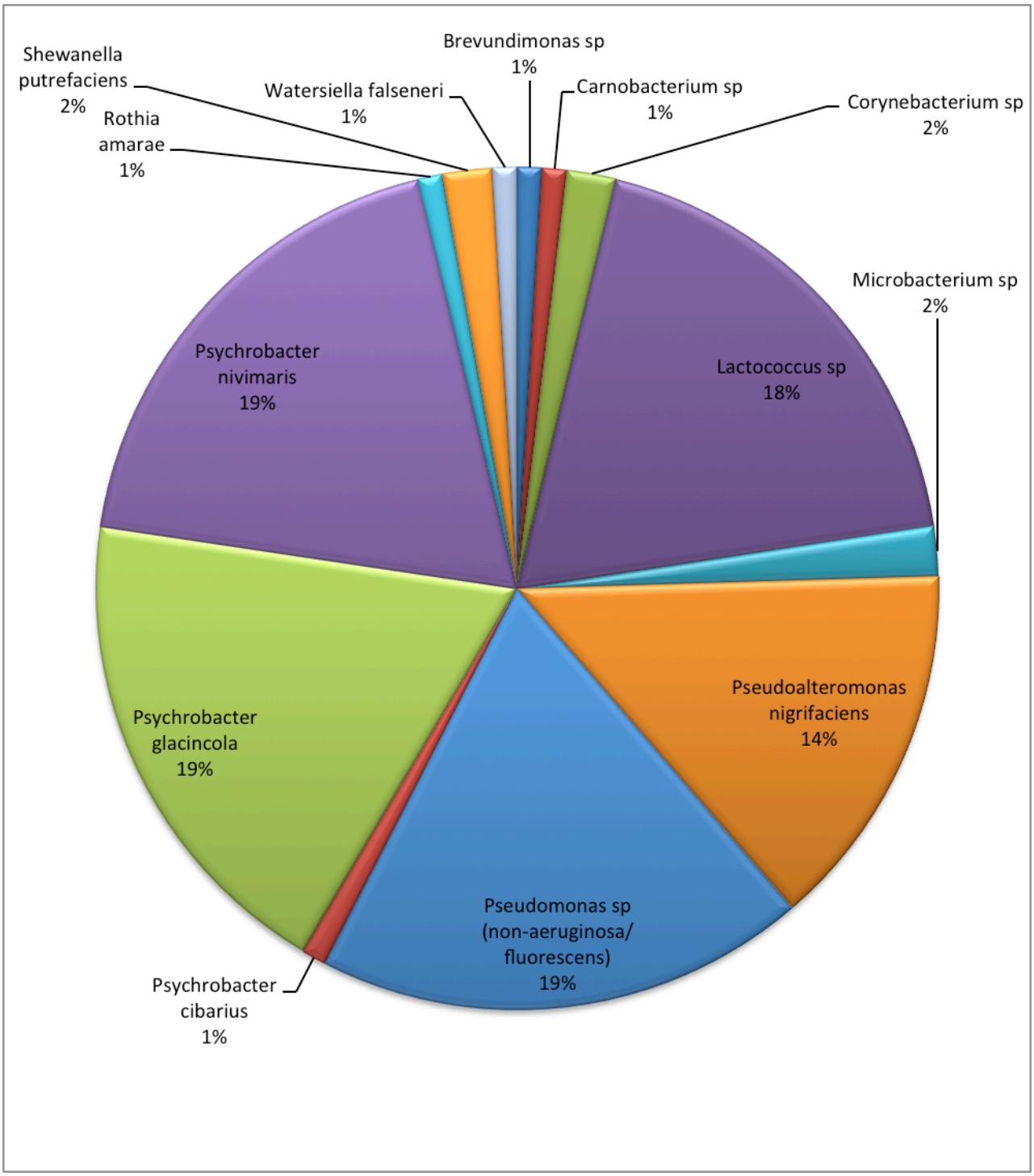


Figure 1.5.1 Major bacterial isolates isolated from freshly caught Sea Mullet

Psychrobacter nivimaris, *Psychrobacter glacincola*, and *Pseudomonas* species other then *Ps. aeruginosa*, and *Ps. flourescens* were the bacteria most commonly isolated fresh sea mullet at retail. Collectively they represent 57% of all bacteria present. *Lactococcus* was the next most commonly isolated bacterial strain representing 18% of all isolates. The other bacterial species found in abundance on unspoilt mullet was *Pseudoalteromonas nigrifaciens* (14%).

Microbacterium sp., *Corynebacterium* and *Shewanella putrifaciens* represent approximately 2% of the major bacterial isolates present on fresh retail sea mullet. Other species such as *Psychrobacter cibarius*, *Rothia* sp., *Watersiella falseneri*, *Brevundimonas* sp., and *Carnobacterium* sp. represent 1% each of the major species present.

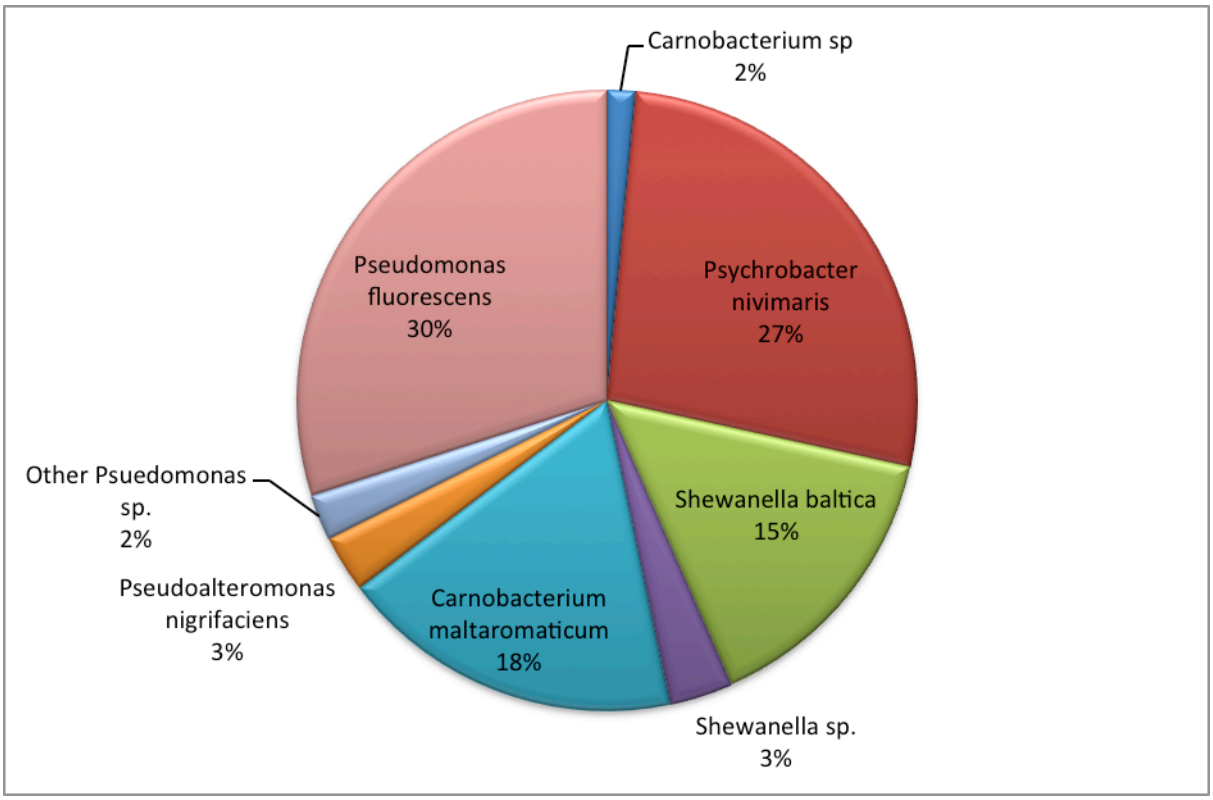


Figure 1.5.2 Major bacterial isolates isolated from spoilt Sea Mullet (Experiment 1).

Following refrigeration in ice for a total of 15 days spoilt sea mullet had a significantly different population profile (see figure 1.5.2 above). The most commonly isolated bacterial species was *Pseudomonas fluorescens* representing 30% of the major bacterial isolates. This represented a total count of approximately $2.0x10^8$ CFU/g when extrapolated from the final total count of $8.0x10^8$ CFU/g. Other *Pseudomonas* sp., including *Ps. aeruginosa*, *Ps. putida*, and *Ps. stutzeri* made up only 2% of the major species present on spoilt sea mullet. *Psychrobacter nivimaris* was the second most common bacterial species on spoilt sea mullet representing 27% of the major species. Eighteen percent of the isolates from spoilt mullet were *Carnobacterium maltaromaticum*, another 2% were other *Carnobacterium* species (mainly *C. divergens*). *Shewanella baltica* was not found to be a major isolate on unspoilt mullet, but represented 15% of the major isolates on spoilt sea mullet. A further 3% were

found to be other *Shewanella* species. This group included *Shewanella putrefaciens* and *Shewanella frigidimarina*. Three percent of the major isolates were *Alteromonas nigrifaciens*.

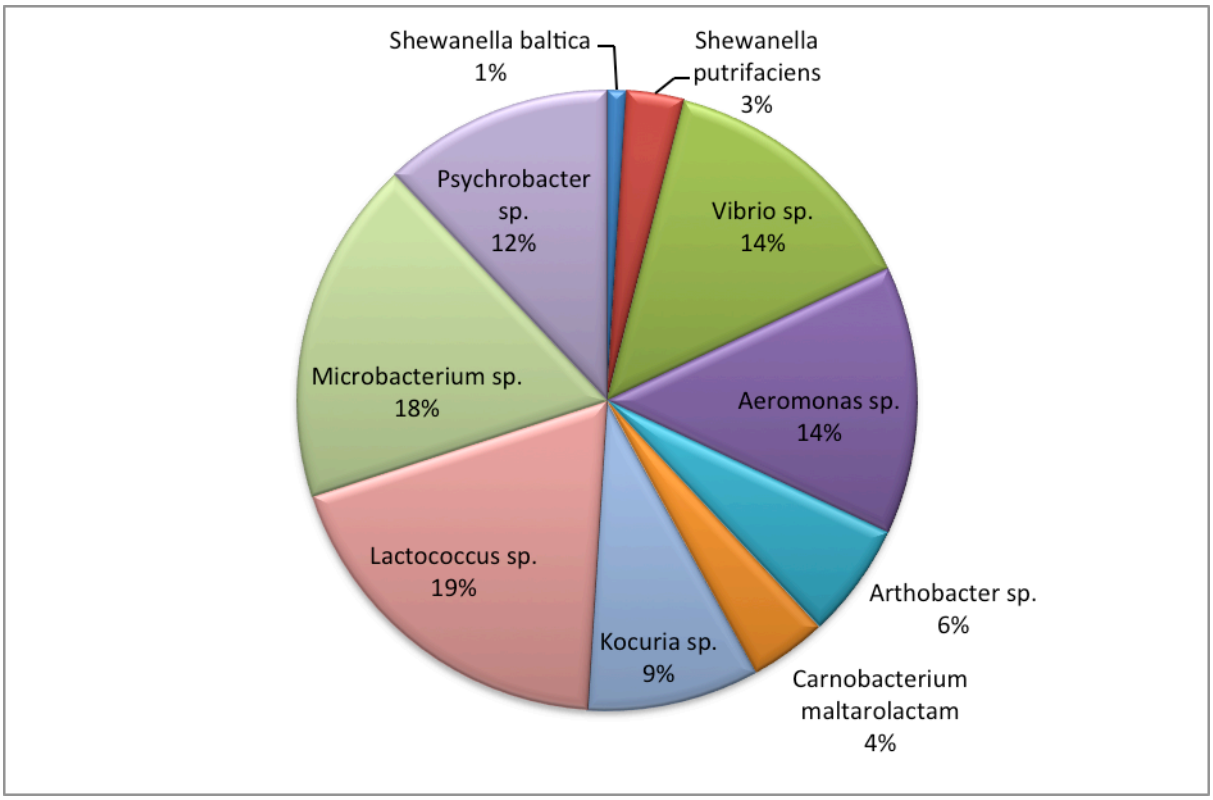


Figure 1.5.3 Major bacterial isolates from freshly caught Sea Mullet (Green Head).

Experiments on Sea Mullet freshly caught on beaches found a slightly differing initial micro-flora to that found on fresh Sea Mullet at retail. The bacteria shown in Figure 1.5.3 were initially isolated using contact (RODAC) plates. Three plates per fish were used containing either PCA, IA, or mL&H agar. The most numerous bacteria were *Lactococcus* species representing 19% of the total isolates, followed by *Microbacterium* sp. (18%), *Aeromonas* sp. (14%), *Vibrio* sp. (14%), and *Psychrobacter* sp. (12%). Of the 12% *Psychrobacter* species, *P. nivimaris* was the most common species representing about 3/4 of all *Psychrobacter* isolates with the other 1/4 being *Psychrobacter glacincola*. The remaining isolates were *Kocuria* sp. (9%), *Arthobacter* sp. (6%), *Canobacterium maltarolactam* (4%), *Shewanella purtifaciens* (3%), and *Shewanella baltica* (1%).

The beach caught fish were transported directly to the laboratory on ice, and were stored in a freely draining container on ice in a refrigerator. Following storage for 29 days, the spoilt Sea

Mullet had a Standard Plate Count of $1.5x10^8$ CFU/g and Specific Spoilage Organism (IA) count of $2.4x10^7$ CFU/g, and a Psychrotrophic Plate Count (mL&H) of $2.3x10^8$ CFU/g. Figure 1.5.4 below details the major bacterial species isolated from the spoilt sea mullet. *Pseudomonas fluorescens* was the most commonly isolated bacterial species following spoilage with 38% of all isolates. Other *Pseudomonas* species only represented 3% of the isolates. *Pseudoalteromonas nigrifaciens* was the next most commonly isolated bacterial species representing 20% of the isolated population, then *Psychrobacter nivimaris* (15%), *Shewanella baltica* (14%), and *Canobacterium maltaromaticum* (10%).

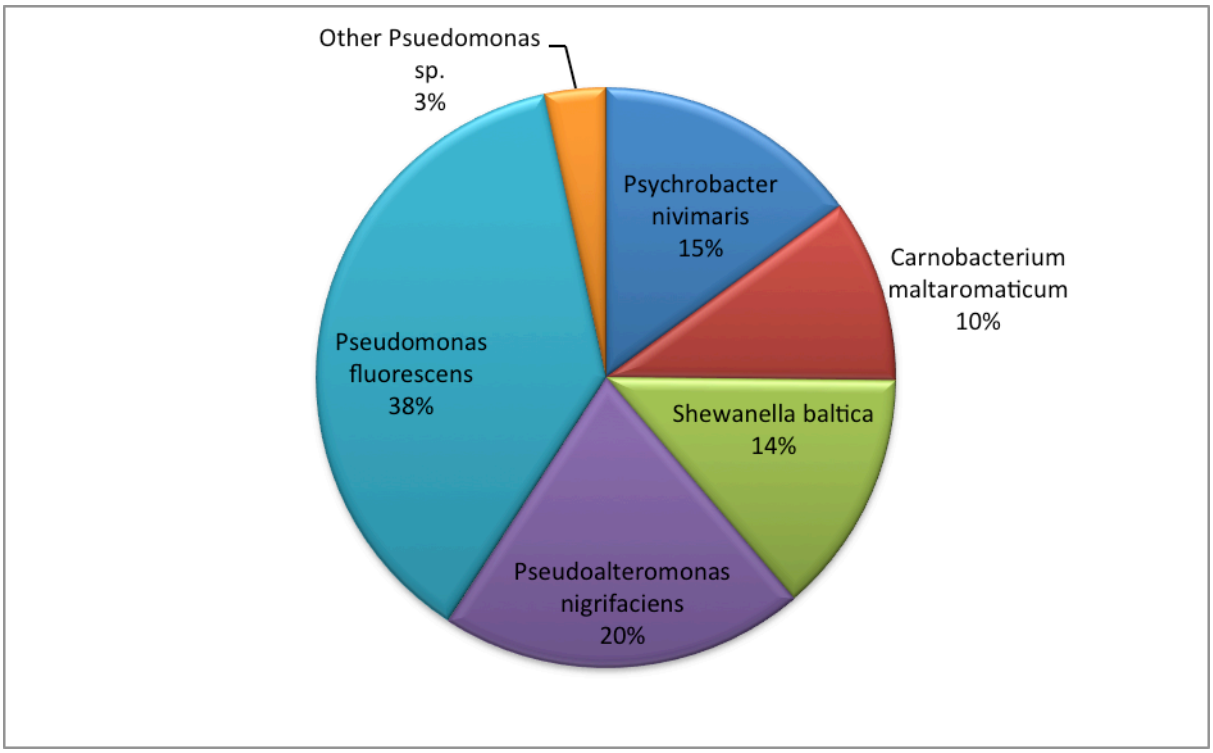


Figure 1.5.4 Major bacterial isolates from spoilt Sea Mullet caught at Green Head.

The initial micro-flora isolated from fresh retail and freshly caught Sea Mullet appears to vary quite considerably. This variation may be due to the fact that the retail mullet had spent at least 1 day in an ice slurry prior to testing. Significantly, there were several different *Vibrio* species isolated from the freshly caught Sea Mullet, but there was no *Vibrio* species present on the retail Sea Mullet. This was also true of *Aeromonas* species, *Kocuria* species and *Arthrobacter* species. *Psychrobacter* species represented 39% of the isolates on the retail sea mullet, but was only 12% of the isolates on freshly caught mullet. On freshly caught mullet *Pseudomonas* species were only isolated very rarely (representing less than 1% of isolates), where as they represented 19% of the isolates on retail mullet. Similarly, *Pseudoalteromonas*

species represented approximately 20% of isolates from retail mullet, but made up less than 1% of the population on fresh mullet. *Microbacterium* species represented 18% of the isolates from freshly caught Sea Mullet, but only represented 2% of the isolates from retail mullet. Interestingly the major bacterial isolates on the beach caught mullet following spoilage was once again *Pseudomonas flourescens* (38%), *Psychrobacter nivimaris* (15%), *Shewanella baltica* (14%) and *Carnobacterium maltaromaticum* (10%). The major difference between the two sets of spoilt mullet, was the presence of a significantly higher incidence of *Pseudoalteromonas nigrifaciens* on spoilt beach caught mullet (20%) compared with the spoilt retail mullet(3%).

Figure 1.5.5 below shows the major bacterial isolates found on spoilt mullet following treatment with chlorine dioxide. Once again *Pseudomonas fluorescens* was the major bacterial isolate on spoilt mullet. There was only minimal difference in the types and proportions of bacterial isolates on the spoilt mullet compared with the spoilt untreated beach caught mullet (Figure 1.5.4, above). The difference in the rate of spoilage of sea mullet on chlorine dioxide treated mullet over untreated mullet therefore appears to be due to an overall decrease in the total number of spoilage bacteria present, and not the elimination of a particular genus or species of bacteria. A significant finding was the presence of *Photobacterium phosphoreum* on one mullet. This was detected on one of one beach caught mullet. The presence of *Photobacterium phosphoreum* is of very high significance if the mullet was to be packed into a modified atmosphere. *Photobacterium phosphoreum* has been found to cause significant spoilage of modified atmosphere packed (MAP) finfish (Dalgaard, Mejlholm et al. 1997; Dalgaard, Madsen et al. 2006).

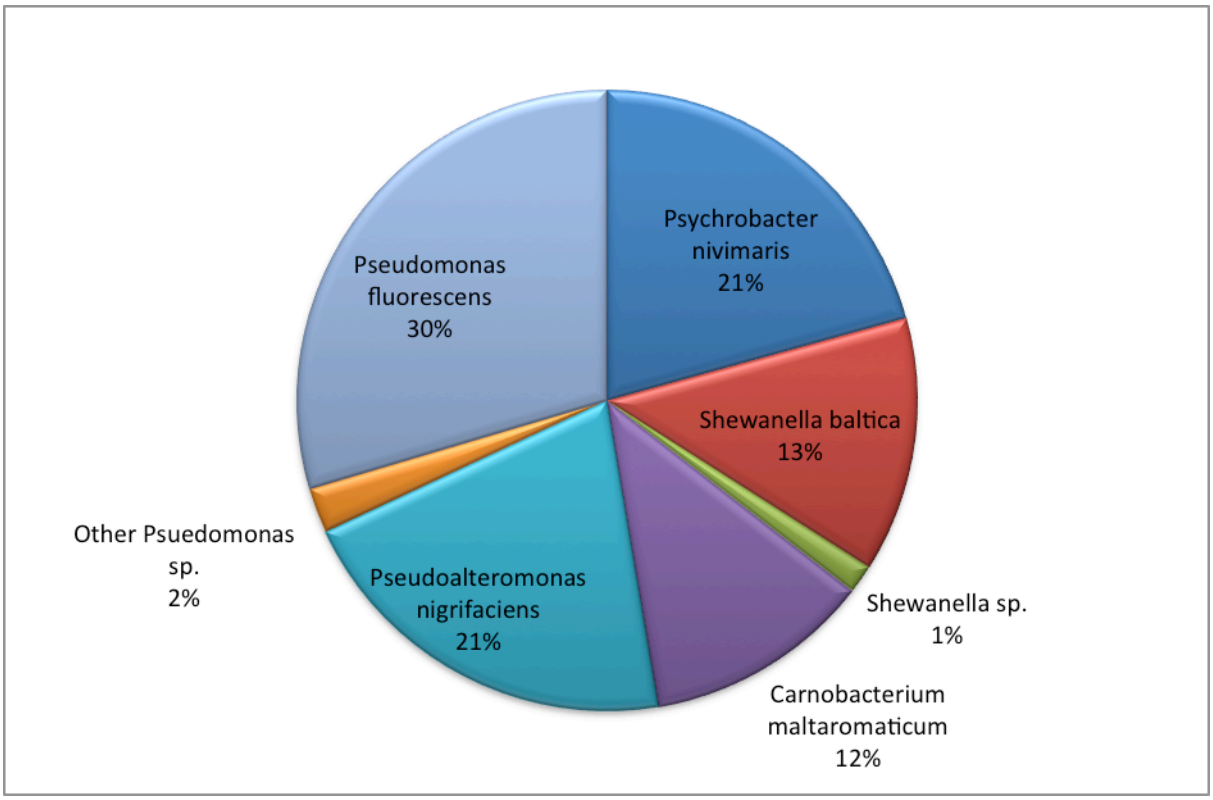


Figure 1.5.5 Major bacterial isolates from spoilt chlorine dioxide treated Sea Mullet caught at Green Head.

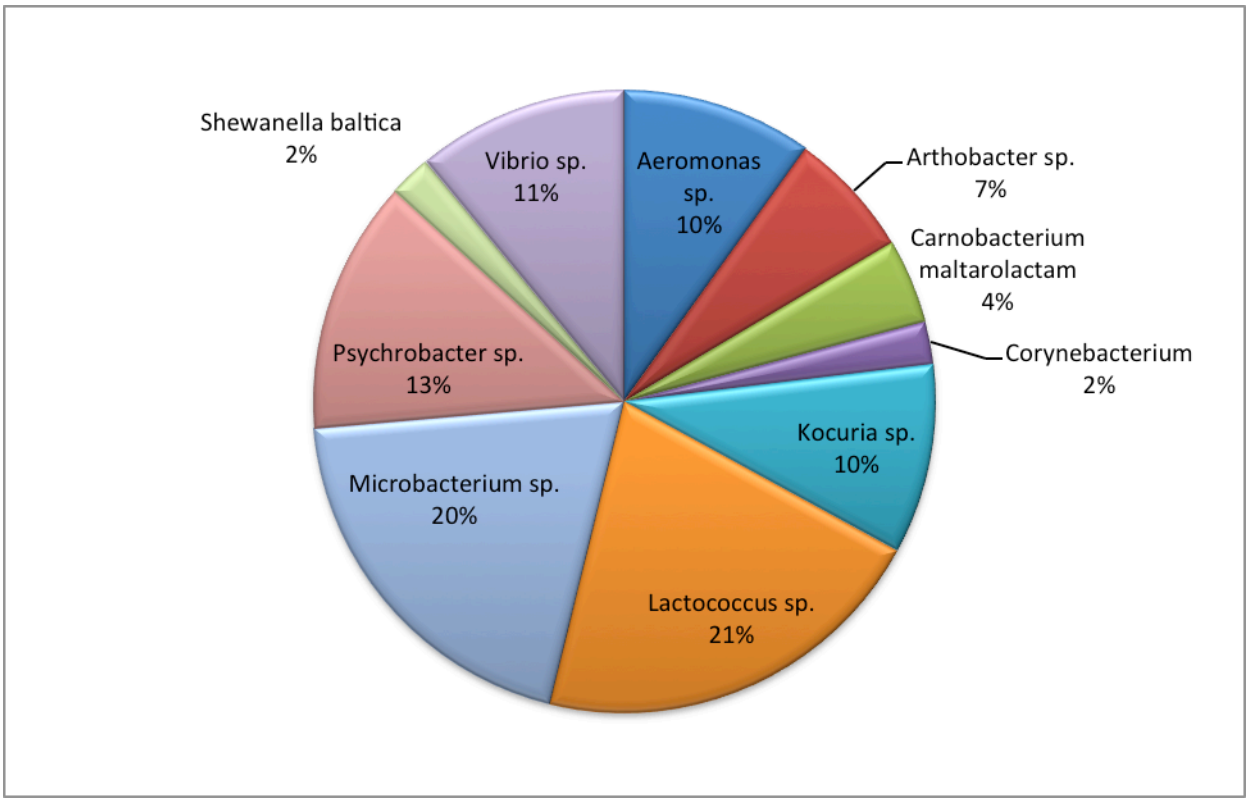


Figure 1.5.6 Major bacterial isolates from freshly caught Sea Mullet caught at Rockingham.

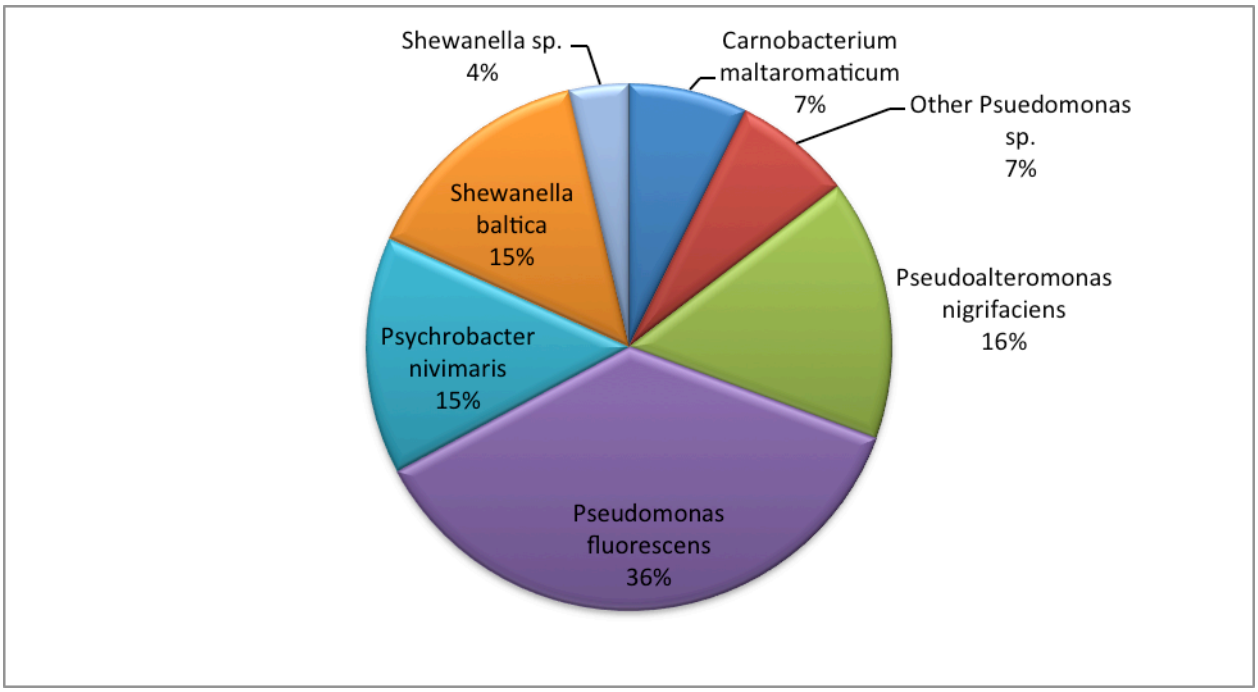


Figure 1.5.7 Major bacterial isolates from spoilt Sea Mullet caught at Rockingham.

The major bacterial isolates from fresh and spoilt Sea Mullet caught at Rockingham are shown in Figures 1.5.6 and 1.5.7 above. These results compared to the results of the mullet caught at Green Head showed no major difference between isolates from freshly caught and spoilt fish.

1.6 Summary of Important Findings

- Low numbers of mesophilic and psychrotrophic bacteria observed at point of capture.
- Rapid growth of mesophilic and psychrotrophic bacteria observed between days 5 and 20 of storage on ice.
- Storage of Sea Mullet in containers which allow for the melted ice water to freely drain gives best results.
- Use of sanitisers such as chlorine dioxide in initial ice slurry can have a significant impact on the microbiological quality and the quality index score of ice stored sea mullet.
- Un-activated chlorine dioxide gives best results for sanitiser treatment.

- The bacterial species most likely to be in involved in spoilage are *Pseudomonas fluorescens, Psychrobacter nivimaris, Carnobacterium maltaromaticum,* and *Shewanella baltica*.
- Geographic location does not appear to have an impact on the rate of spoilage or the major bacterial species present on Sea Mullet.
- Treatment of Sea Mullet with sanitisers results in an overall reduction in all bacterial levels and does not appear to target particular bacterial groups or species.

2. SPOILAGE OF GOLDBAND SNAPPER

2.1. Bacterial Growth on Retail Goldband Snapper

Fresh whole Goldband Snapper was obtained from a retail shop on the day the fish had arrived from Broome WA. The fish were transported to the laboratory in ice. On arrival the fish were stored on ice in a refrigerator. The container of iced fish was set up with a drain to ensure the fish did not stand in water. Samples were taken at days 1, 5, 9, 15 and 21.

Total mesophilic bacterial numbers (as assessed using the Standard Plate Count method) started at a relatively low level of around 10,000 CFU/cm^2, rising steadily to a count of just under 310,000 CFU/g at day 21. Total psychrotrophic bacterial numbers were assessed using the Long & Hammer agar method and started at around 100,000 CFU/g rising to a maximum count of around 7x10^8 CFU/g. The total number of SSOs (enumerated using Iron Agar) started at levels of around 40000 CFU/g rising to approximately 1.3x10^7 CFU/g at day 21 (see Figure 2.1 below).

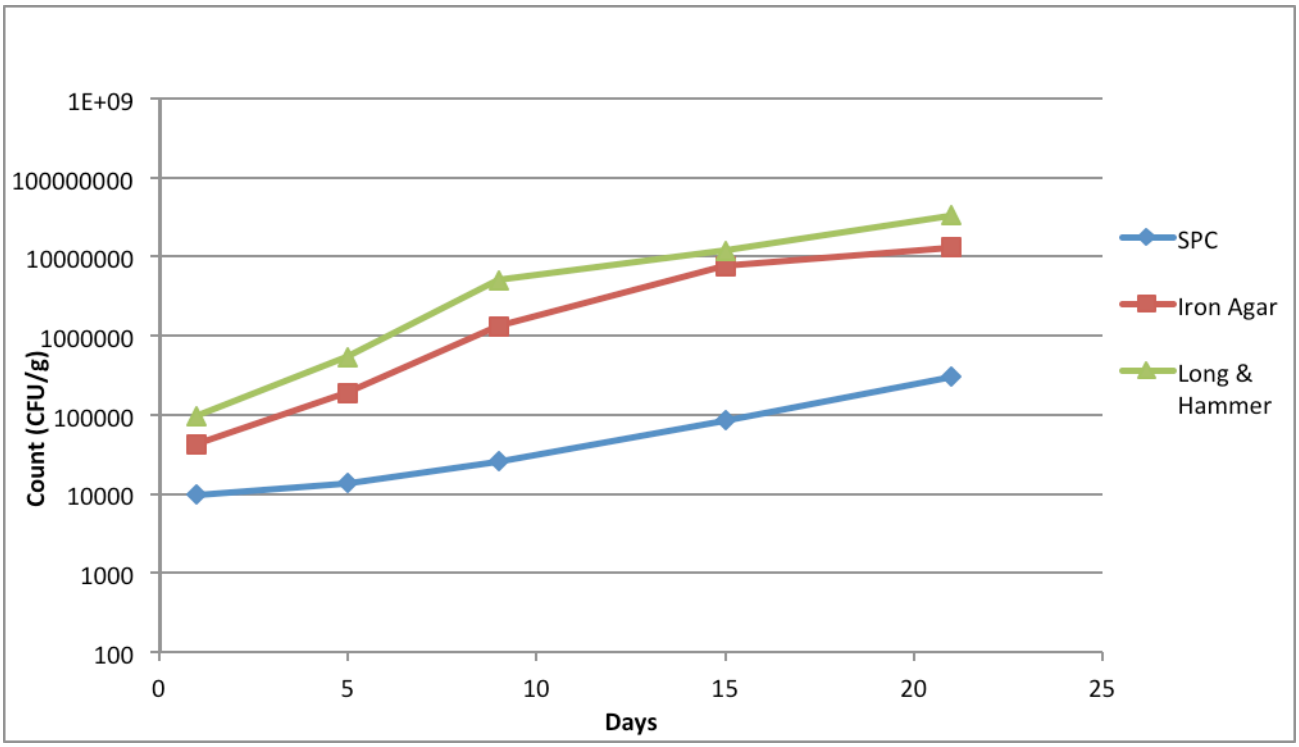


Figure 2.1 Microbiological quality of retail whole Goldband Snapper

These results were markedly different to the results of sea mullet. The rate at which bacteria grew on Goldband Snapper was significantly slower than the rate seen on mullet (See Figure 2.2 below). While the initial counts for all tested bacteria (i.e. aerobic mesophilic bacteria, aerobic psychrophilic bacteria, and specific spoilage organisms) were approximately 10 times higher on Goldband Snapper, by day 9 the counts on sea mullet were higher on sea mullet than the corresponding counts on Goldband Snapper on day 21. The cause of the initial higher counts on Goldband Snapper was likely to be due to the fact that Goldband Snapper purchased in Perth were likely to be approximately 5 days since capture, while the mullet was caught the day prior to purchase. This delay will allow psychrotrophic bacteria and specific spoilage organisms to grow to higher levels before reaching retail. As a result of these findings, and previous studies indicating Goldband Snapper's tendency to have a long shelf life compared to other tropical snapper species, a PhD project was funded by the Seafood CRC. Results of this PhD project will be submitted shortly in a thesis by Rachel Tonkin.

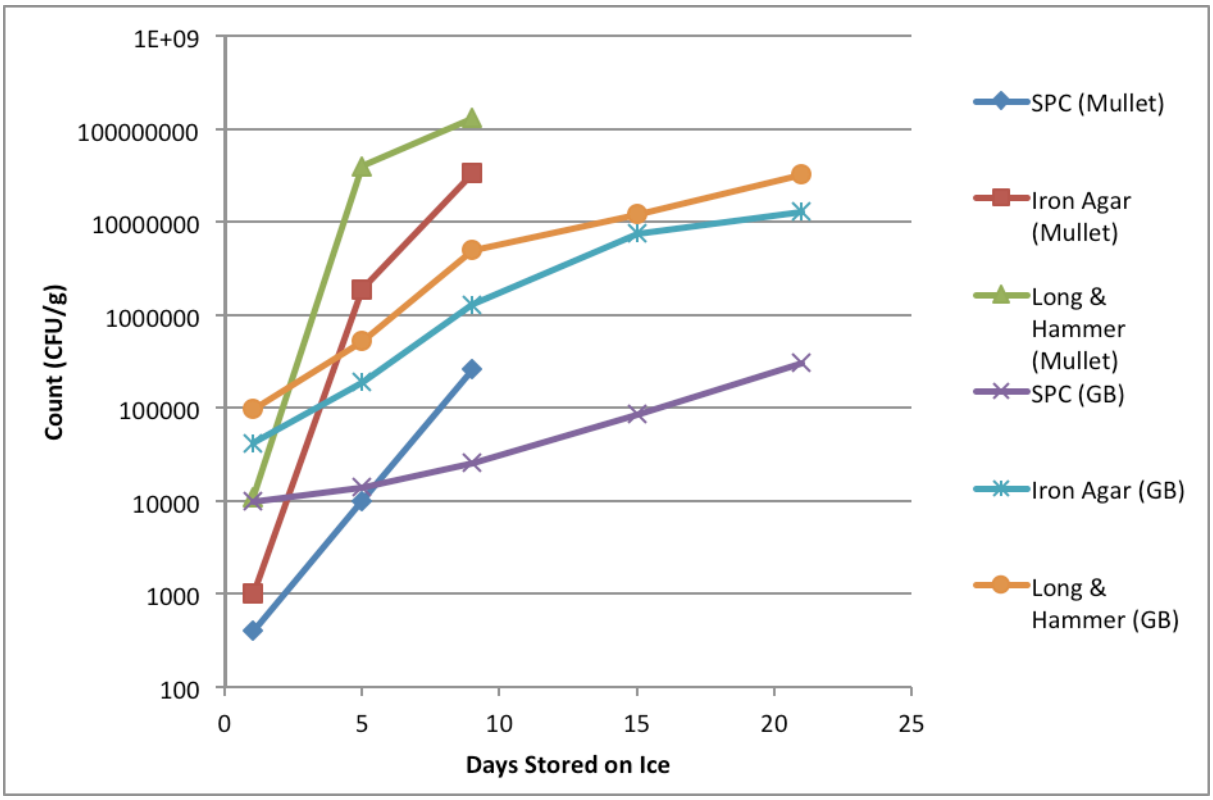


Figure 2.2 Comparison of microbiological counts on retail Goldband Snapper and Sea Mullet.

2.2. Use of Sanitisers to Extend the Shelf-life of Whole Chilled Goldband Snapper

Figure 2.2.1 details a comparison of using activated and un-activated chlorine dioxide on the Quality Index score of Goldband Snapper. Previous studies in Sea Mullet indicated that the use of un-activated chlorine dioxide in an ice slurry increased the shelf life of mullet (as determined by the Quality Index) more than using activated chlorine dioxide.

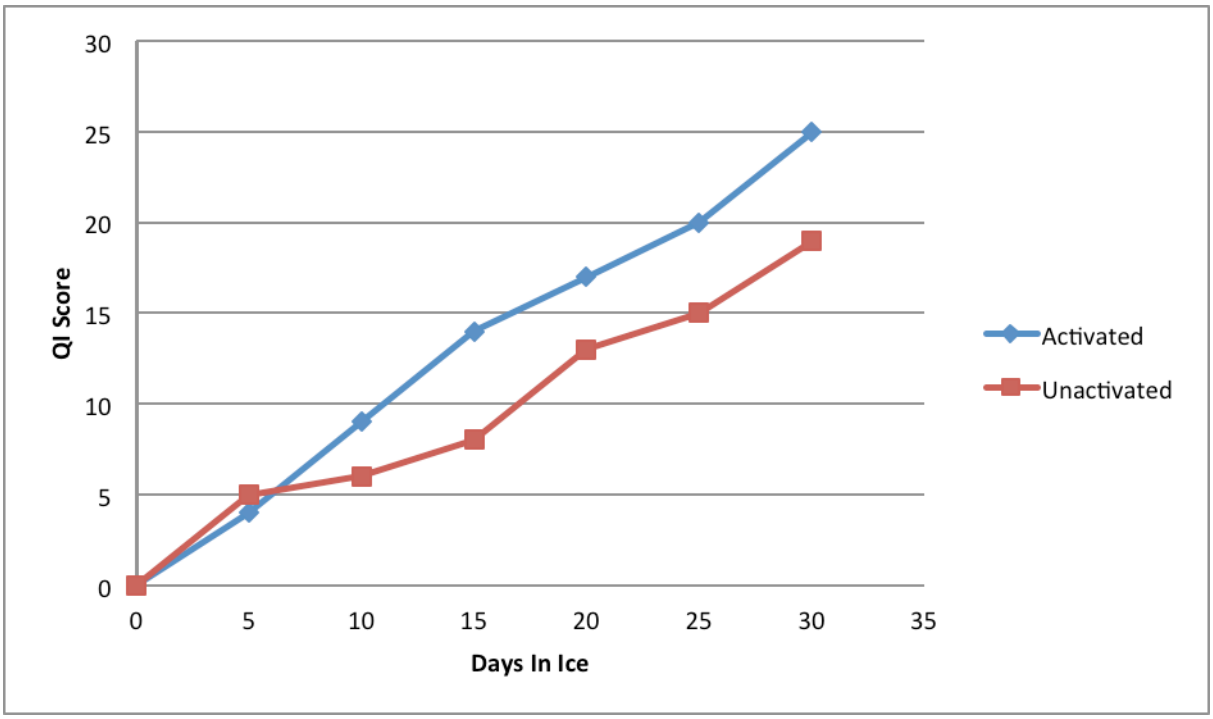


Figure 2.2.1 Effect of activating chlorine dioxide on the Quality Index score of Goldband Snapper.

The effect of using chlorine dioxide based sanitisers in ice slurries prior to storage in refrigerated ice was investigated. Un-activated chlorine dioxide was used at the manufacturers recommended rate of 20mL/ L of slurry. The fish were held in the ice slurry for up to 15 minutes. Following treatment the fish were assessed by the Quality Index method and sampled for microbiological analysis, and stored in ice in a refrigerator. The plastic containers used were free draining such that the fish were not standing in melted water. Figure 2.2.2 to 2.2.5 summarise the effect of chlorine dioxide on the rate of spoilage of Goldband Snapper as determined using the Quality Index method and the levels of mesophilic bacteria, psychrotrophic bacteria and specific spoilage organisms (SSOs). These initial trials indicated that the use of un-activated chlorine dioxide in an ice slurry reduced the rate of spoilage and lowered the levels of spoilage bacteria on Goldband Snapper. The major impact of the use of chlorine dioxide was the reduction in levels of *Shewanella* (an SSO) as this was the only group of microorganisms which were consistently reduced to a statistically significant level (see Figure 2.2.4).

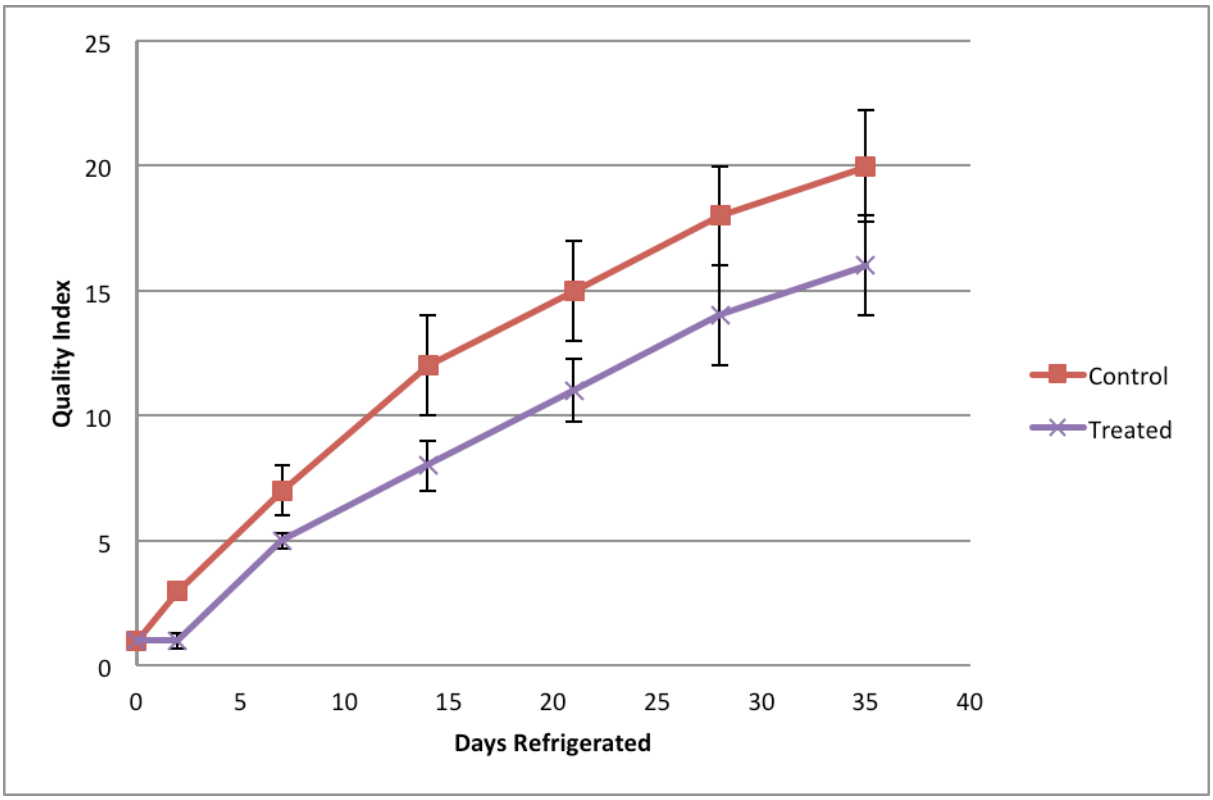


Figure 2.2.2 The effect of using un-activated chlorine dioxide ice slurry dips on the Quality Index score of Goldband Snapper.

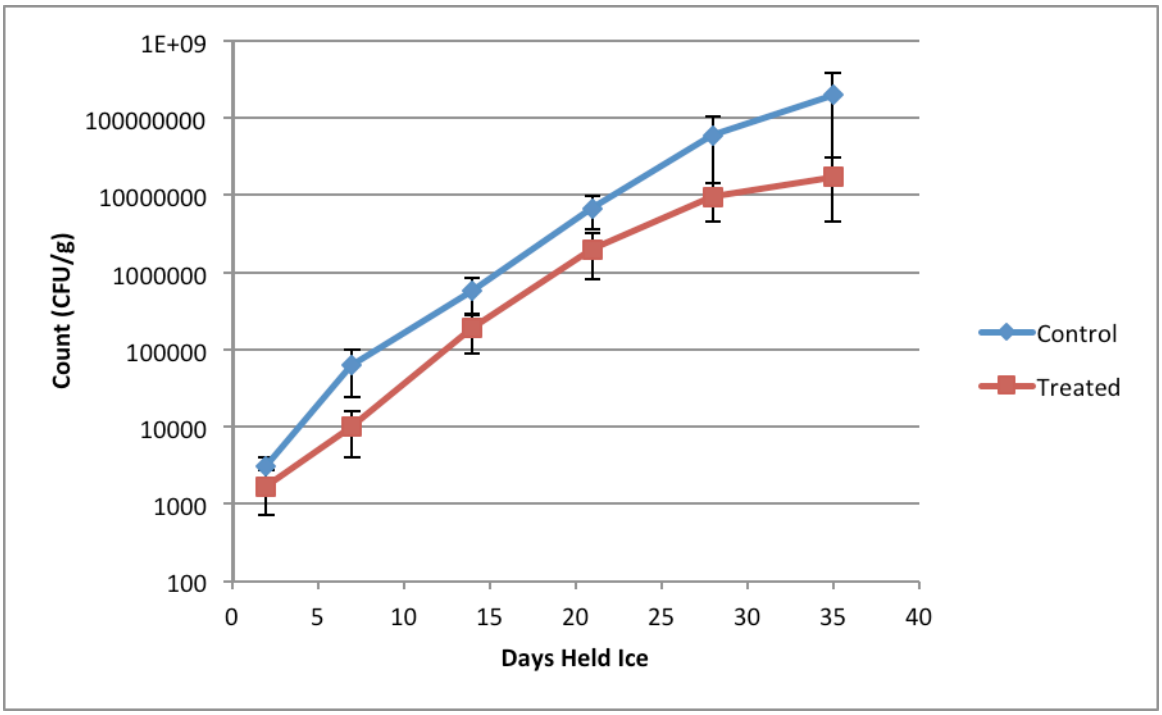


Figure 2.2.3 The effect of using un-activated chlorine dioxide ice slurry dips on the Standard Plate Count of Goldband Snapper.

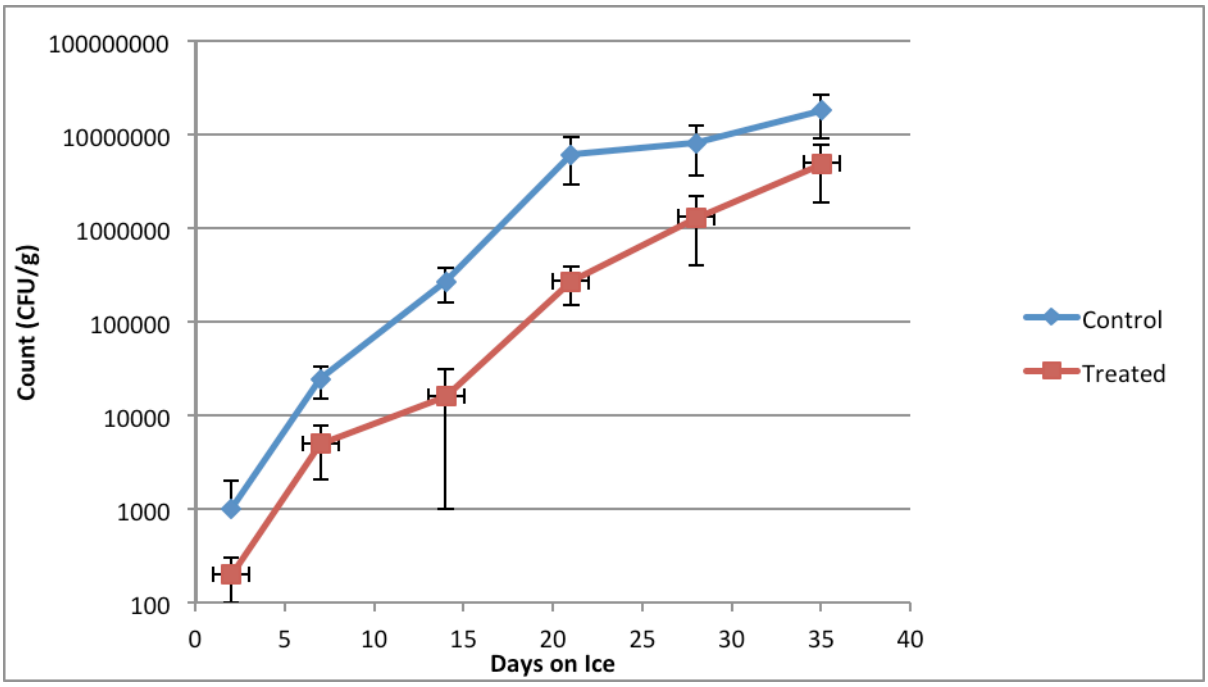


Figure 2.2.4 The effect of using un-activated chlorine dioxide ice slurry dips on the Specific Spoilage Organisms (Iron Agar count) on Goldband Snapper.

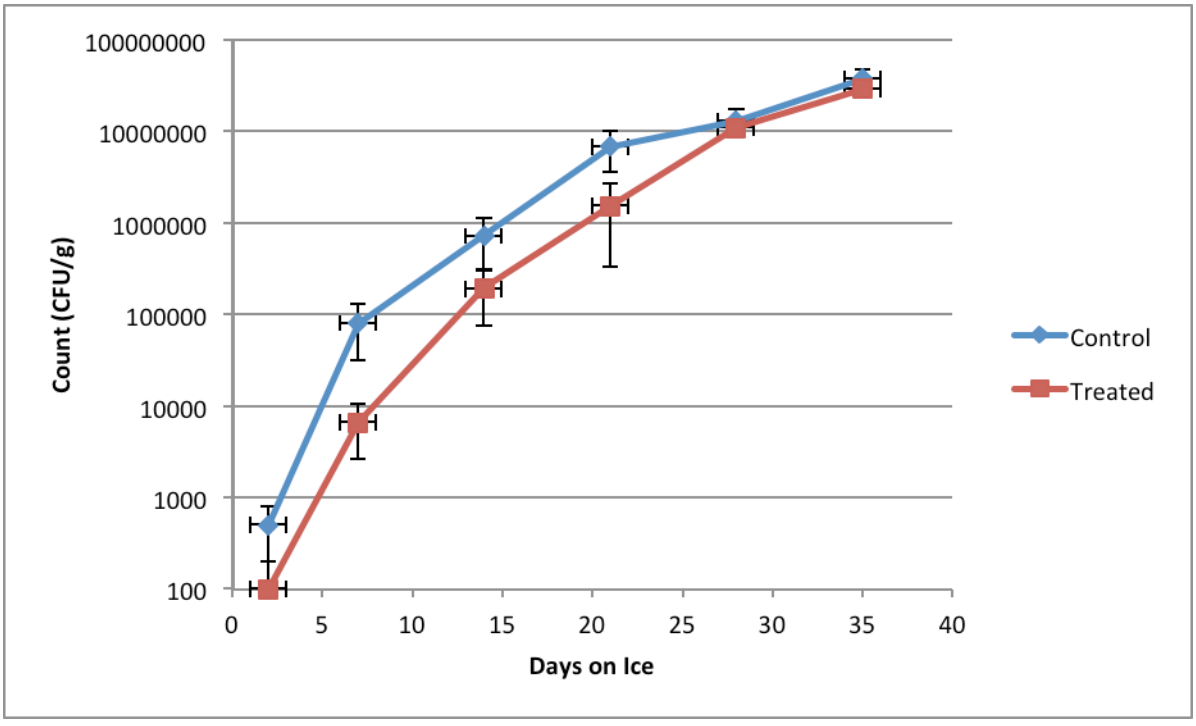


Figure 2.2.5 The effect of using un-activated chlorine dioxide ice slurry dips on the Psychrotrophic Bacteria (modified Long & Hammer count) on Goldband Snapper.

Trials of application of chlorine dioxide to Goldband Snapper prior to transport to Perth were performed. Fish were placed into an ice slurry containing 200ml/L of un-activated chlorine dioxide and held for 15 minutes prior to packing for transport as per normal procedures. The fish were transported to Perth in refrigerated trucks and samples were tested for Standard Plate Count, Iron Agar count and modified Long & Hammer count. The quality index for each fish was also assessed. Figures 2.2.6 to 2.2.9 summarise the results of these trials. These trials found that while there was a difference in the rate of spoilage of Goldband Snapper, these differences were not statistically significant. Given Goldband Snapper's inherent long shelf life, the application of chlorine dioxide is probably of limited advantage to the producer. However, the application of chlorine dioxide to other tropical snapper species with significantly shorter shelf life should be further investigated.

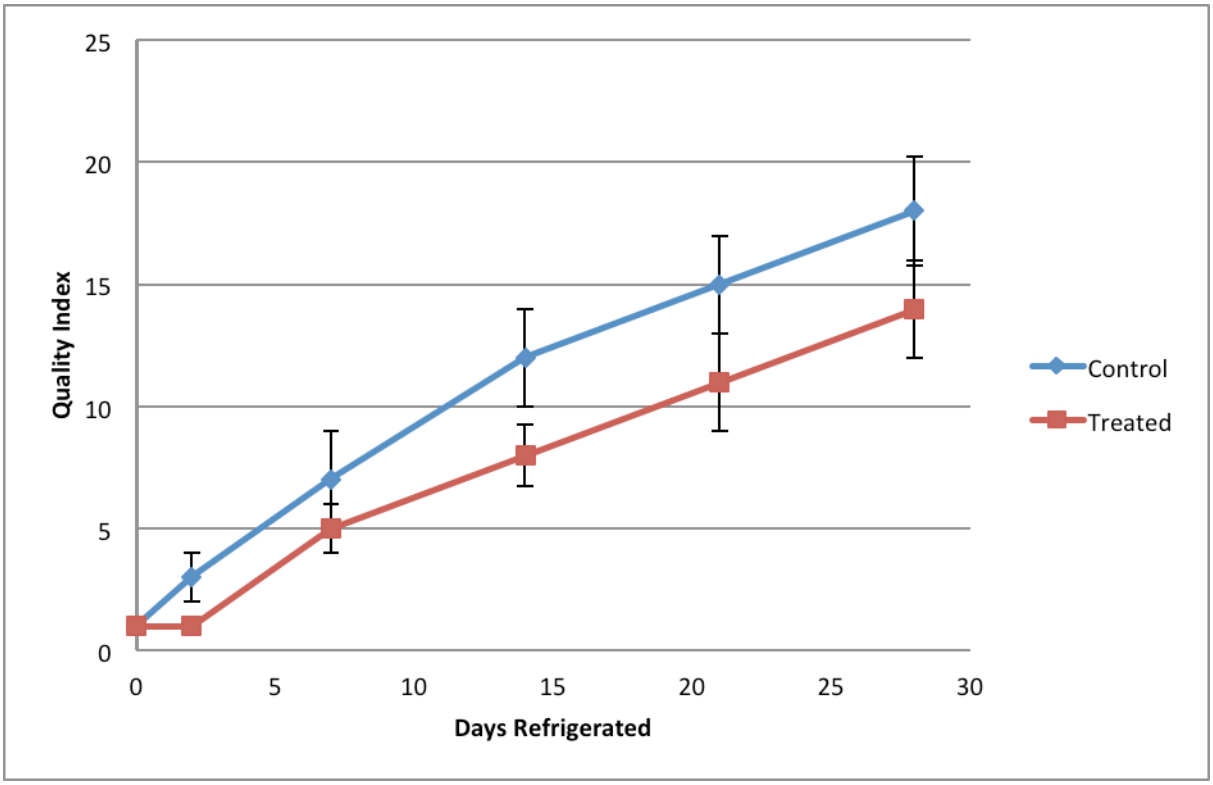


Figure 2.2.6 Effect of chlorine dioxide treatment on QIM scores for Goldband Snapper.

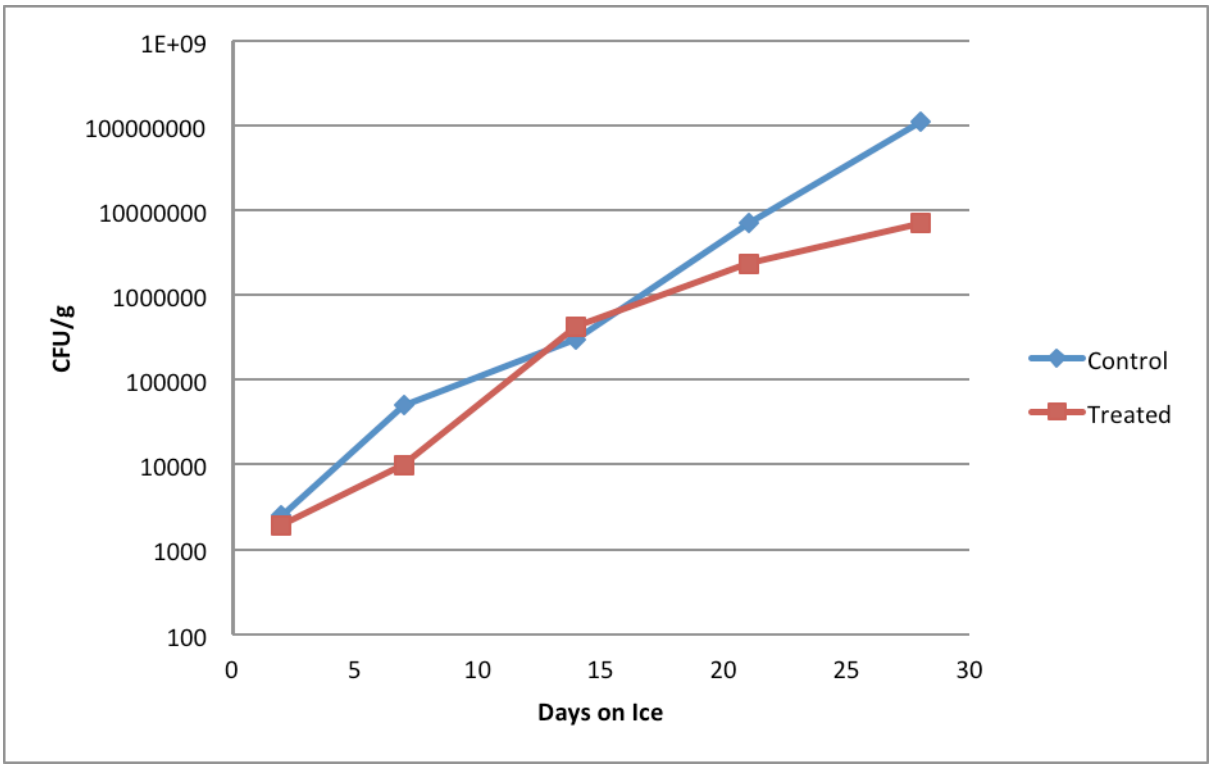


Figure 2.2.7 Effect of chlorine dioxide treatment on Standard Plate Count measuring aerobic mesophilic organisms on Goldband Snapper.

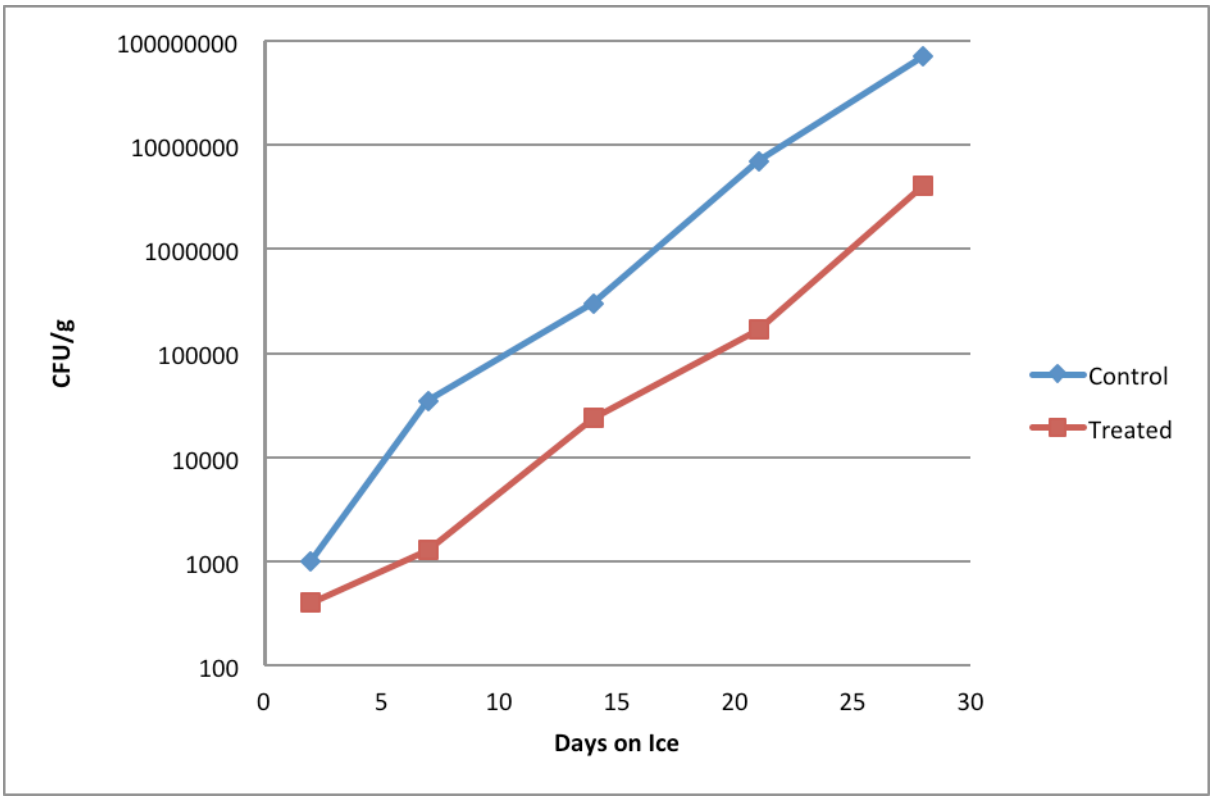


Figure 2.2.8 Effect of chlorine dioxide on the levels of Specific Spoilage Organisms on Goldband Snapper.

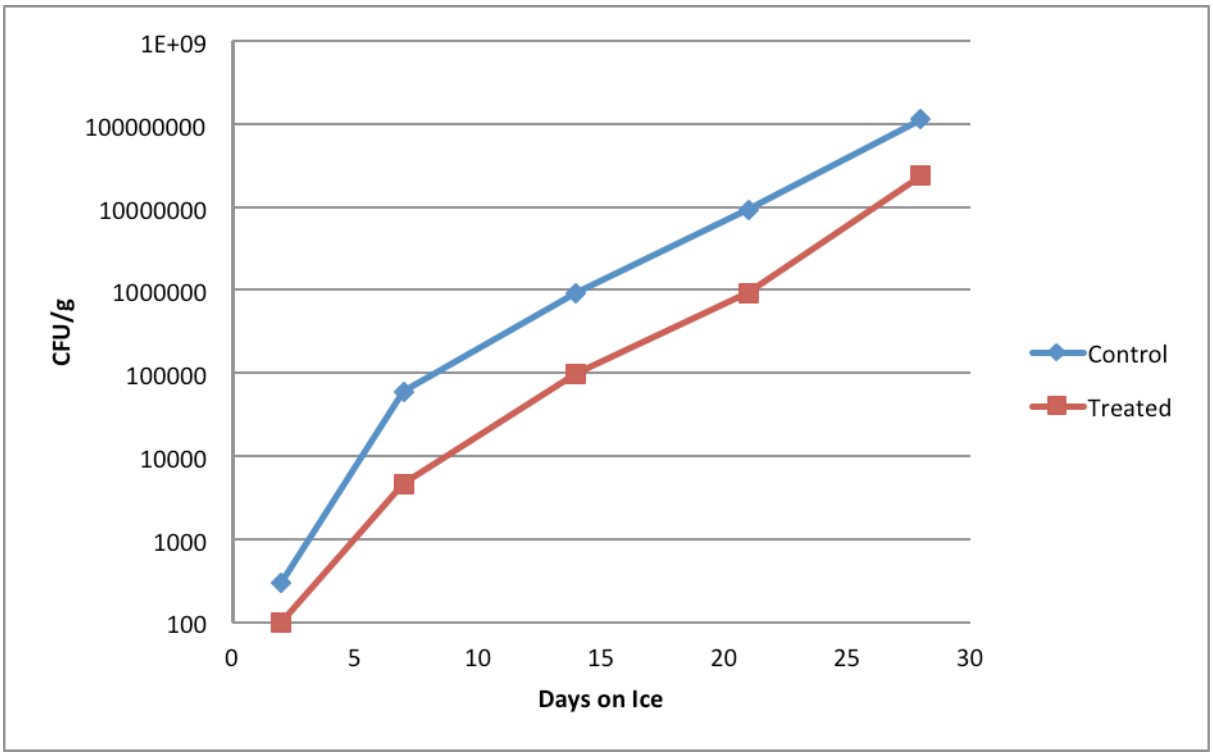


Figure 2.2.9 Effect of chlorine dioxide on the levels of psychrotrophic bacteria on Goldband Snapper.

2.3. Bacterial Species Isolated From Goldband Snapper.

The major colony types from each agar plate (i.e. Plate Count Agar, Iron Agar & modified Long & Hammer Agar) were selected and tested as described in the methods section (up to 10 colonies per plate). Figure 2.3.1 shows the proportion of major genera and species present on fresh Goldband snapper at retail. The figure represents those bacterial species which were present on mullet on at least two of the three experiments.

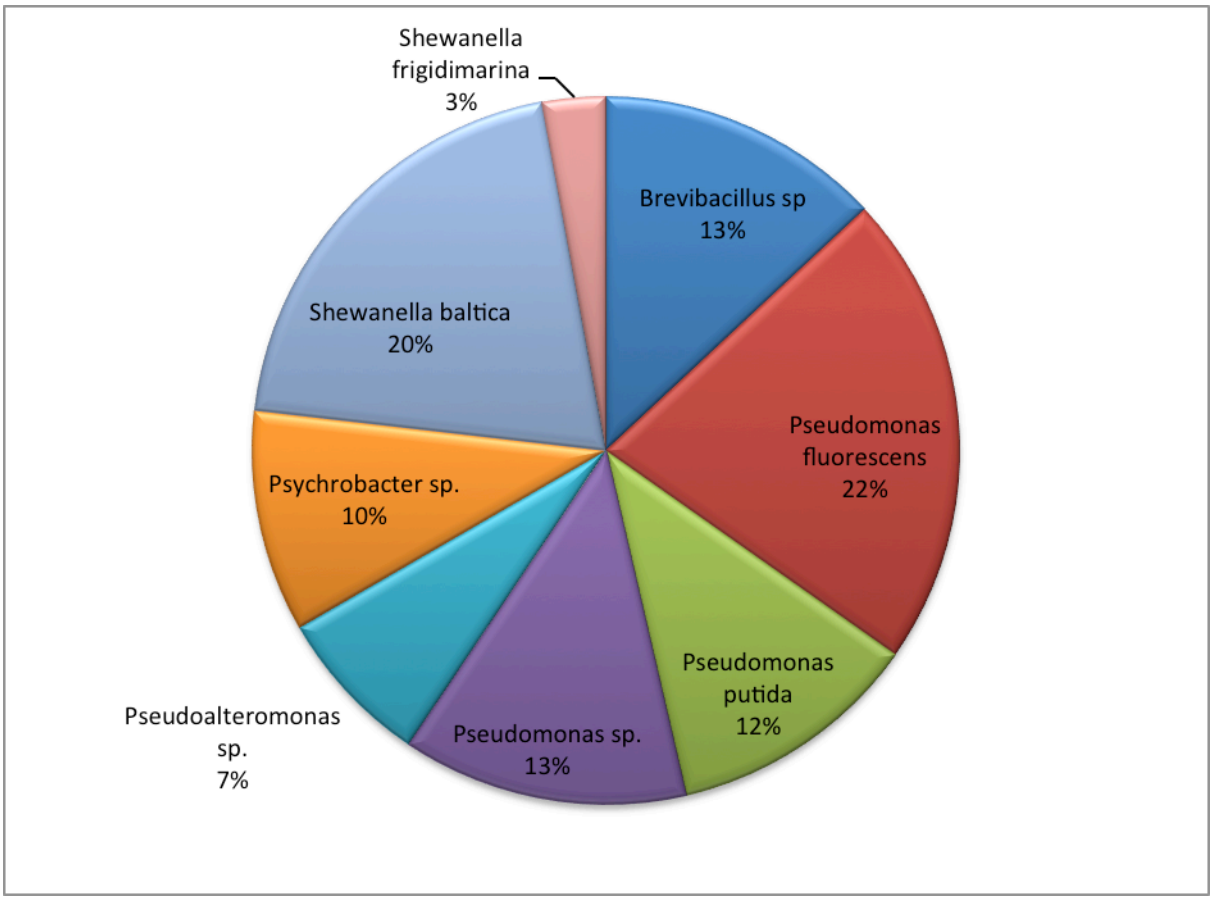
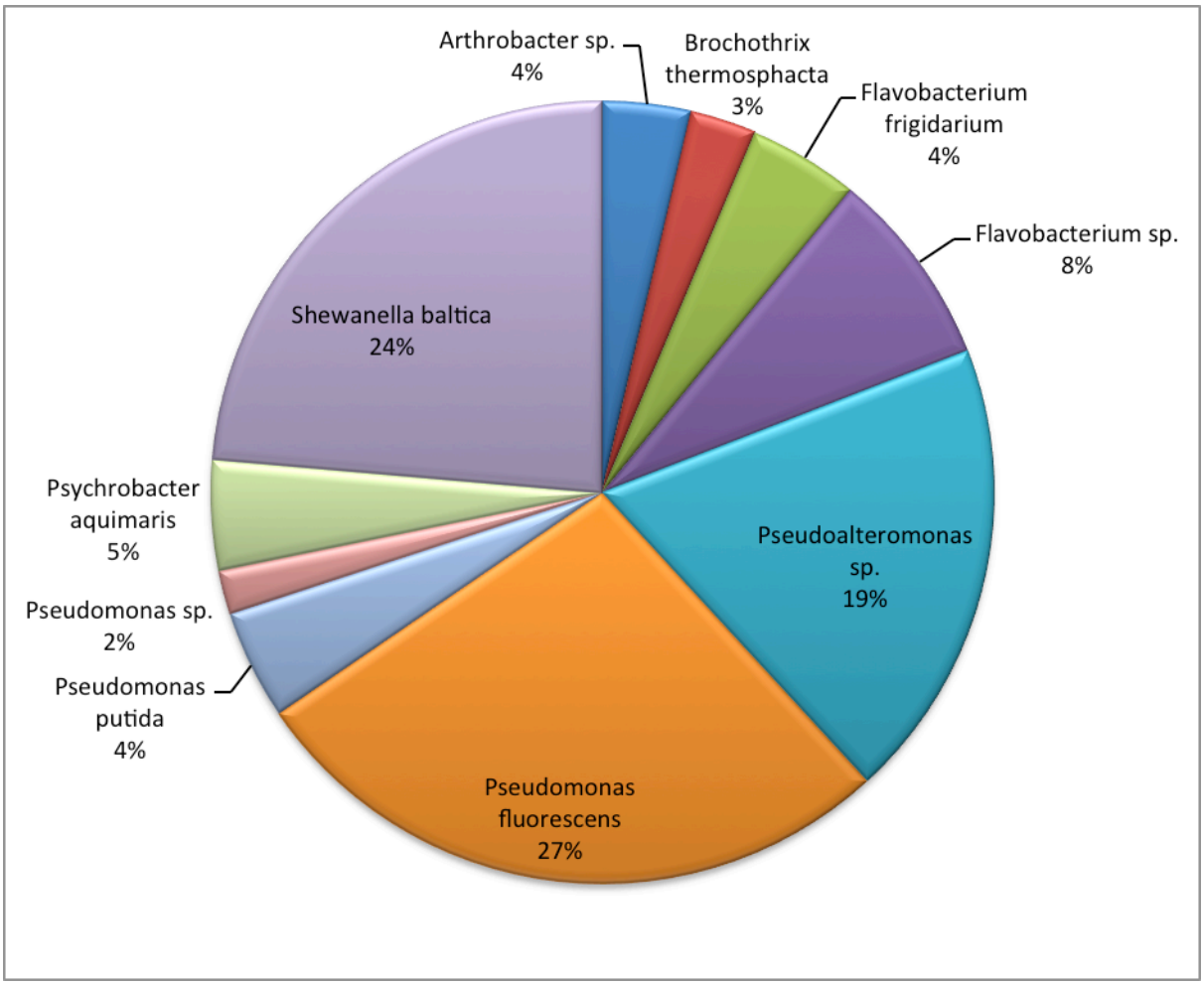


Figure 2.3.1 Major bacterial isolates cultured from fresh Goldband Snapper.

Pseudomonas fluorescens was the most commonly isolated species found on retail whole chilled Goldband Snapper, representing 22% of all major bacterial isolates. Other *Pseudomonas* species represent another 25% of all isolates including 12% of all isolates being *Pseudomonas putida.* The next most common isolates were *Shewanella,* with *Shewanella baltica* representing 20% of all major bacterial isolates, and *Shewanella frigidimarina* being 3%. The other major isolates were *Brevibacillus* sp., *Psychrobacter* sp. and *Pseudoalteromonas* sp. The majority of these bacterial species have been found to be involved in bacterial spoilage of foods. The presence of so many spoilage organisms indicates a high probability that the fish were starting to undergo bacterial spoilage by the time they were placed on sale in Perth. Goldband Snapper has a long cold chain to Perth as the fish may be caught up to 1 week prior to their arrival in Perth.



2.3.2 Major bacterial isolates from spoilt Goldband Snapper.

The major bacterial species isolated on spoilt retail purchased Goldband Snapper (Figure 2.3.2) was *Pseudomonas fluorescens* representing 27% of the major bacterial isolates. The second most commonly isolate species was *Shewanella baltica* representing 24% of the major isolates. Other major bacterial isolates included *Pseudoalteromonas* sp., *Flavobacterium* sp., other *Pseudomonas* species, *Psychrobacter*, *Arthrobacter*, and *Brochothrix thermosphacta*. From these results it would appear that the most likely causes of bacterial spoilage of Goldband Snapper are *Pseudomonas fluorescens*, *Shewanella baltica,* and *Pseudoalteromonas* sp. Each of these species increased in both total numbers, and proportion of the major bacterial species present on Goldband Snapper when compared to the fresh retail Goldband Snapper. The emergence of *Flavobacterium* species, including *F. frigidarium* and *Brochothrix thermosphacta,* as major bacterial isolates also indicates their importance in bacterial spoilage of Goldband Snapper.

Figure 2.3.3 summarises the effect that chlorine dioxide had on the major bacterial species isolated from spoilt Goldband Snapper. As was noted for Sea Mullet the use of chlorine dioxide in ice slurries prior to transport did not have a major effect on the proportion of major bacterial isolates present, but did see an overall reduction in total numbers. This indicates that the chlorine dioxide does not specifically target spoilage organisms, but has a bacteriostatic effect on the bacteria present on the Goldband Snapper thus causing a delay in spoilage, rather than eradication of spoilage organisms.

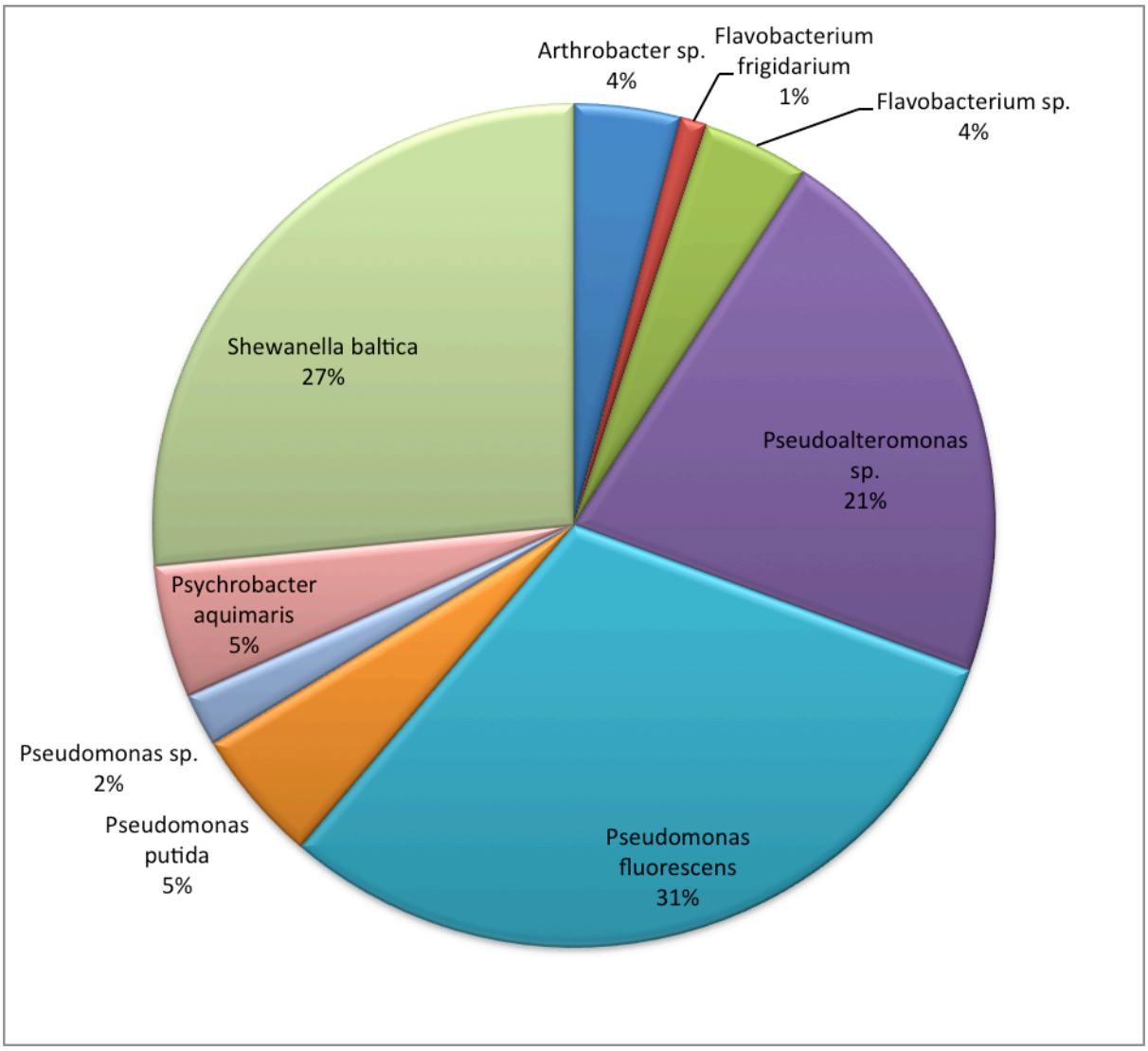


Figure 2.3.3 Major bacterial isolates on chlorine dioxide treated Goldband Snapper.

2.4 Summary of Important Findings

- Rate of bacterial growth was slower on Goldband Snapper than on Sea Mullet.
- The use of chlorine dioxide based sanitisers results in a marginal improvement in quality index score and bacterial levels. This marginal increase would possibly not be financially viable for Goldband Snapper, but may be of use for other tropical snappers with a shorter shelf-life.
- *Shewanella baltica, Pseudomonas fluorescens,* and *Pseudoalteromonas* species appear to have the greatest impact on spoilage of Goldband Snapper.
- Treatment of Sea Mullet with sanitisers results in an overall reduction in all bacterial levels and does not appear to target particular bacterial groups or species (as also seen in Sea Mullet treatment).
- The microbial population on Goldband Snapper appears more diverse than that found on Sea Mullet
- A PhD project funded by the CRC for Seafood Quality was commenced following preliminary results of this project. A thesis by Rachel Tonkin (Curtin University) has been submitted.

3. SPOILAGE OF OCTOPUS

3.1. Bacterial Spoilage of Octopus

Freshly caught and decapitated octopus was tested on site and then transferred to the laboratory in and esky with ice. The octopus was transferred to a freely draining plastic container and covered in ice. This container was then transferred to a refrigerator. The octopi were sampled and for aerobic mesophilic bacteria, Specific Spoilage Organisms, and psychrotrophic bacteria suing the Standard Plate Count, Iron Agar count and modified Long & Hammer count methods. Figure 3.1.1 details the results of these analysis. The microbiological growth patterns observed for the octopus differed quite significantly from those previously observed for the fin fish. Most significantly was the lack of growth of Specific Spoilage Organisms (SSOs) i.e. *Shewanella* species. Whilst low levels of *Shewanella baltica* were detected these levels are very unlikely to cause spoilage issues. Unlike Sea Mullet, there was not a problem with background micro-flora causing interference with the counts on Iron Agar and the SSO counts were not likely to be underestimates of the true numbers.

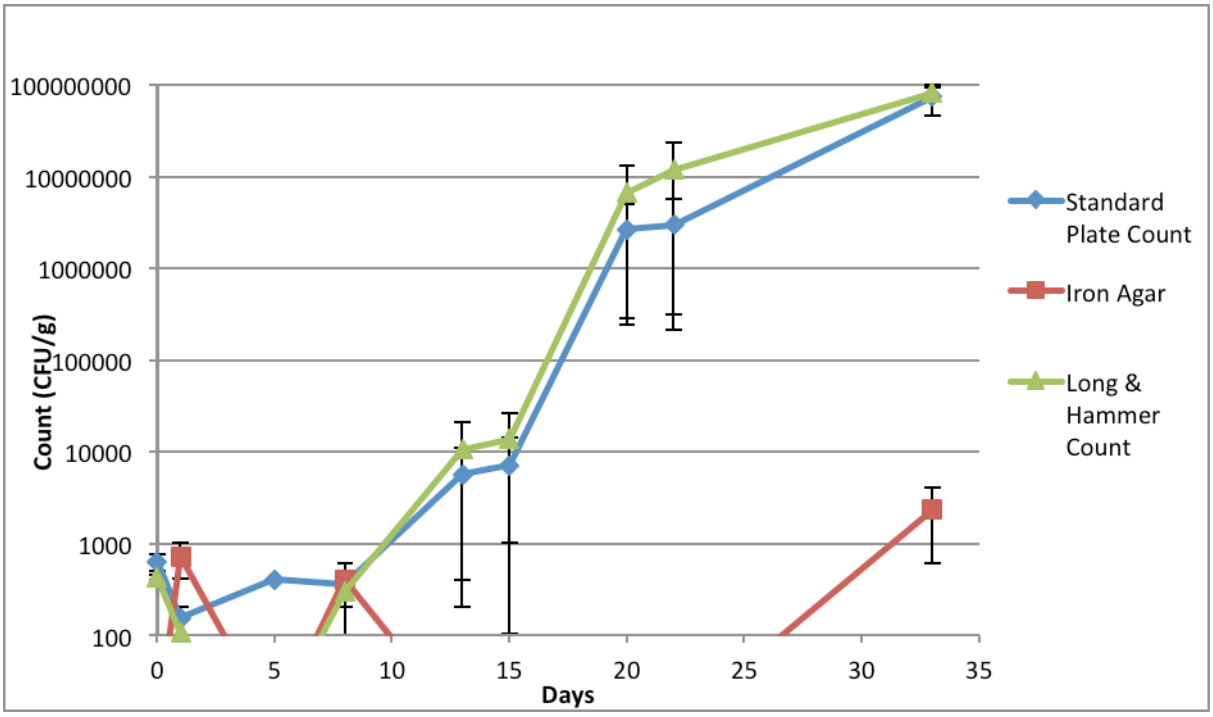


Figure 3.1.1 Growth of aerobic mesophilic bacteria (Standard Plate Count), Specific Spoilage Organisms (Iron Agar count) and psychrotrophic bacteria (modified Long & Hammer count) on refrigerated octopus held in ice.

The rate of bacterial growth was relatively slow up to around day 15. In all cases a dip in numbers of aerobic mesophilic, and aerobic psychrotrophic bacteria occurred between initial sampling following capture, and samples taken following 1 days storage in ice. On day 5 no bacteria were counted before detectable levels of bacteria were counted following 7 days of storage on ice. Investigations of the predominant bacterial isolates found that a large majority of the bacteria initially present were *Acinetobacter johnsonii* (see section 3 below). This species was not detected in the octopus after 1 days storage on ice.

A rapid increase in the number of aerobic mesophilic bacteria and aerobic psychrotrophic bacteria occurred between 15 days and 21 days of storage on ice. An increase of approximately 3 log (1000 times) occurred in both the Standard Plate Count and modified Long & Hammer counts, with a slowdown in this rate occurring between days 21 and 33, while the final Standard Plate Count and modified Long & Hammer counts were approximately $8x10^7$ CFU/g. No significant odours were detected, and only very small amounts of slime were noted.

Microbiological spoilage did not appear to play a major role in the spoilage of octopus. Storage on ice does appear to have a detrimental effect on the physical appearance of the octopus though. Significant damage appears to occur to the extremities of the tentacles around day 5 of storage on ice due to dehydration (see Figure 3.1.2). The appearance of the flesh of the octopus tends to significantly deteriorate between days 14 and 21 possibly due to proteolytic enzymes. By day 25, the skin on the tentacles had a loose gelatinous appearance (see Figure 3.1.3 below).

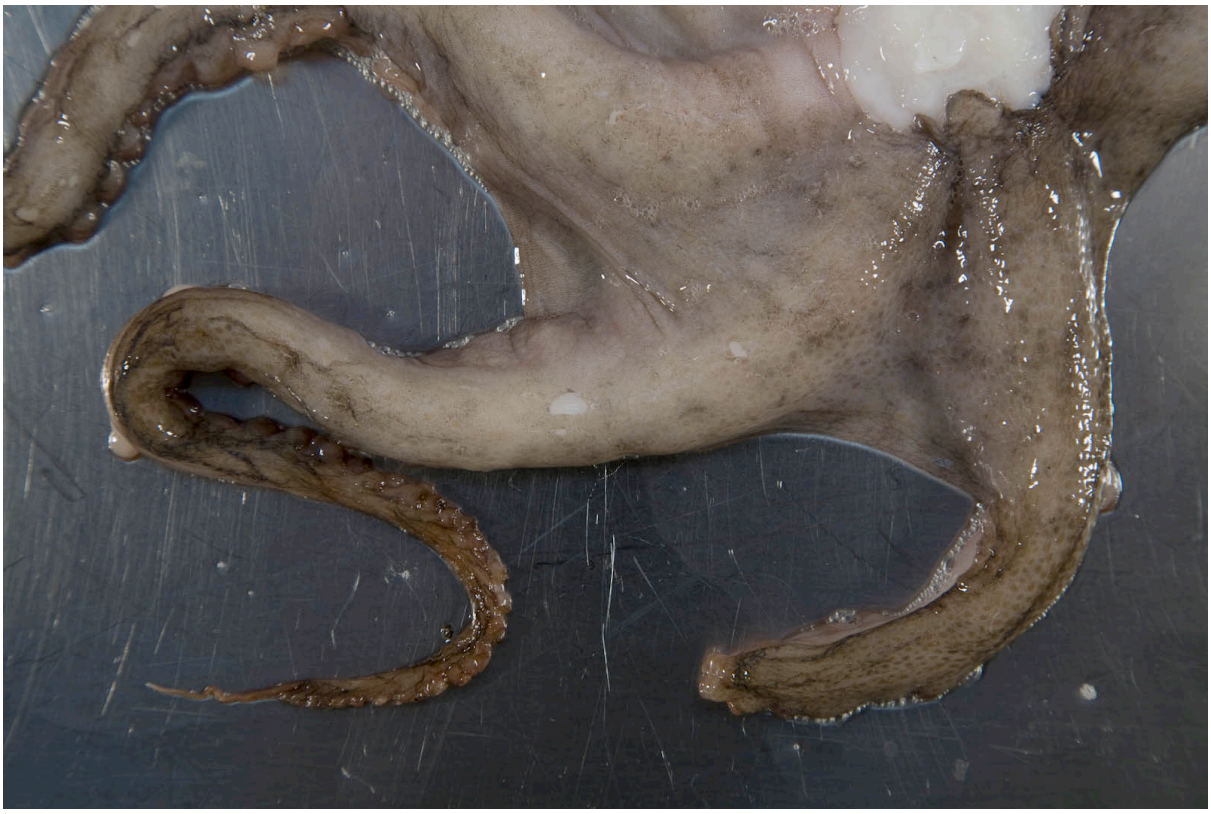


Figure 3.1.2 Example of the dehydration of the ends of tentacles occurring on after 5 days storage in ice.

Figure 3.1.3 Example of gelatinous blebs forming on tentacles following storage in ice for 21 days.

Experiments were performed to investigate the impact of bacteria on the formation of the gelatinous blebs octopus was treated with un-activated and activated chlorine dioxide for 2 hours prior to storage in refrigerated ice (Figure 3.1.4). This treatment resulted in a final Standard Plate Count and modified Long and Hammer count of around 10^4 CFU/g after 35 days. However, significant deterioration of the skin still occurred after 14 days in ice, with significant gelatinous bleb formation occurring between day 14 and day 21.

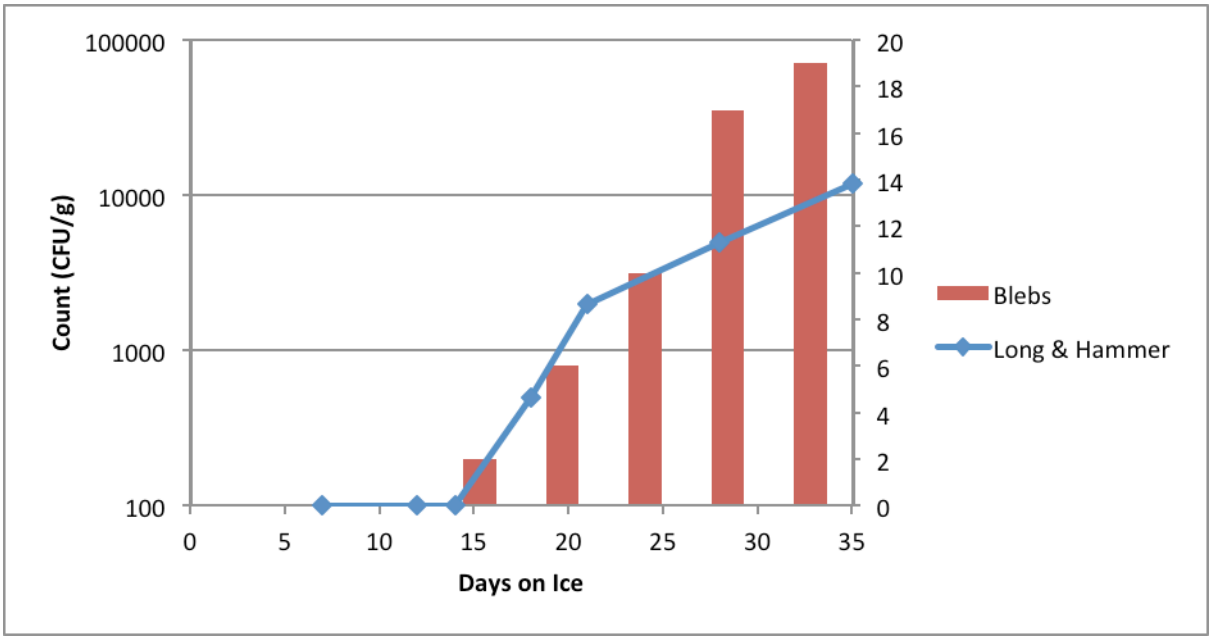


Figure 3.1.4 Effect of chlorine dioxide sanitiser treatment on bleb formation - comparison of modified Long & Hammer count and bleb formation.

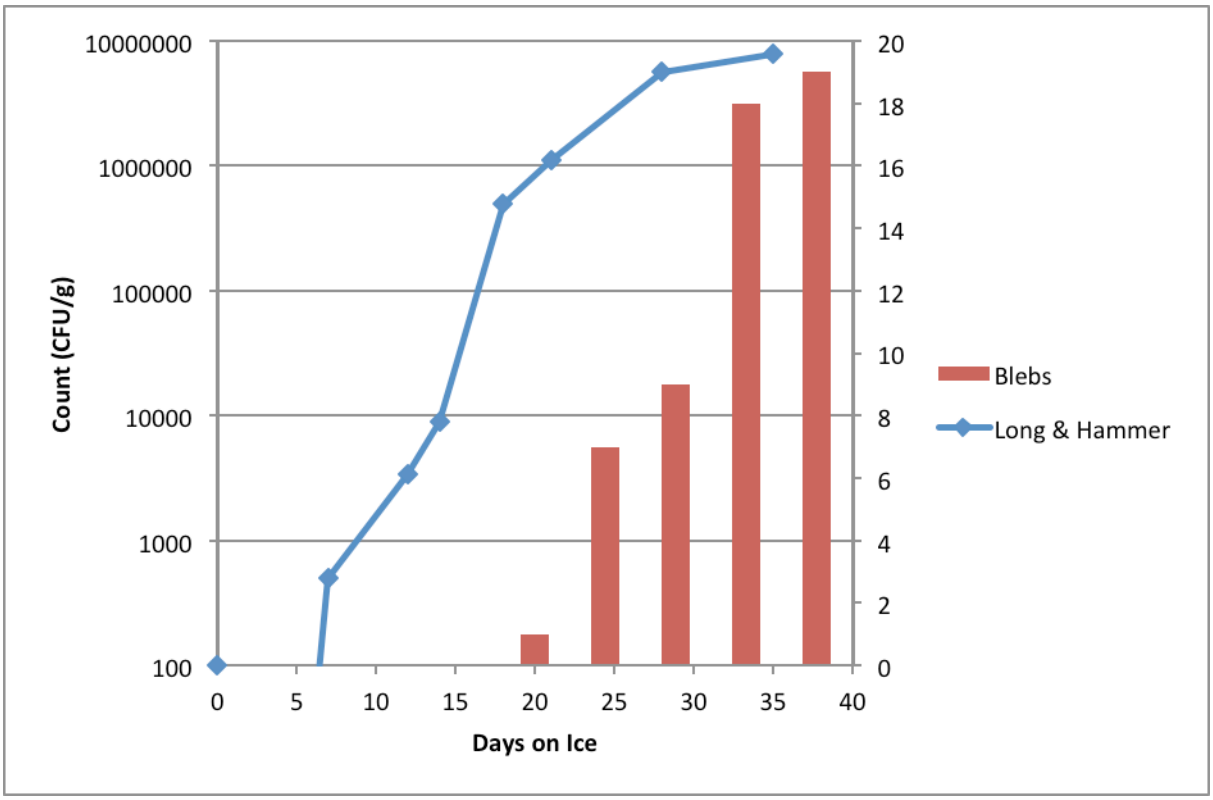


Figure 3.1.5 Modified Long & Hammer count and gelatinous bleb formation on untreated octopus.

No significant difference was observed in bleb formation and presence of large numbers of psychrotrophic and mesophilic bacteria. This indicates that the formation of gelatinous blebs is due to a physicochemical reaction occurring in the tentacles.

The use of an ice slurry held under refrigeration to store the octopi instead of ice under refrigeration was also investigated (see Figures 3.1.6 and 3.1.7). There was no impact on the bacterial counts with the counts on octopi held in an ice slurry virtually identical to the counts of octopi held in ice only. The use of an ice slurry did have a positive effect on dehydration of tentacle tips, with dehydration occurring on octopus held in ice, but not occurring in octopus held in an ice slurry. However there was a negative effect on the formation of gelatinous blebs (see Figure 3.1.7). Bleb formation occurred earlier in storage, and with greater frequency.

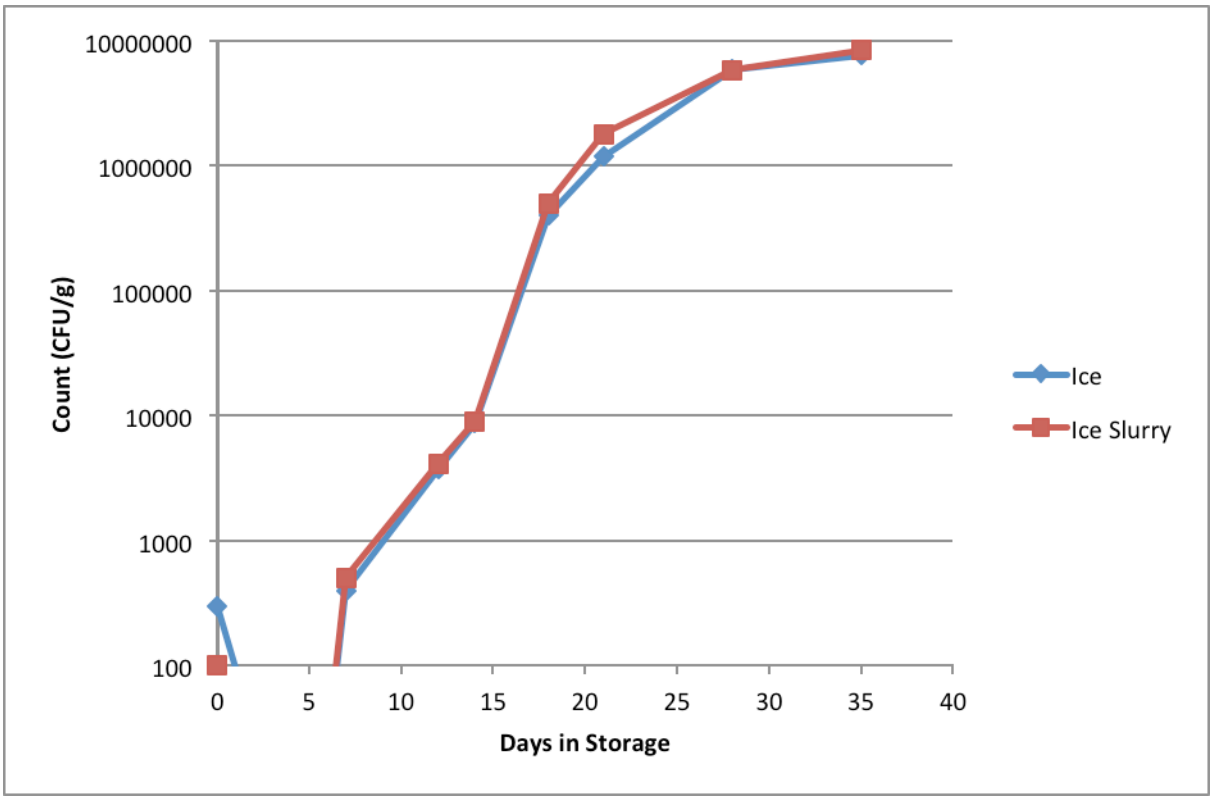


Figure 3.1.6 Effect of storage conditions on modified Long & Hammer count for octopus.

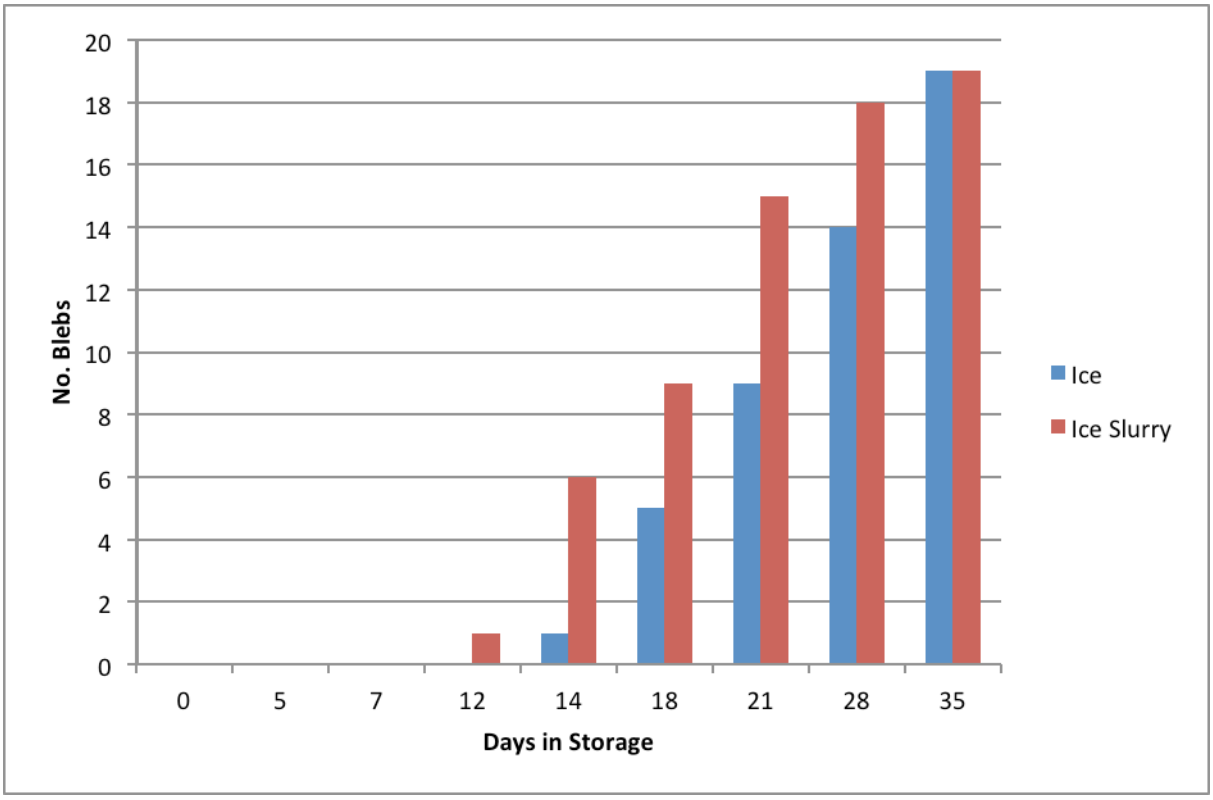


Figure 3.1.7 Effect of storage conditions on gelatinous bleb formation for octopus.

3.2. Bacteria isolated from Octopus

The major colony types from each agar plate (i.e. Plate Count Agar, Iron Agar & modified Long & Hammer Agar) were selected and tested as described in the methods section (up to 10 colonies per plate). Figure 3.2.1 shows the proportion of major genera and species present on fresh decapitated octopus. The figure represents those bacterial species which were present on octopi on at least two of the three experiments.

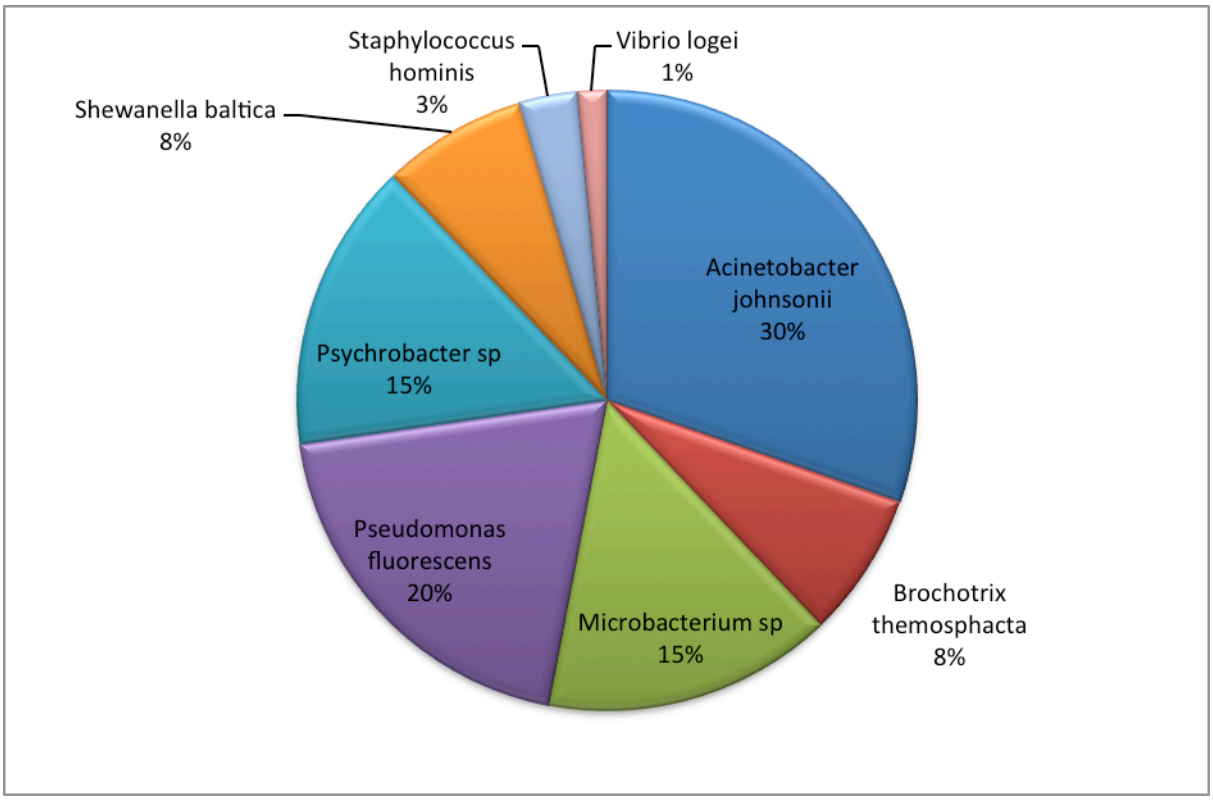


Figure 3.2.1 Major bacterial isolates on fresh octopus.

Acinetobacter johnsonii was the most common bacterial isolate on fresh octopus representing 30% of all isolates. Twenty percent of isolates were *Pseudomonas fluorescens*. With *Psychrobacter* sp. and *Microbacterium* sp. the next most common isolates making 15% of all major isolates each. *Brochothrix thermosphacta* and *Shewanella baltica* represented 8% of the major isolates. *Staphylococcus hominis* and the bioluminescent *Vibrio logei* made up the remainder of the isolates.

After 35 days the major bacteria isolates were predominantly *Pseudomonas* species (Figure 3.2.2). Fifty four percent of all isolates were *Pseudomonas fluorescens*. The next most

commonly isolated species was *Pseudomonas putida* representing a further 27% of isolates, while other *Pseudomonas* species made up 8% of the major isolates. *Brochotrix thermosphacta* (6%) and *Shewanella baltica* (5%) were the remaining major isolates. While all of these species are commonly associated with food spoilage their impact on the spoilage of octopus is doubtful, as there was no evidence of bacterial spoilage such as significant slime formation and off odours. The Standard Plate Count and modified Long and Hammer counts were always very similar. The fact that the major organisms which were growing on chilled octopus were *Pseudomonas* species and most predominantly *Pseudomonas fluorescens* would account for this as they will readily grow on both Plate Count agar and modified Long & Hammer agar.

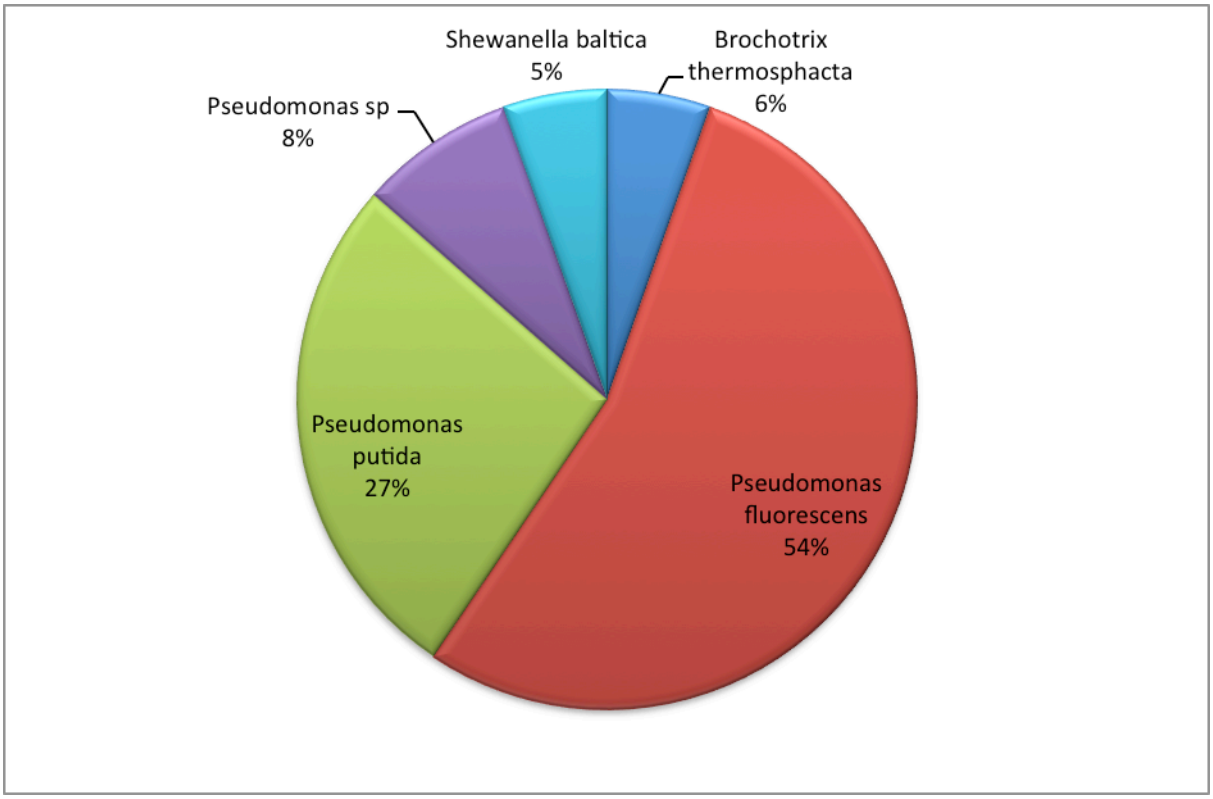


Figure 3.2.2 Bacterial isolates on octopus held in refrigerated ice for 35 days.

3.3 Summary of Important Findings

- Microbiological growth does not appear to have a significant impact on the rate of spoilage of octopus held in ice under refrigeration.
- Rapid growth of aerobic mesophilic and aerobic psychrotrophic bacteria occurs between 15 and 21 days of storage.
- The growth of "Specific Spoilage Organisms" (*Shewanella*) on octopus was insignificant.
- Dehydration of the tips of the tentacles and the formation of gelatinous blebs impact on the quality of octopus stored in drained ice under refrigeration.
- Storage of octopus in undrained ice slurries under refrigeration eradicated tip dehydration but worsened gelatinous bleb formation.
- Gelatinous bleb formation was not influenced by the levels of bacteria present.

4. SPOILAGE OF BLUE MUSSELS

4.1 Microbiological Spoilage of Blue Mussels

Freshly harvested Blue Mussels were transported to the laboratory in an esky with ice bricks. The mussels were transferred to a plastic container, placed into a refrigerator, and covered with a damp tea towel. Samples were tested for microbiological quality on the day of harvest (day 0) and then after 3, 5, and 9 days storage. Only mussels with closed shells (live mussels) were tested for microbiological quality. Figure 4.1.1 summarises the microbiological quality of refrigerated Blue Mussels.

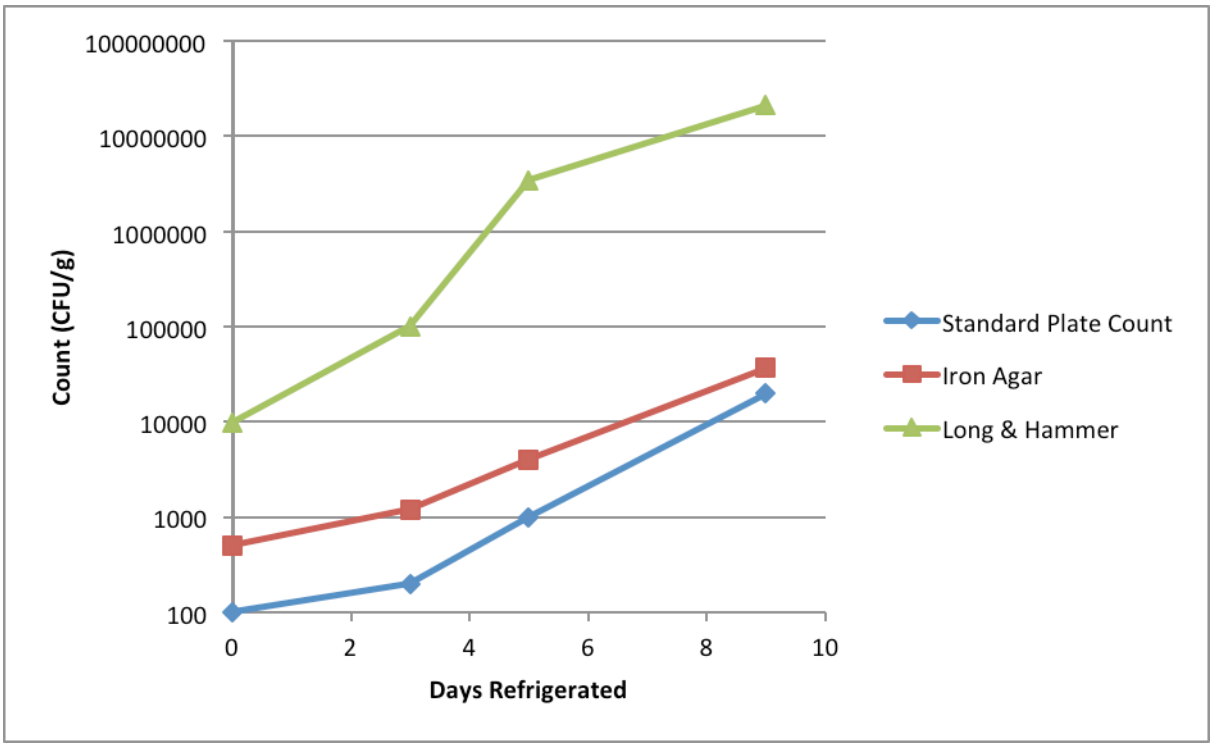


Figure 4.1.1 Growth of mesophilic, psychrotrophic, and specific spoilage organisms in Blue Mussels.

The initial concentration of aerobic mesophilic bacteria, as measured by the Standard Plate Count (SPC) was a very low 100 CFU/g on day of harvest. The SPC grew very slowly to 20,000 CFU/g by day 9. The growth of *Shewanella* on Iron Agar showed a very similar growth pattern to that of the mesophilic bacteria. Initial levels were approximately 500 CFU/g rising to 37,000 CFU/g. Significantly, the levels of psychrotrophic bacteria, as measured by the modified Long & Hammer count, was significantly higher than those of both the SPC and Iron Agar. Initial counts were 9,900 CFU/g rising to 21,000,000 CFU/g. A

significant off odour was detected on day 5 of storage. This odour increased significantly from this time to the final sampling on day 9. From the data in Figure 4.1.1 it would appear that the odour is most likely due to the growth of psychrotrophic organisms as determined by the modified Long & Hammer count. On day 5 both the SPC and Iron Agar counts were below 10,000 CFU/g. At these levels the microorganisms growing on these plates are highly unlikely to cause off odours. However, the modified Long & Hammer counts were 3,400,000 CFU/g, while this count was high it is unlikely to account for the intensity of the off odour. On day 9 a pooling of the shell liquor (water withheld in the mussel post-harvest) in the plastic container was noted and decanted off. The liquor had an intense off odour. Microbiological tests were performed on the liquor and is summarised in Table 4.1.1 below. These counts were significantly higher than those found in the Blue Mussels tested on the same day.

Table 4.1.1 Microbiological counts of mussel meat and collected shell liquor after storing blue mussels for 9 days in refrigeration.

	Standard Plate Count (CFU/g)	**Iron Agar Count (CFU/g)**	**modified Long & Hammer Count (CFU/g)**
Mussels	20,000	37,000	21,000,000
Collected Liquor	5,500,000	2,300,000	140,000,000

A series of experiments was performed to compare the levels of bacteria in the pooled expressed liquor (i.e. the water released by the mussels into the holding container) and the levels of bacteria present in mussel meat summarised in Figures 4.1.2 to 4.1.4. The counts for all tested bacterial groups were similar to that seen in Figure 4.1.1 above. The growth of aerobic mesophilic bacteria and SSOs was relatively low, with a total increase of only 2 log (100 times) from low starting points. Once again the levels of aerobic psychrotrophic bacteria were over 10 times higher on the day of harvest. They grew very rapidly between harvest and 5 days refrigerated storage and after 9 days storage had approximately 3 log (10,000 times) to a final average level of 9,400,000 CFU/g.

In comparison the pooled expressed liquor generally showed higher levels of bacteria by 9 days of mussel storage (Figures 4.1.3. and 4.1.4). On the day of harvest the liquor had

similar levels of all tested bacterial groups. As observed in the analysis of the mussel meat, by the end of the storage period the levels of aerobic mesophilic bacteria and "specific spoilage organisms" were similar to each other, but significantly less than the levels of psychrotrophic bacteria detected. In fact this difference was approximately 2 log (100 times).

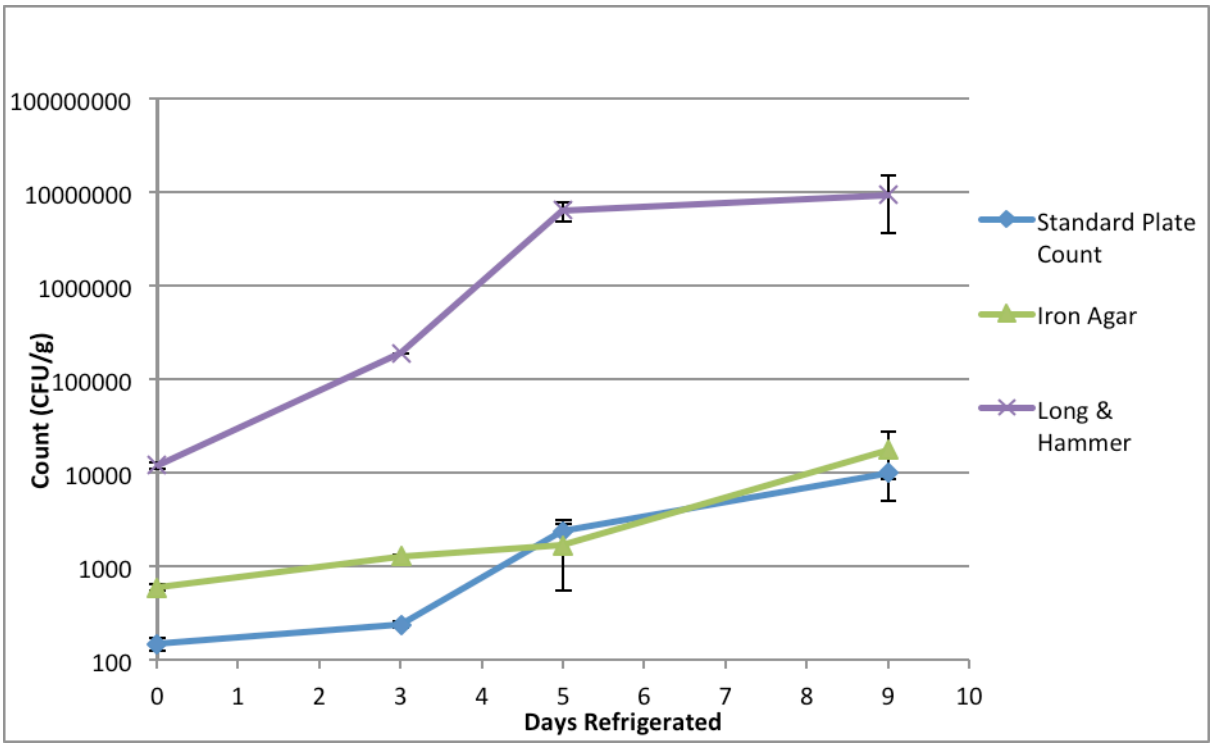


Figure 4.1.2 Levels of aerobic mesophilic bacteria (Standard Plate Count), "Specific Spoilage Organisms" (Iron Agar Count), and aerobic psychrotrophic bacteria (Long & Hammer Count) in refrigerated Blue Mussel meat.

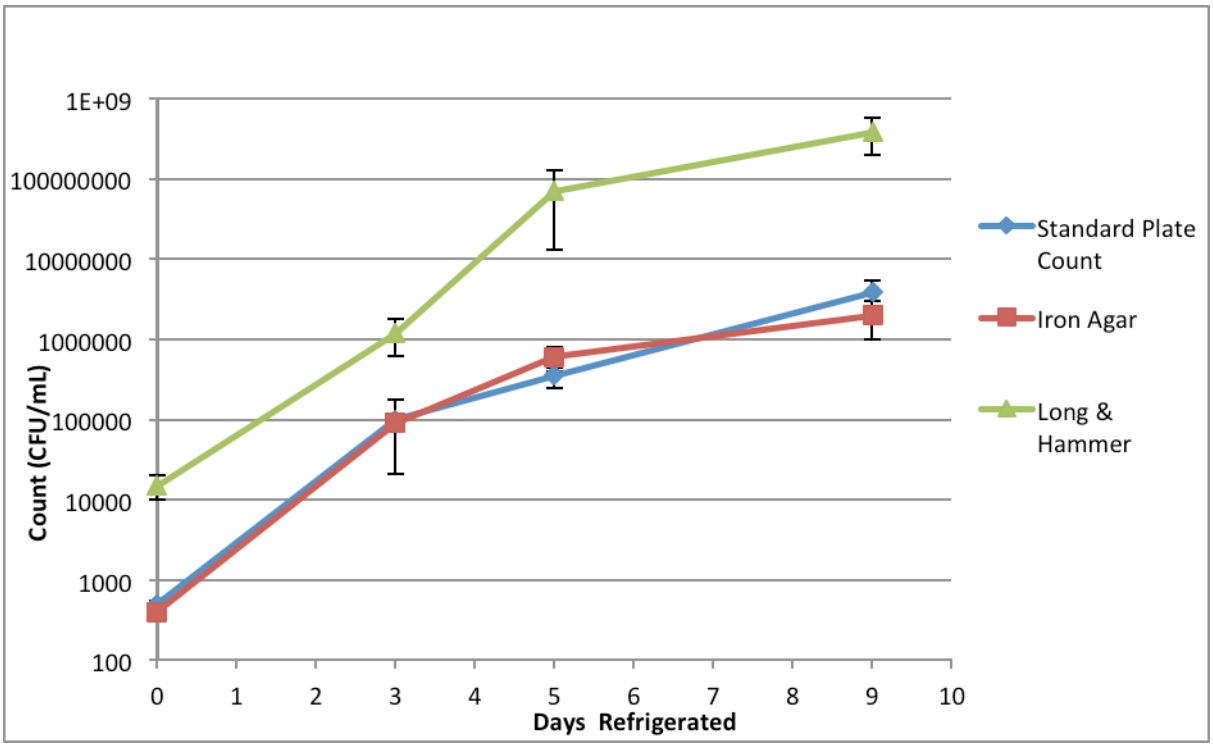


Figure 4.1.3 Levels of aerobic mesophilic bacteria (Standard Plate Count), "Specific Spoilage Organisms" (Iron Agar Count), and aerobic psychrotrophic bacteria (Long & Hammer Count) in refrigerated Blue Mussel "Pooled Expressed Liquor".

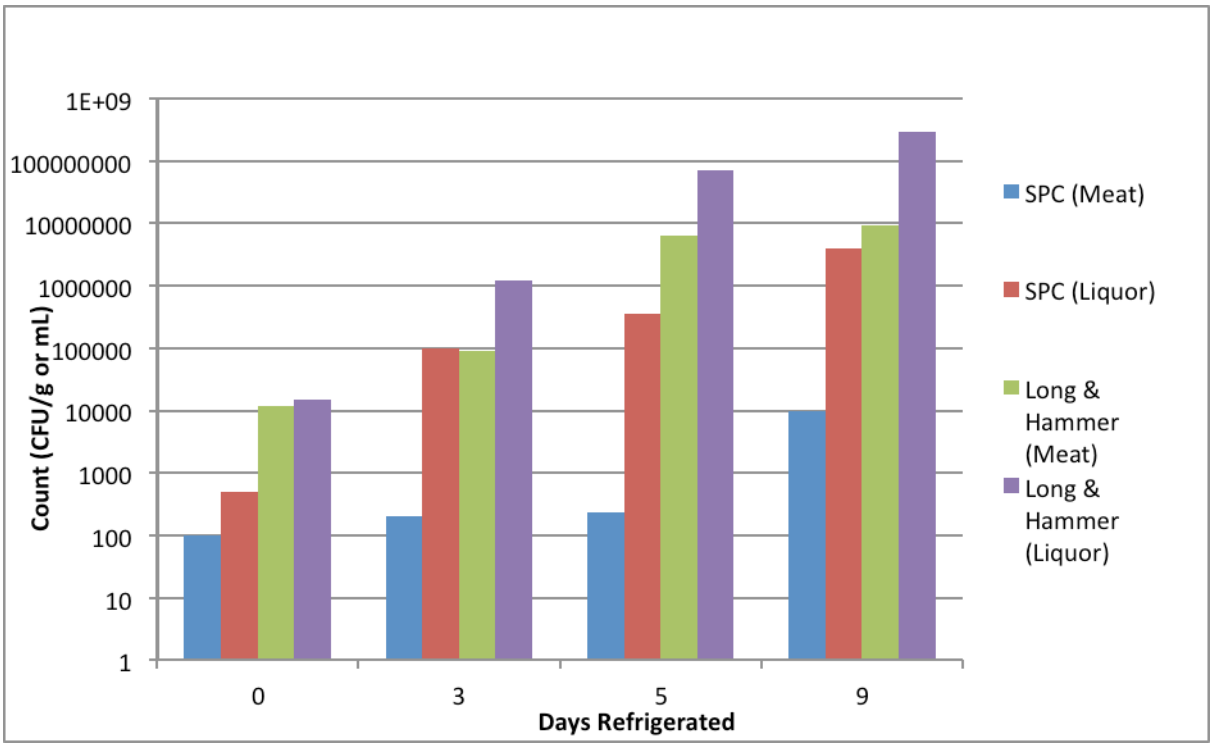


Figure 4.1.4 Comparison of Standard plate count and modified Long & Hammer Count in refrigerated Blue Mussel meat and pooled mussel liquor.

While the pooled expressed liquor, SPC and Iron agar counts showed some similarity to those seen in meat, the overall levels for both of these tests were over 3 log (1000 times) higher than those found in the mussel meat. Similarly, the counts of aerobic psychrotrophic bacteria showed similar differences between the mussel meat counts and counts in the pooled expressed liquor (Figure 4.1.4). The initial levels of psychrotrophs were similar in both the meat and liquor, however, following 9 days storage in refrigeration there was a difference of approximately 1.5 log between the levels present in the mussel meat and liquor. Off odours were consistently detected around 5 days of storage in refrigeration.

The effect of draining the expressed liquor was investigated. The mussels were placed on a raised metal grate in a plastic container with a drain and covered with a damp towel. Liquor was collected and removed from the refrigerator each day. A second set of mussels were held under the same conditions as previously described. Figure 4.1.5 shows a comparison of the bacterial counts of the mussel meat for mussels which were held in draining containers and non-draining containers (stored in liquor).

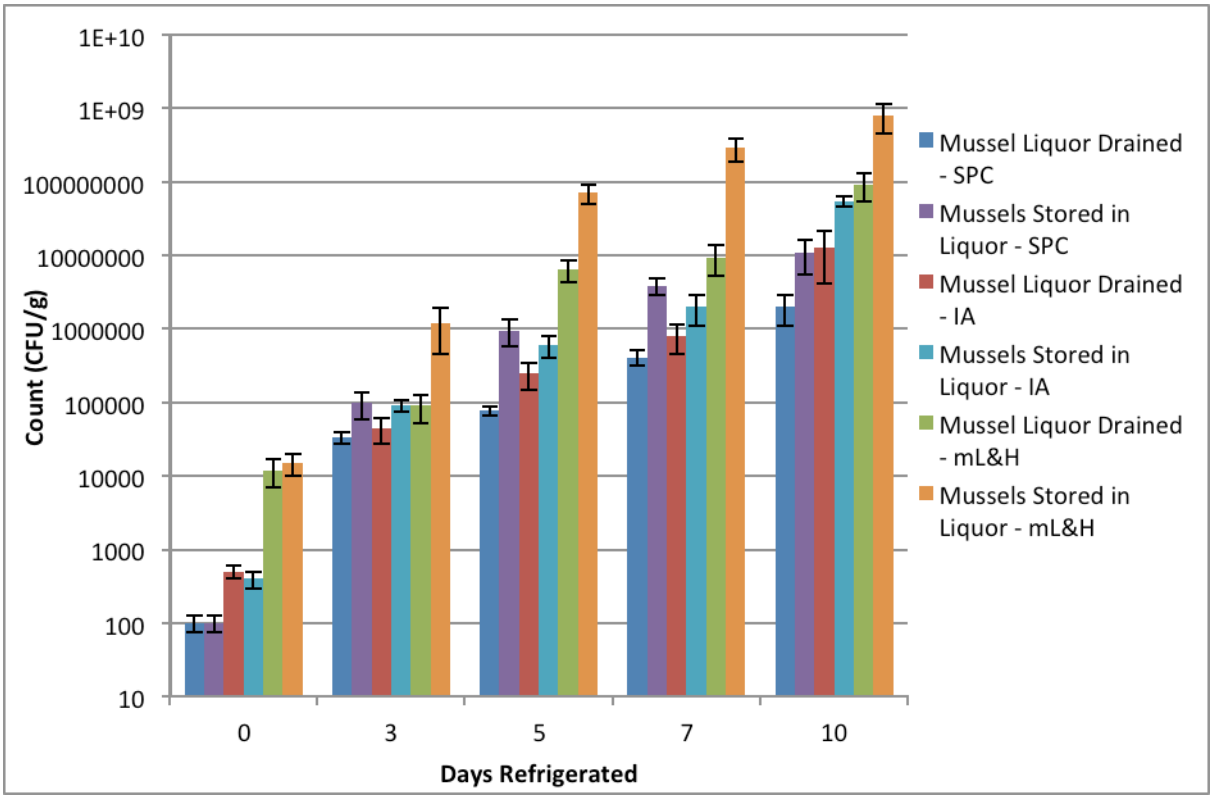


Figure 4.1.5 Comparison of microbiological counts of mussel meat of mussels held in drained plastic containers and non-drained containers (stored in liquor).

Refrigeration of mussels in well drained containers had a significant impact on both the microbiological quality of the mussel meat and the onset of off odours. Off odours were not detectable until 7 days of refrigeration on mussels held in freely draining conditions compared to day 5 in non-draining conditions. There was also a significant reduction in levels of aerobic mesophilic bacteria, aerobic psychrotrophic bacteria, and specific spoilage organisms in the meat of mussels stored in freely draining conditions compared to those held in non-draining conditions. One detrimental effect noted was an increase of about 25% in the number of dead mussels when compared to holding the mussels in non-draining conditions.

4.2. Development of a simple quality assessment test

A simple to perform test to assess the potential microbiological quality of a mussel was assessed. The Quality Index method was developed as a simple means to assess the freshness of fin fish (Bremner 1985). Unfortunately, there are very few external morphological features which may be assessed under a QI scheme. As mussels are held alive, a process to assess the physiological status of the stored mussels was looked for. The float test was investigated to see if there was a correlation between the number of floating mussels and the microbiological quality of mussels.

The float test was performed by selecting 20 mussels which were not showing obvious signs of death (i.e. the mussel shells were closed). The mussels are placed into a container of water and the number of mussels which were floating at certain periods of time were noted. The percentage of floating mussels was calculated. In the initial tests, mussels which were store for up to 9 days were assessed. Figure 4.2.1 summarises the percentage of mussels floating at a 1 min and the effect days of storage in refrigeration had on this result.

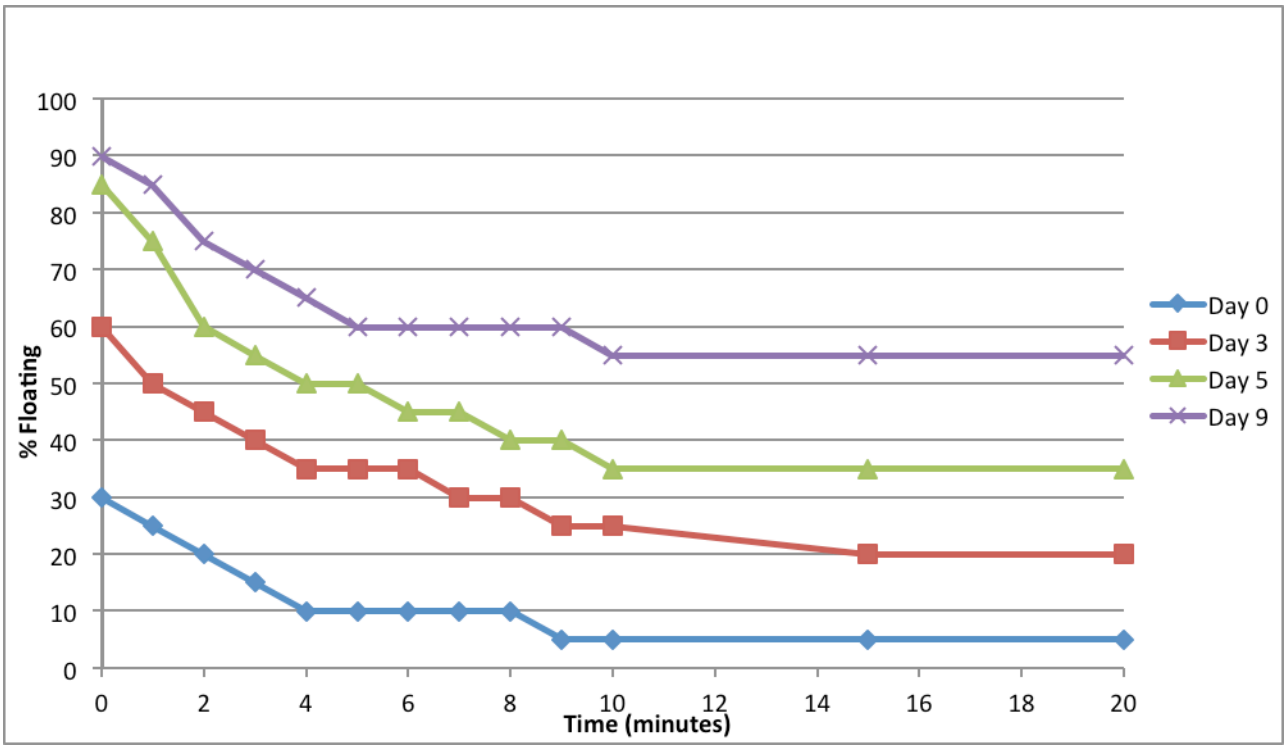


Figure 4.2.1 Impact of days stored in refrigeration on the percentage of mussels floating over time.

The percentage of mussels floating decreased rapidly from the start of the test to 2 min of immersion in water. From 2 min immersion there was a slow reduction in the number of floating mussels up to 10 min for each storage time. From 10 min only mussels refrigerated for 3 days had a reduction in numbers floating. From this data it was determined that assessing the number of mussels floating after 10 min was the time which was most discriminating. All further tests were performed by calculating the percentage of mussels floating after 10 min.

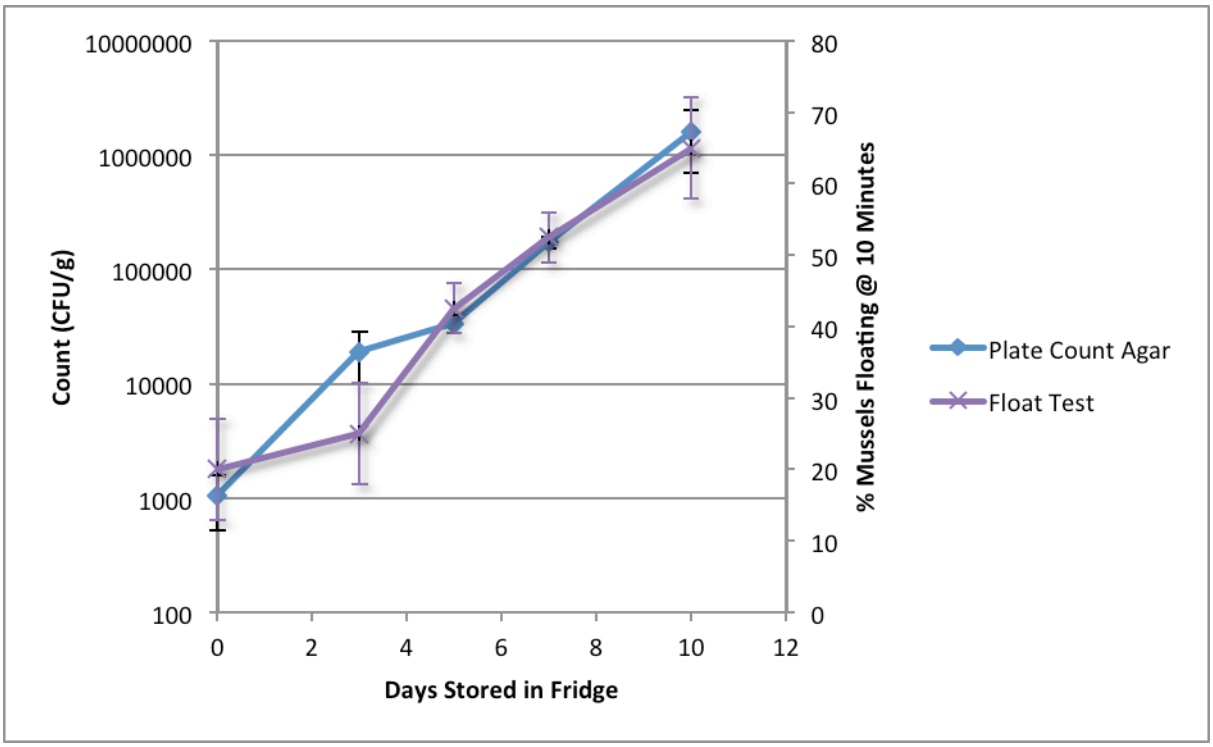


Figure 4.2.2 Comparison of float test and Standard Plate Count (aerobic mesophilic bacteria) for mussels.

A comparison of the average bacterial counts and the average float test (percentage of mussels floating at 10 min) showed very similar curves when plotted against the days stored in refrigeration (Figures 4.2.2 to 4.2.4). This was maintained for all of the bacterial groups tested (i.e. aerobic mesophilic bacteria, aerobic psychrotrophic bacteria, and specific spoilage organisms). Figures 4.2.5 to 4.2.7 show the correlation between the float test and bacterial counts. The average float test results were highly correlated with the bacterial counts with R^2 = 0.9585 for Standard Plate Count, R^2 = 0.953 for Iron Agar Count, and R^2 = 0.9612 for modified Long and Hammer Count. The float test proved to be a good means of estimating the quality (freshness) of Blue Mussels.

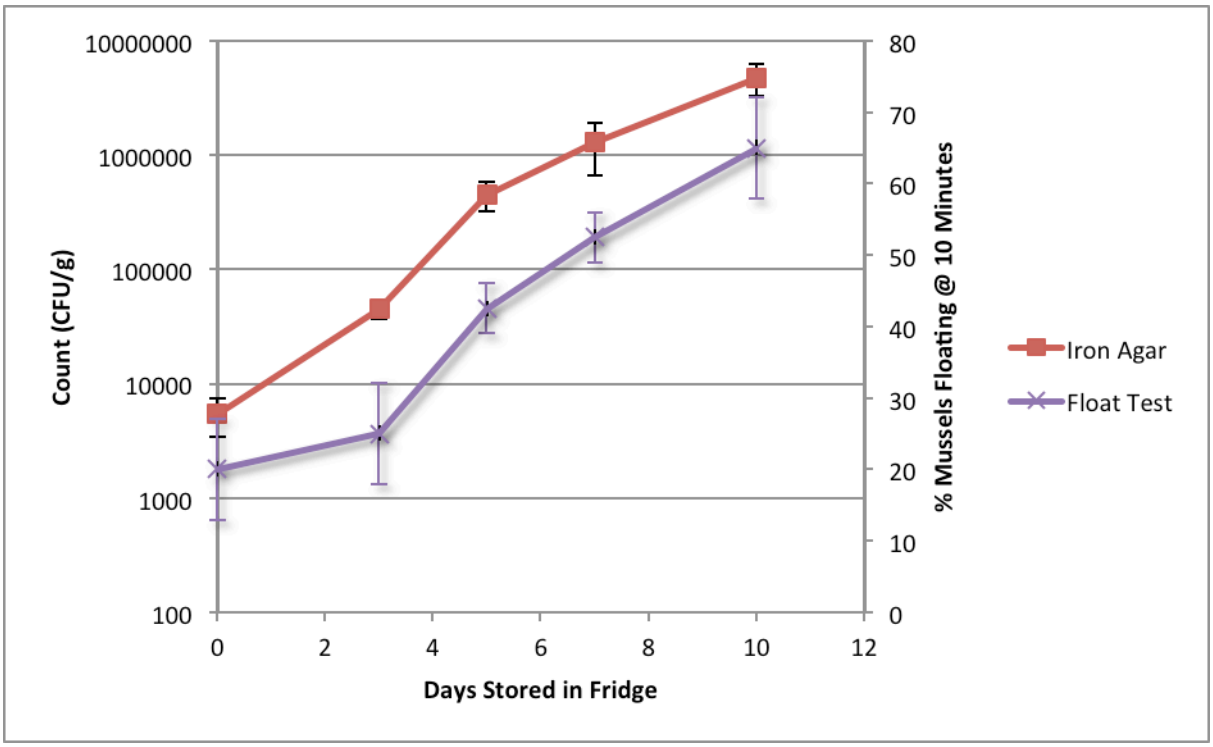


Figure 4.2.3 Comparison of float test and Iron Agar Count ("Specific Spoilage Organisms) for Blue Mussels.

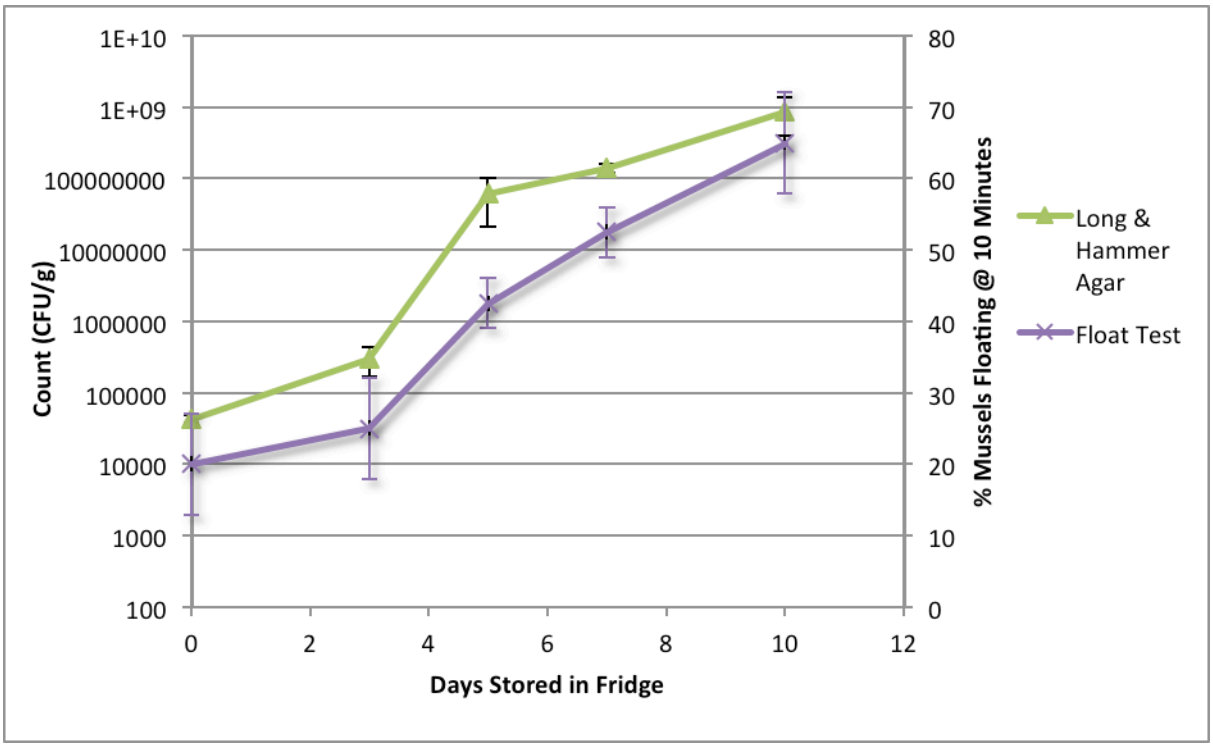


Figure 4.2.4 Comparison of float test and modified Long & Hammer Count (aerobic psychrotrophic bacteria) for Blue Mussels.

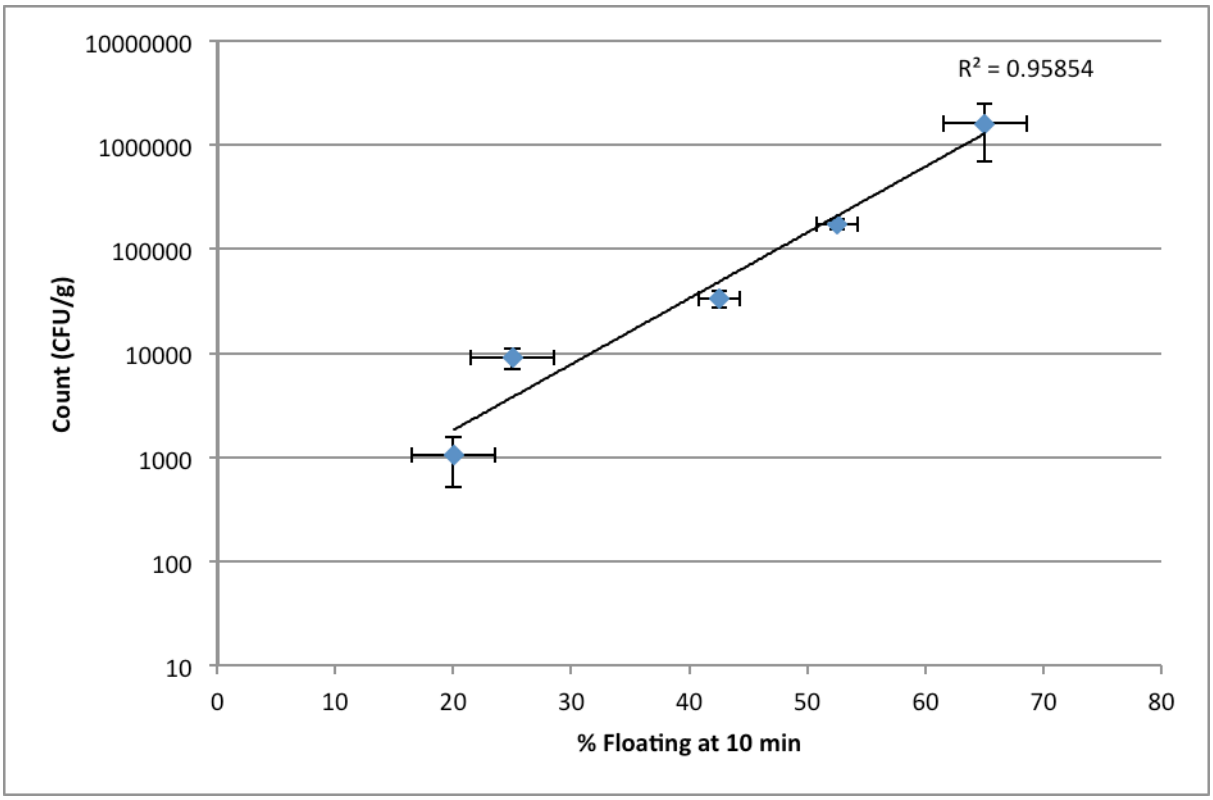


Figure 4.2.5 Correlation between aerobic mesophilic bacteria (Standard Plate Count) and float test for Blue Mussels.

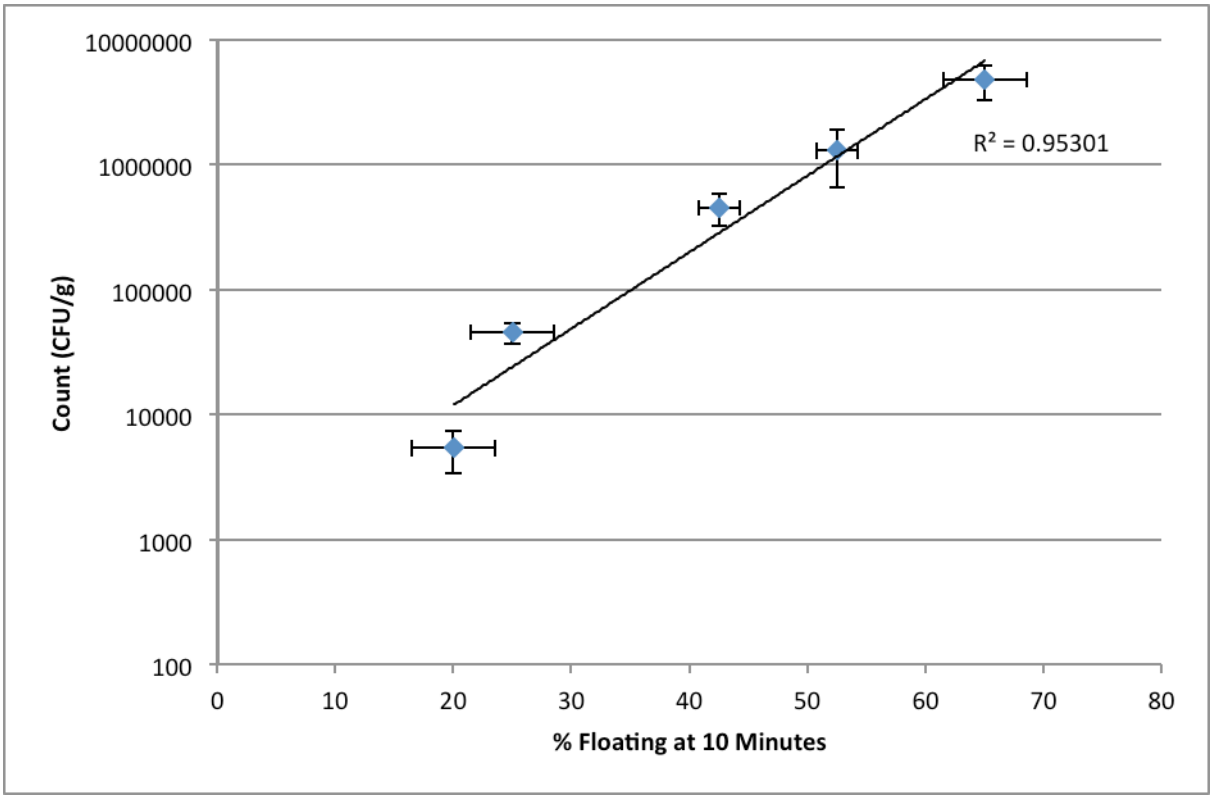


Figure 4.2.6 Correlation between specific spoilage organisms (Iron Agar Count) and float test for Blue Mussels.

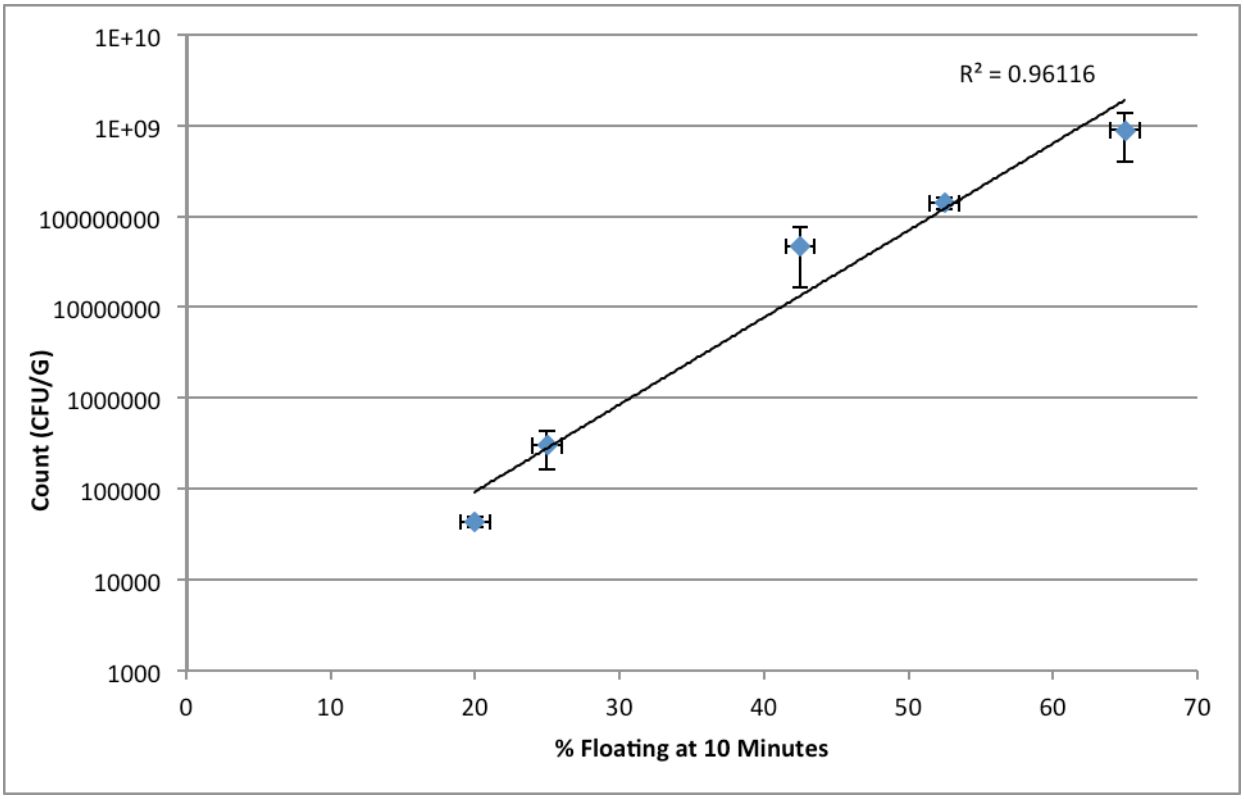


Figure 4.2.7 Correlation between aerobic psychrotrophic bacteria (Standard Plate Count) and float test for Blue Mussels.

4.3. Bacteria isolated from Blue Mussels

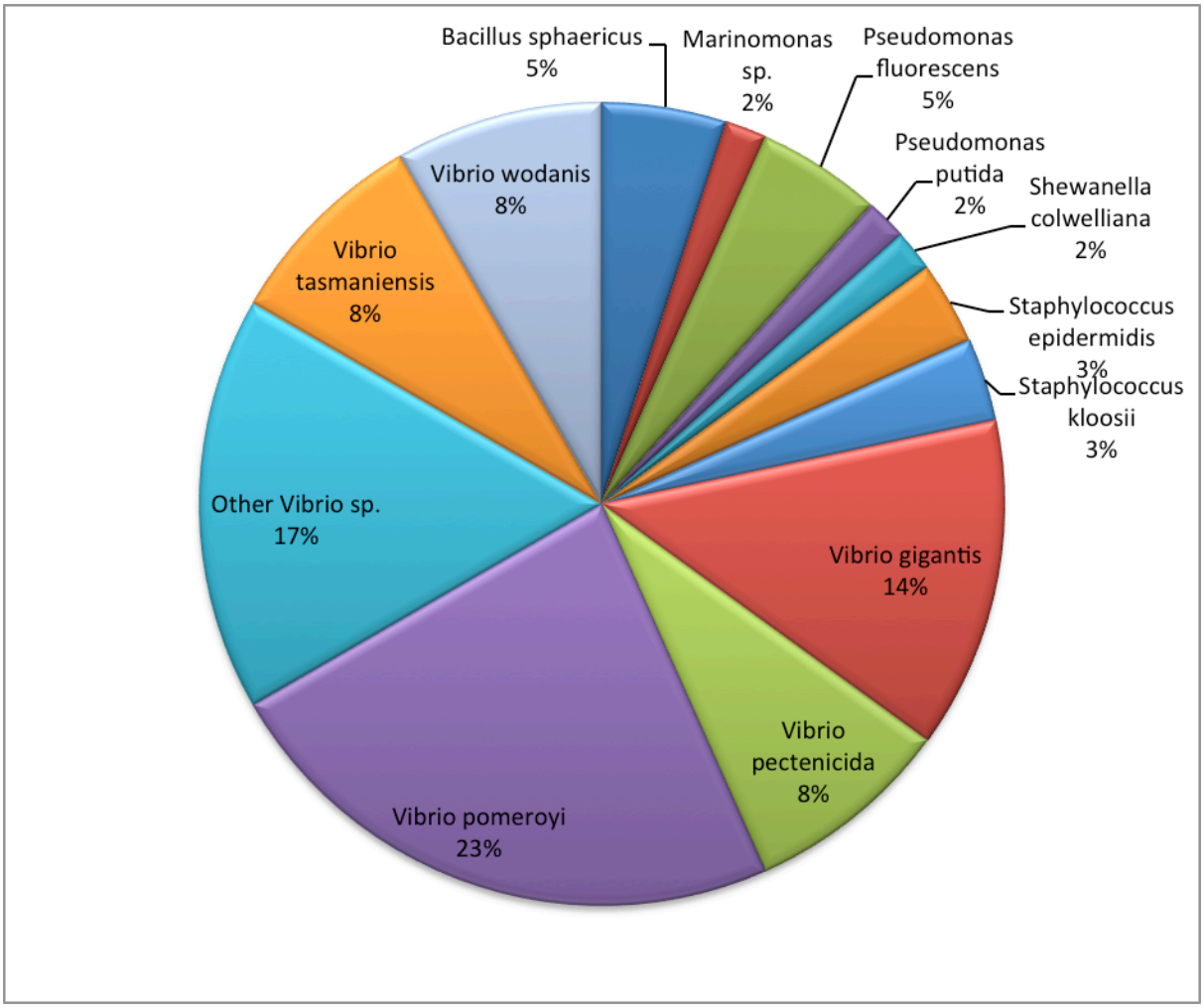


Figure 4.3.1 Major bacterial isolates from freshly harvested Blue Mussels.

The most commonly isolated bacteria in freshly harvested blue mussel meat (Figure 4.3.1) were *Vibrio* species representing 78% of the major isolates. *Vibrio* are commonly found in sea water, and as Blue Mussels are filter feeders it is not unusual for find them present in mussel meat. At no stage were pathogenic species of *Vibrio,* such as *Vibrio parahaemolyticus,* detected in any of the samples. *Pseudomonas* species were the next most commonly isolated bacterial species representing 7% of all major isolates. Non-pathogenic staphylococci represented 6% of the major isolates, followed by the spore-former *Bacillus sphaericus* (3%), *Marinomonas* (2%) and *Shewanella colwelliana* (2%).

The major bacterial isolates on Blue Mussel meat held under refrigeration for 10 days shows a very significant shift in the bacterial population (Figure 4.3.2). Where *Vibrio* species represented 78% of the major isolates in freshly harvested mussels, after 10 days refrigeration

Vibrio only represented 34% of isolates. *Pseudoalteromonas* sp. and *Psychrobacter nivimaris* represented 20% each of the major isolates and were both the most commonly isolated major isolate after 10 days storage. *Pseudomonas fluorescens* was the next most common isolate representing 17% of isolates. The last major isolate was *Shewanella colwelliana* making up 9% of the isolates.

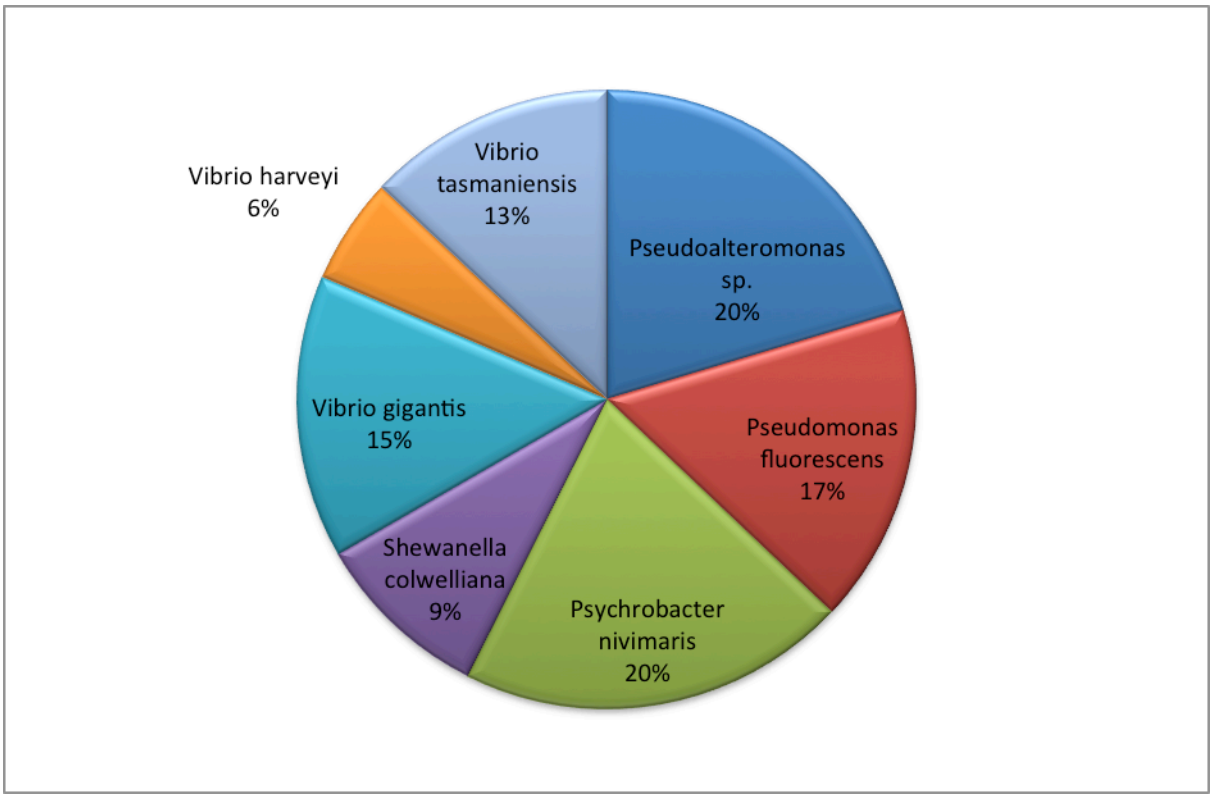


Figure 4.3.2 Major bacterial isolates from Blue Mussels refrigerated for 10 days.

Investigation of the major bacterial isolates in the liquor expressed by Blue Mussels found that the liquor after 10 days refrigerated storage had a very different make up than that found in the live mussel meat after 10 days storage (Figure 4.3.3). *Pseudoalteromonas* species were made up approximately one third of all major isolates in 10 day old liquor. This is 13% of the total isolates more than that found in the mussel meat. The other significant increase in the proportion of isolates present is found in the levels of *Shewanella colwelliana* which represents 22% of all major isolates in liquor, but is 9% of the isolates in the corresponding mussel meat. The number of *Vibrio* species present in the liquor was significantly lower than that found in the corresponding mussel meat. *Vibrio* species represented 17% of the isolates in the expressed liquor, half of the levels found in the mussel meat (34%). *Marinomonas* made up 5% of the isolates in the expressed liquor even though it was not found to be a major isolate in the corresponding meat samples.

The presence of *Shewanella colwelliana* in mussel meat is of interest as it has been found that biofilms of this organism are involved in the set of larva of oysters in the United States. (Weiner, Walch et al. 1989; Labare and Weiner 1990).

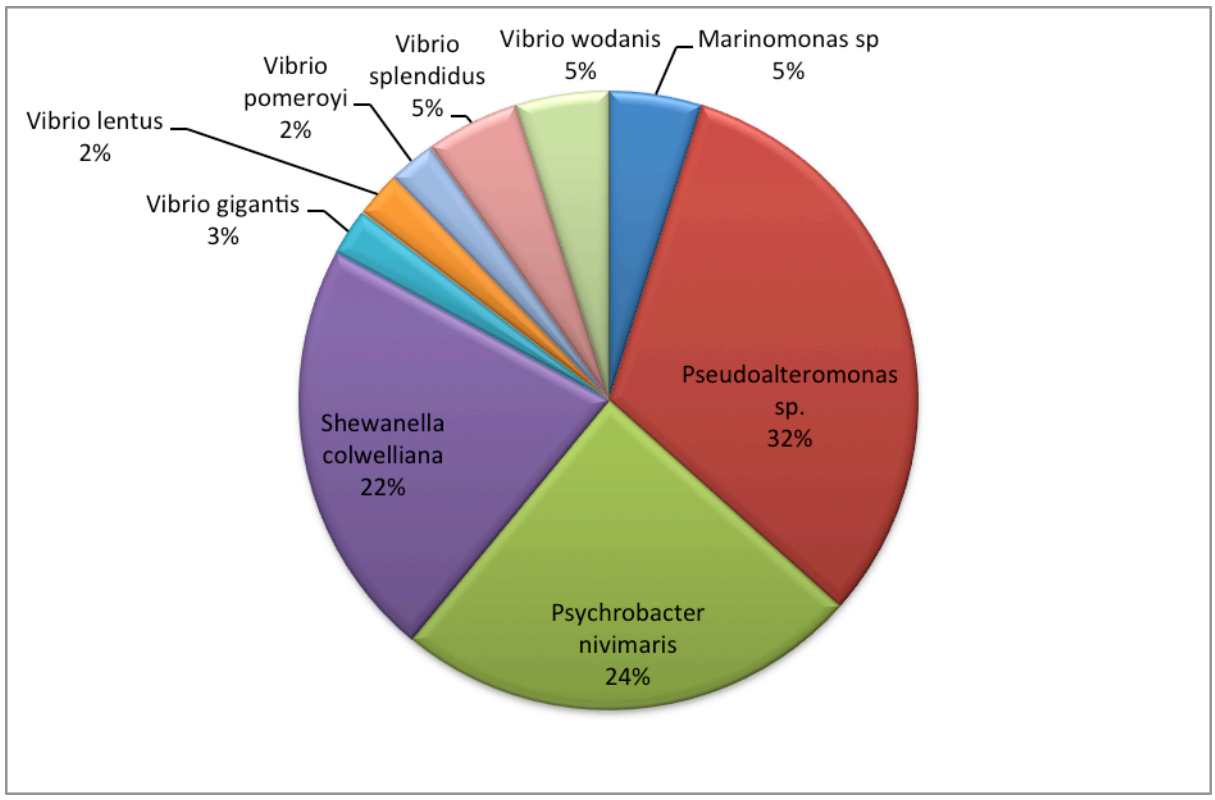


Figure 4.3.3 Major bacterial isolates from pooled expressed mussel liquor after 10 days storage in refrigeration.

4.4 Extension of Blue Mussel Shelf-Life by Vacuum Packaging.

An attempt to extend the shelf-life of Blue Mussels was made by vacuum packaging the mussels. The mussels were packaged by a mussel producer and transported to the laboratory in an esky with ice bricks. The packaged mussels were held in a refrigerator for up to 13 days. Separate packages were opened on each day of testing and tested for Standard Plate Count, Iron Agar Count, modified Long & Hammer count, plus anaerobic Standard Plate Count (anaerobic mesophilic bacteria), and anaerobic Long & Hammer count. Results are summarised in Figures 4.4.1 and 4.4.2.

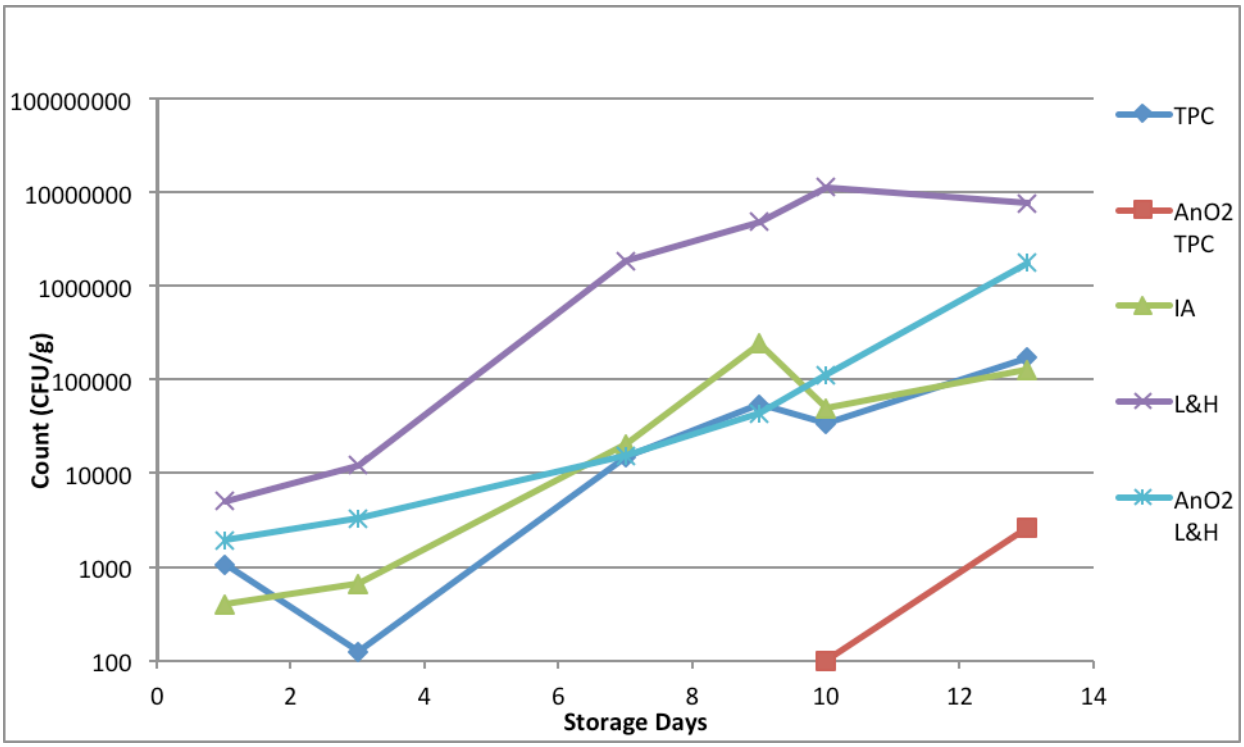


Figure 4.4.1 Bacterial quality of vacuum packed Blue Mussels stored in refrigeration.

As seen previously, the levels of bacteria in mussel meat were much lower than that of the liquor. Off odours were detected after 7 days of storage. This odour was associated with the expressed liquor pooling in the package. The producer did not follow up on these findings.

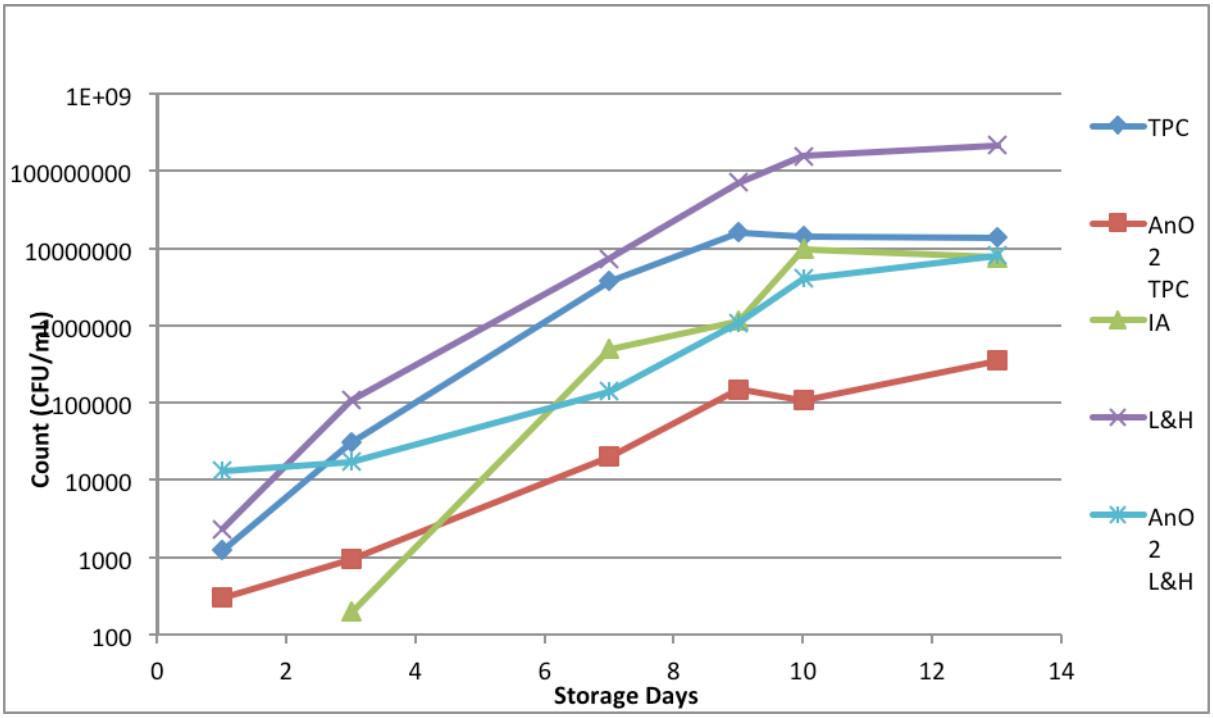


Figure 4.4.2 Bacterial quality of expressed liquor vacuum packed Blue Mussels stored in refrigeration.

4.5 Summary of Important Findings

- Transport temperature did not have a major impact on the microbiological quality of mussels, but storage temperature does.
- Bacterial growth occurs at a higher rate in the expressed liquor of mussels and is the initial source of off odours for refrigerated mussels.
- The aerobic psychrotrophic bacteria counts were much higher in mussel meat than the counts for aerobic mesophilic bacteria and "specific spoilage organisms".
- The growth of Specific Spoilage Organisms (*Shewanella*) was much higher in expressed liquor that in mussel meat.
- Refrigeration of mussels in freely draining conditions while washing mussels frequently reduced overall bacterial levels.
- The Float Test was developed to assess freshness (quality) of mussels.
- There is a good correlation between float test and microbiological counts.
- Mussels had a very diverse micro-flora dominated by *Vibrio* species.
- *Shewanella colwelliana* the species of "specific spoilage organism" most commonly isolated.
- The growth of bacteria in expressed liquor had a major impact on vacuum packed mussels.

5. SPOILAGE OF BLUE SWIMMER CRABS

5.1 Bacterial Growth on Blue Swimmer Crabs

Whole uncooked Blue Swimmer Crabs were sourced on day of catch and transported to the laboratory in an esky with ice bricks. Crabs were stored in ice in a refrigerator and crabs removed on each day of sampling. The carapace was removed from the crabs and meat from the body and claws were aseptically removed and tested at a rate of 9:1 (body:claws). Figure 5.1.1 summarises the average levels of aerobic mesophilic bacteria (SPC), "Specific Spoilage Organisms" (Iron Agar count), and aerobic psychrotrophic bacteria (modified Long & Hammer count).

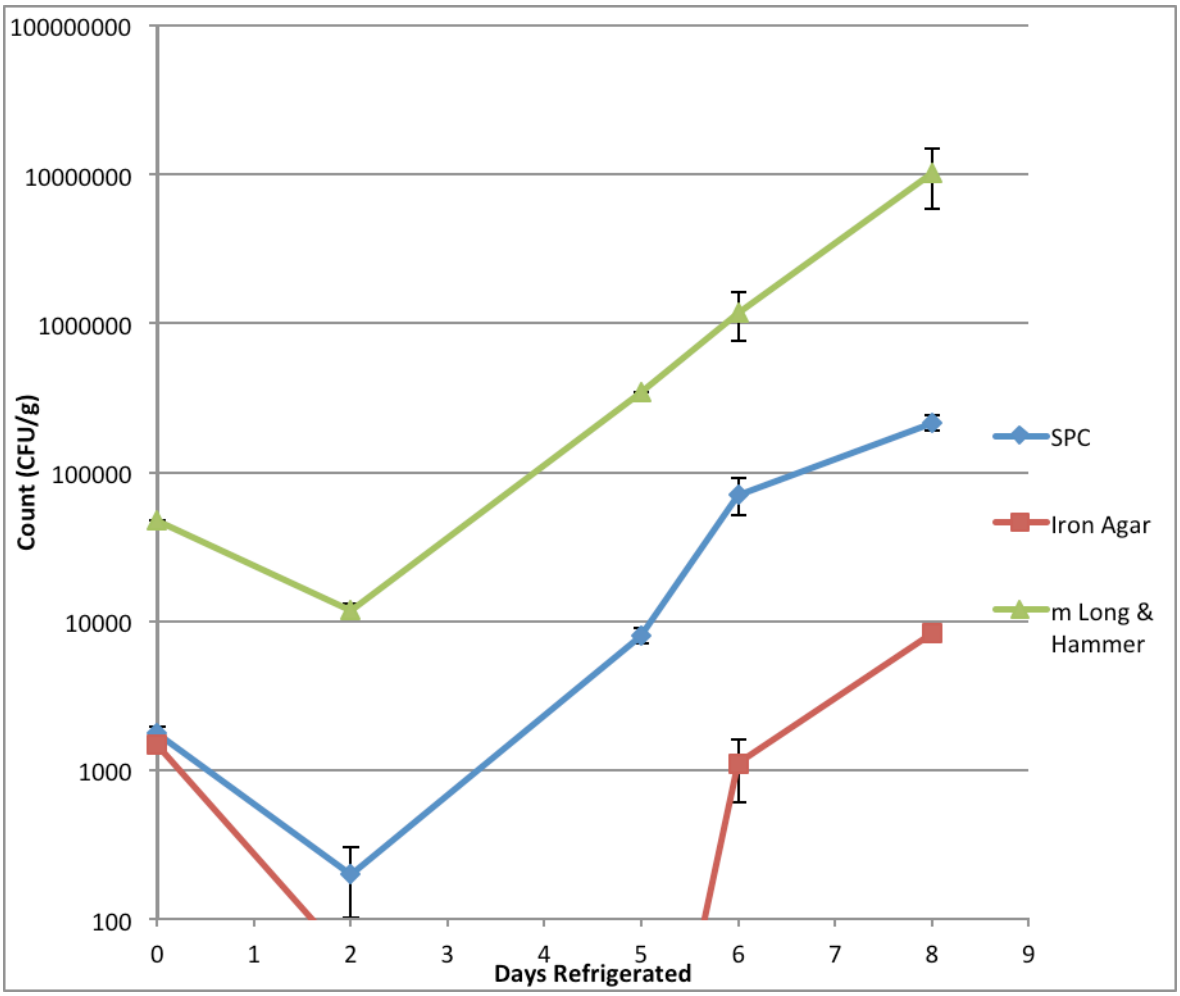


Figure 5.1.1 Average bacterial growth in uncooked whole Blue Swimmer Crab meat.

Uncooked crabs were found to have levels of around 2,000 CFU/g aerobic mesophilic bacteria, 1,500 CFU/g SSOs, and 50,000 CFU/g aerobic psychrotrophic bacteria. The initial levels of psychrotrophic bacteria in relation to the levels of mesophilic bacteria was quite unusual compared to the other seafood tested in this project. These levels were significantly higher than other studies (Faghri, Pennington et al. 1984), although the incubation temperature and medium used in this study differed. All tested bacterial groups showed a decrease in levels between the day of capture and 2 days storage. By day 5 the levels of aerobic mesophilic bacteria and aerobic psychrotrophic bacteria had exceeded initial levels, however the levels of specific spoilage organisms took 8 days to exceed initial levels. By day 8 the final bacterial counts were 2,200,000 CFU/g for aerobic mesophilic bacteria, 8,300 CFU/g for SSOs, and 10,000,000 CFU/g for aerobic psychrotrophic bacteria. Off odours were initially noted at between day 5 and day 8 of storage increasing in intensity from day 6 to day 8. Off odours are usually detected when the modified Long & Hammer count exceeds

1,000,000 CFU/g. SSOs do not appear to have an impact on the spoilage of whole fresh crabs.

It has been previously noted that bacterial numbers in crab haemolymph varied with the sex of the crab (Tubiash, Sizemore et al. 1975). The effect of the sex of the crab on bacterial levels in the crab flesh was investigated. Crabs were sexed and equal numbers of male and female crabs transported and stored separately in the same conditions as detailed previously. Figures 5.1.2 to 5.1.4 summarise the results for the number of aerobic mesophilic bacteria (Standard Plate Count), SSOs (Iron Agar Count) and aerobic psychrotrophic bacteria (modified Long & Hammer count). No significant difference in the tested microbiological parameters was seen between the different sexes of the crabs.

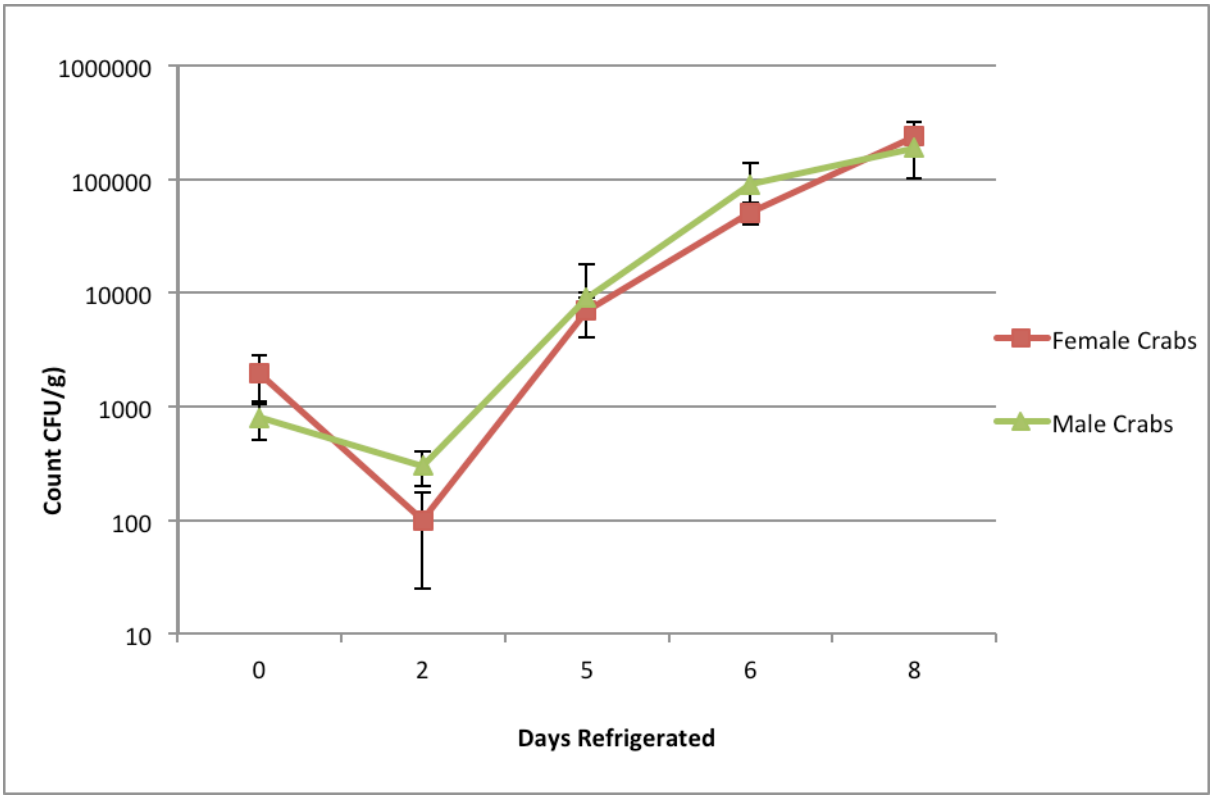


Figure 5.1.2 Comparison of Standard Plate Count of male and female Blue Swimmer Crabs.

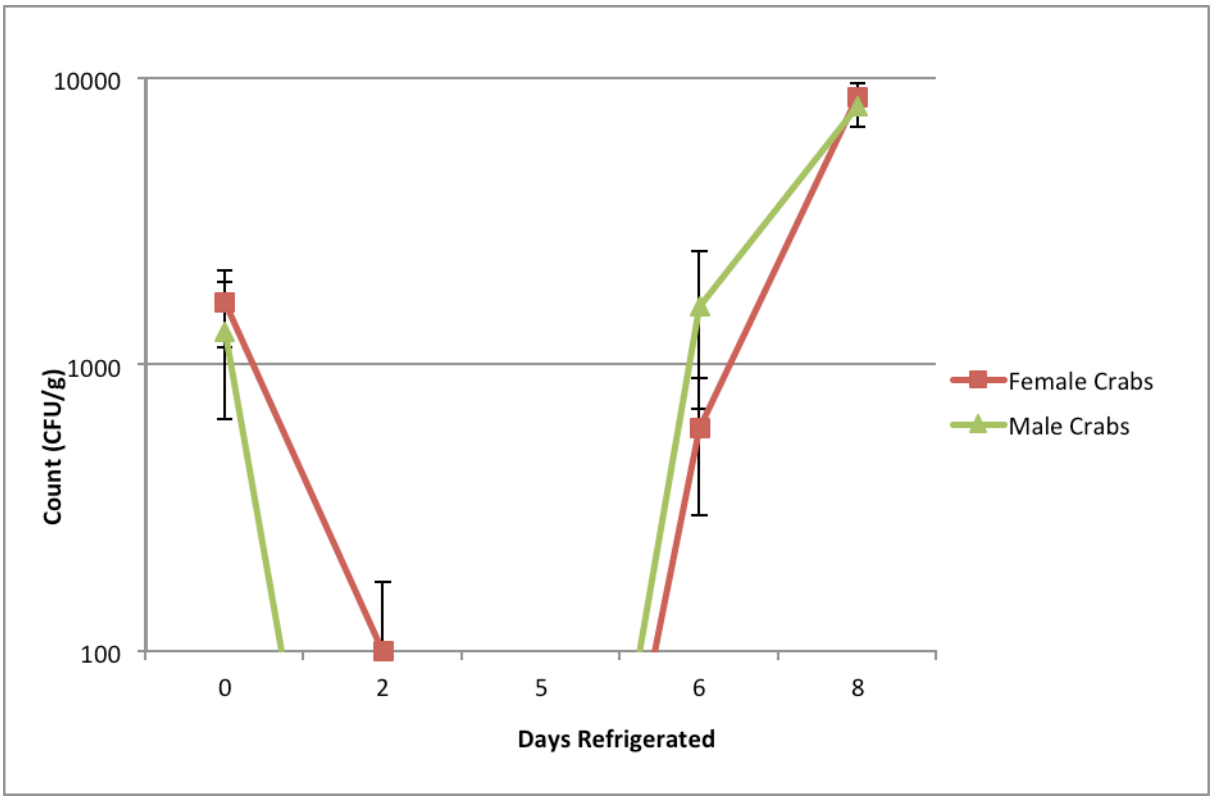


Figure 5.1.3 Comparison of Iron Agar Count of male and female Blue Swimmer Crabs.

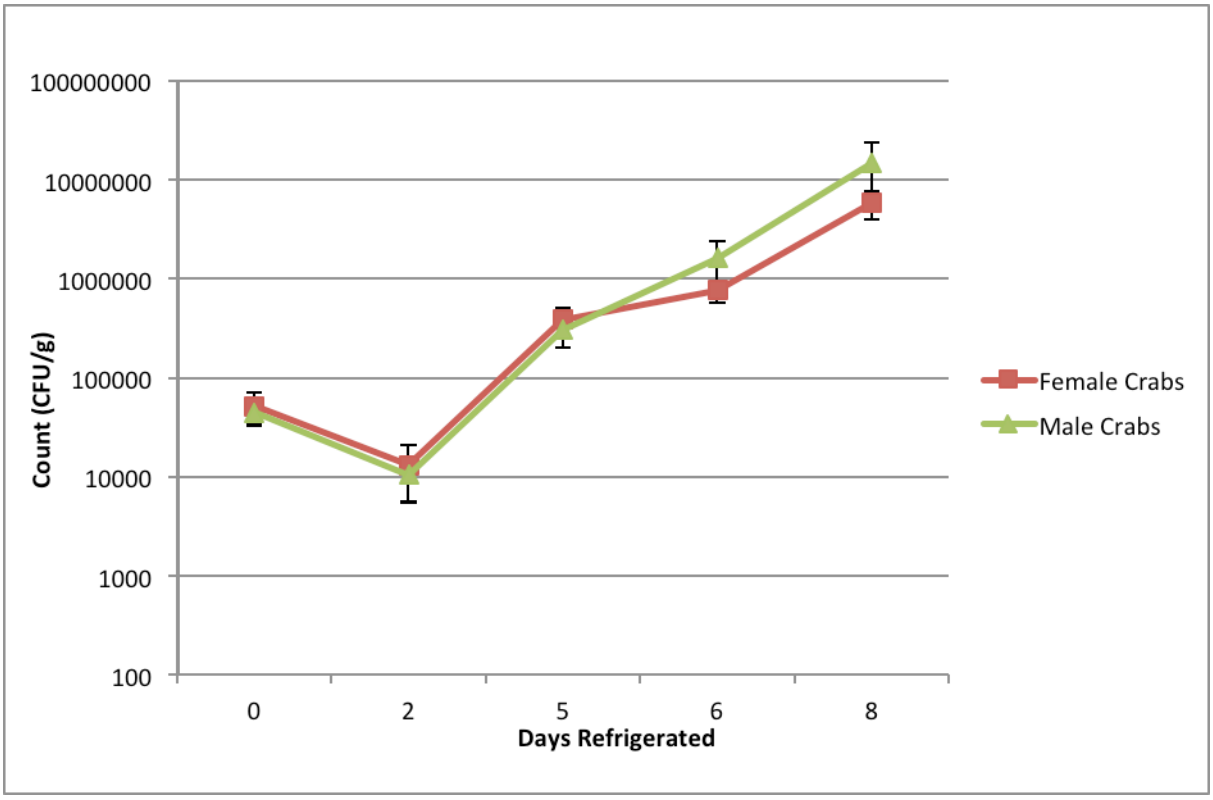


Figure 5.1.4 Comparison of Standard Plate Count of male and female Blue Swimmer Crabs.

5.2 Effect of storage conditions on the rate of bacterial spoilage

A study conducted on crabs in the USA found that some species of crabs had an extended shelf-life when held under refrigeration when compared holding the crabs in ice (Robson, Kelly et al. 2007). To investigate this, groups of crabs were stored under three conditions: 1) held in the refrigerator in plastic containers covered in ice (Ice), 2) held in a refrigerator in ice in a plastic container with a drain to remove ice melt (Drained Ice), and 3) held in a refrigerator in a plastic container covered with a damp cloth (Fridge). Samples were tested by Standard Plate Count and modified Long & Hammer count.

A difference was seen in the levels of bacteria present on the crabs held in ice compared to both the counts of crabs held in drained ice and directly in the refrigerator (Figures 5.2.1 and 5.2.2). The impact of the difference in the number of aerobic mesophilic bacteria was significantly less than that seen in the difference for aerobic psychrotrophic bacteria. The onset of off odours was delayed by 2 days for crabs held in drained ice and in a plastic container with a damp cloth compared to crabs held in the non-drained ice. Off odours were detected on day 6 for crabs held in non-drained ice and day 8 for the other two treatments.

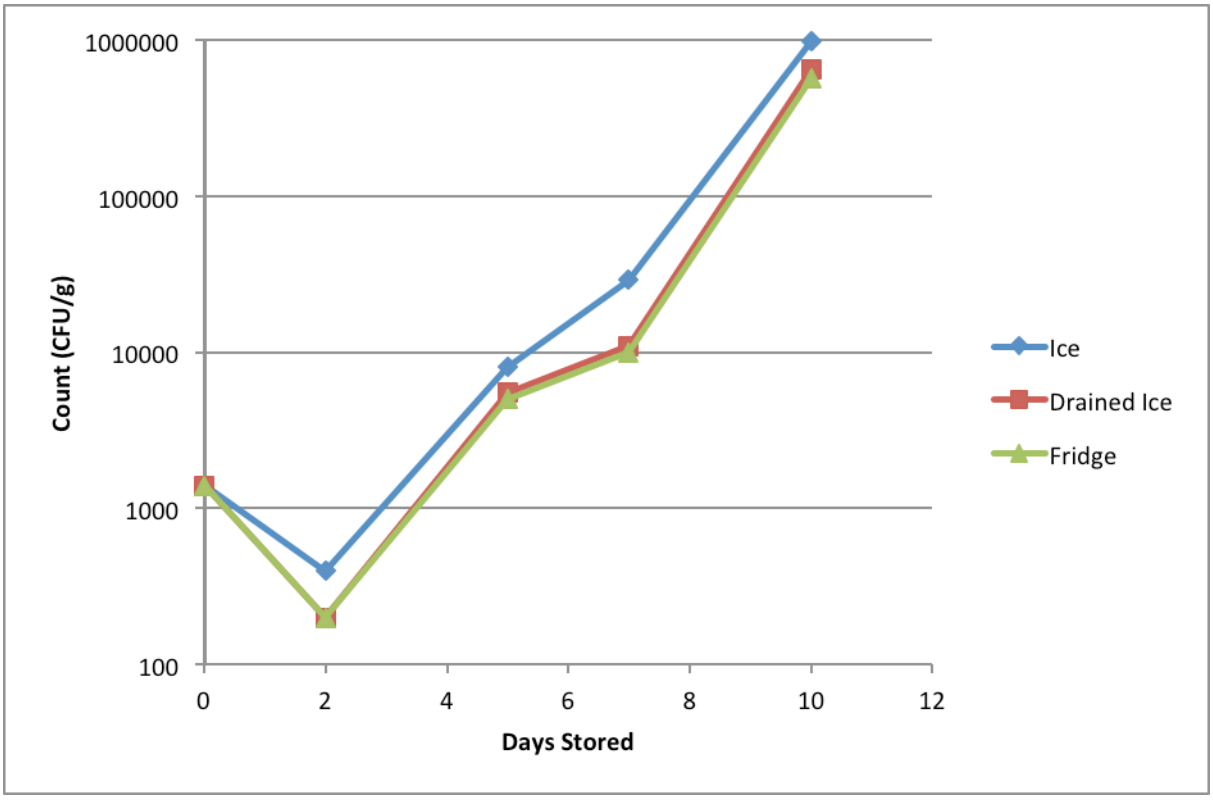


Figure 5.2.1 Effect of storage conditions on the average levels of aerobic mesophilic bacteria (Standard Plate Count) in crabs.

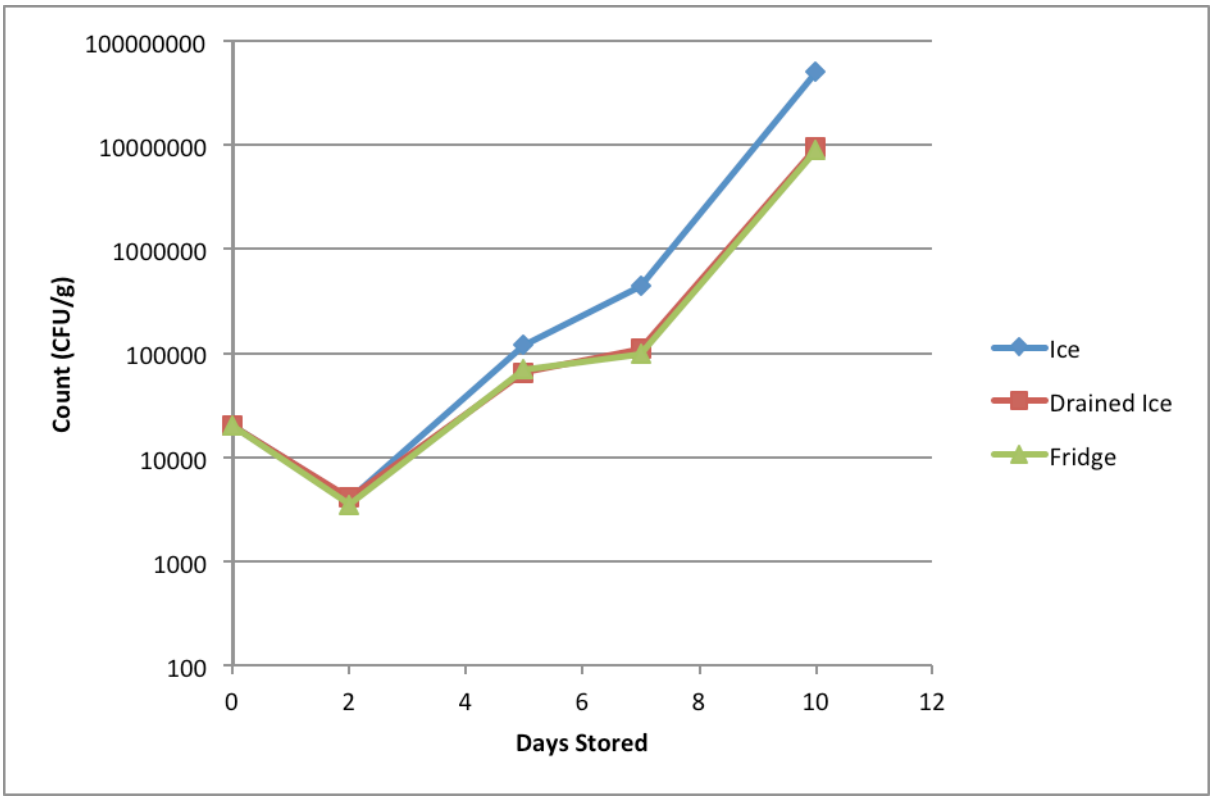


Figure 5.2.2 Effect of storage conditions on average levels of aerobic psychrotrophic bacteria (modified Long & Hammer count) in crabs.

5.3 Treatment of Whole chilled Crabs with Chlorine Dioxide Sanitiser

Crabs were treated by immersing them in an ice slurry containing 20mL/L of un-activated chlorine dioxide for 1 h. Crabs were then stored in a refrigerator in drained ice and tested for standard plate count, iron agar count, and modified Long & Hammer count. A slight but significant difference was found for the aerobic mesophilic bacteria present on chlorine dioxide treated crabs. There was a reduction of approximately 1 log (10 times) in the SPC of the treated crabs on days 6 and 8 of storage (Figure 5.3.1). The levels of specific spoilage organisms were actually increased by 1 log. The total number of SSOs was still significantly less than the total number of psychrotrophic bacteria. There was no significant impact on the average count of aerobic psychrotrophic bacteria between experiments (Figure 5.3.3). Significantly, there was a 1 day impact on the onset of off odours between the treated and untreated crabs (Figure 5.3.2). The untreated crabs had off odours detected on day 6 of storage, while the treated crabs had off odours detected on day 7.

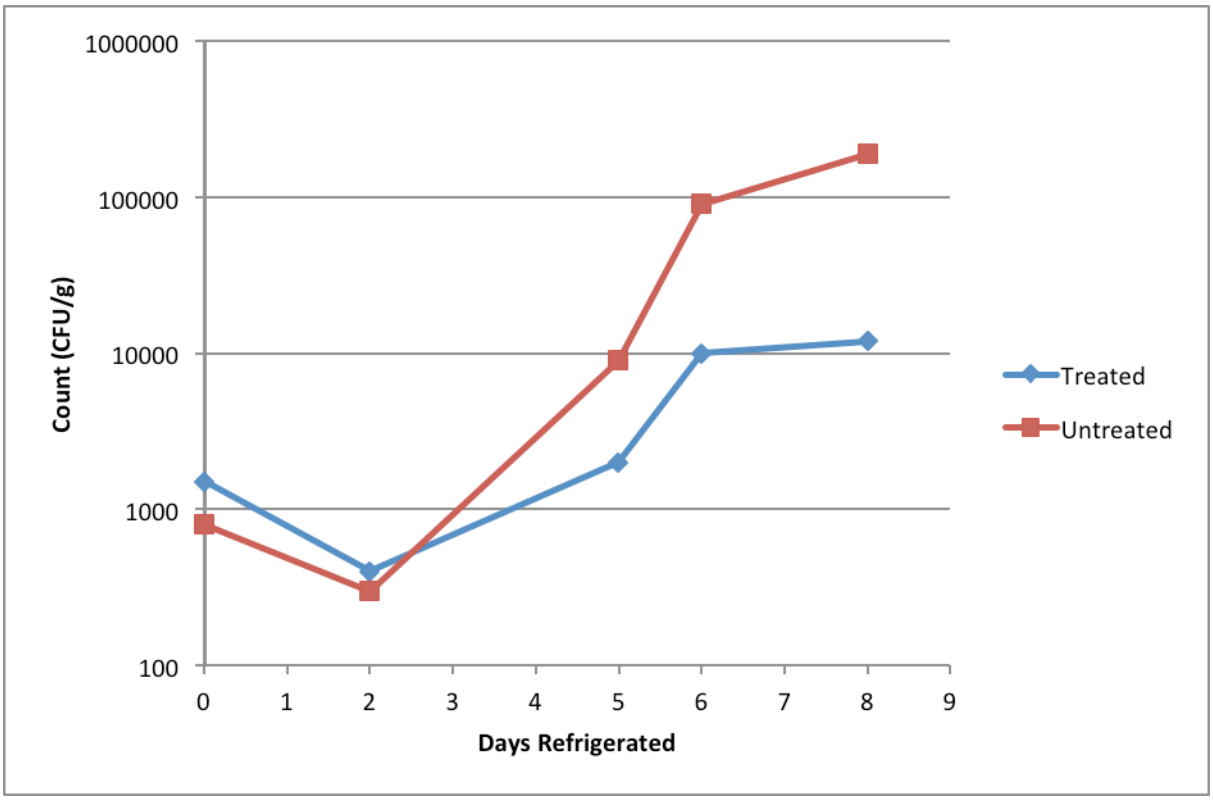


Figure 5.3.1 Comparison of the average levels of aerobic mesophilic bacteria (Standard Plate Count) on un-activated chlorine treated and untreated Blue Swimmer Crabs.

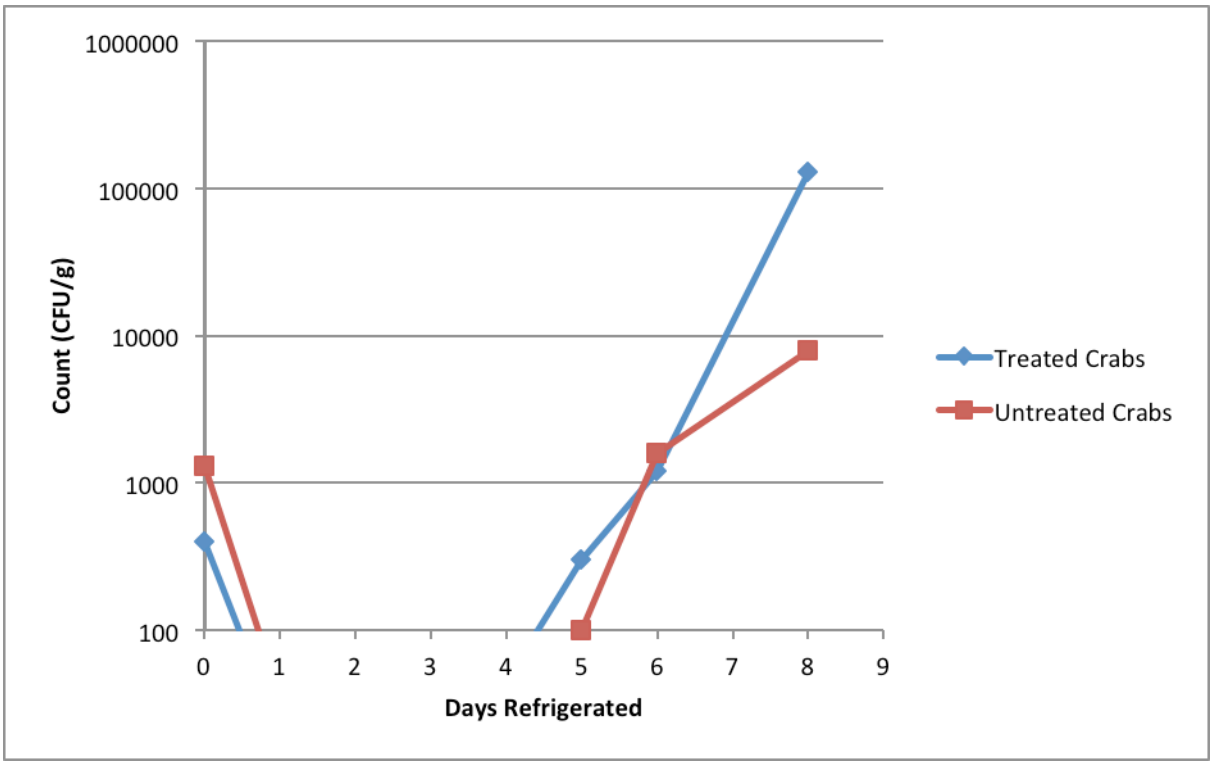


Figure 5.3.2 Comparison of the average levels of specific spoilage organisms (Iron Agar Count) on un-activated chlorine treated and untreated Blue Swimmer Crabs.

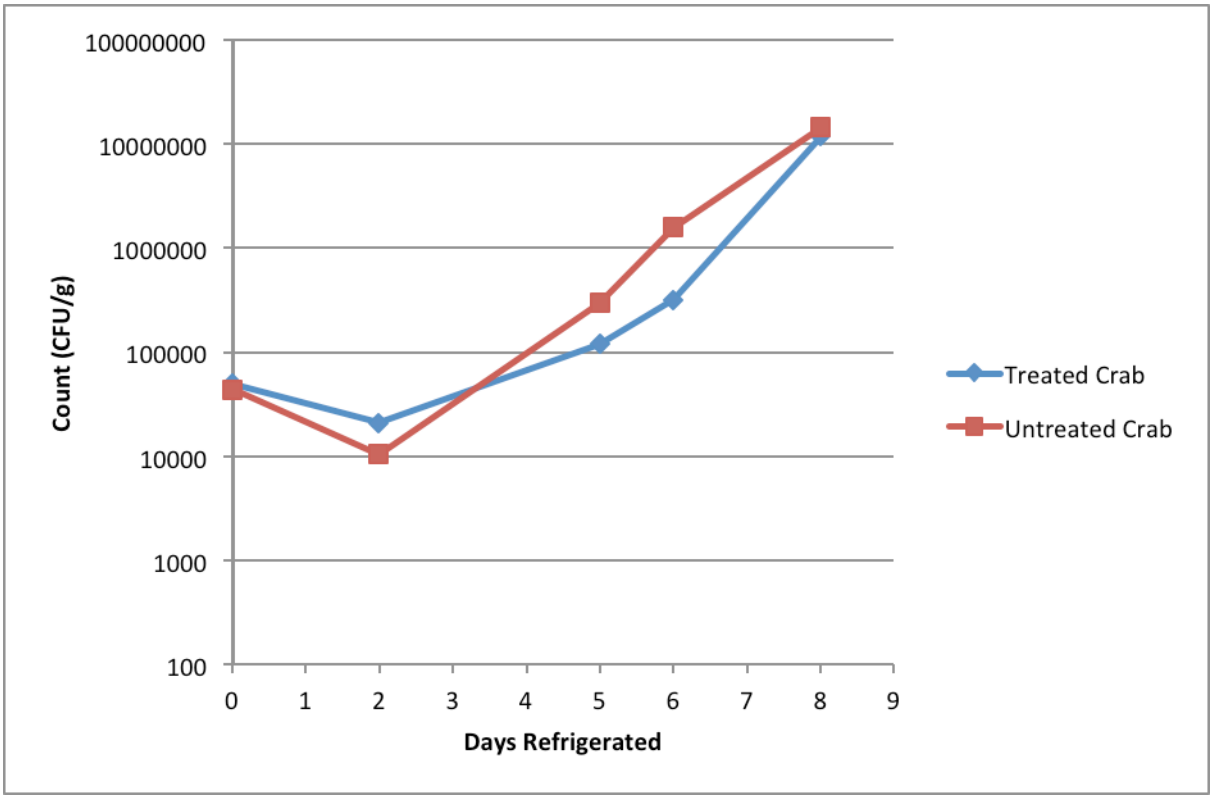


Figure 5.3.3 Comparison of the average levels of aerobic psychrotrophic bacteria (modified Long & Hammer Count) on un-activated chlorine treated and untreated Blue Swimmer Crabs.

5.4 Microbiological spoilage of cooked Blue Swimmer Crabs

Cooked crabs were held under freely draining ice in refrigeration. Testing was performed on cooked crabs for aerobic mesophilic bacteria (Standard Plate Count), "Specific Spoilage Organisms" (Iron Agar Count), and aerobic psychrotrophic bacteria (modified Long & Hammer Count). Testing of cooked crabs on day of cooking was performed 6 hours after cooking. Figure 5.4.1 show the average microbiological counts on cooked blue swimmer crabs over time. On day of cooking the average Standard Plate Count (SPC) and modified Long and Hammer counts L&H) were around 1,000 CFU/g. Levels of Specific Spoilage Organisms were below levels of detection (<100 CFU/g). After 4 days of refrigerated storage there was only a small amount of bacterial growth with the Standard Plate Count and modified Long and Hammer counts increasing by less than 1 log to an average SPC of around 5,000 CFU/g and average L&H count of approximately 6,500 CFU/g. Specific Spoilage Organisms were detected on day 4 with an average Iron Agar Count of 150 CFU/g. By day 8

there was an increase of approximately 1 log in both the SPC (54,000 CFU/g) and L&H count (94,000CFU/g). The Iron Agar counts did not increase significantly with an average count of 400 CFU/g. Rapid growth of bacteria occurred for all bacterial groups between 8 and 14 days of storage. The SPC and L&H counts increased over 2 log to a final counts of 17,000,000 CFU/g (SPC) and 31,000,000 CFU/g (L&H). The number of SSOs increased by over 4 log to a final average count of 19,000,000 CFU/g.

Off odours were detected at around 13 to 14 days of storage. While SSOs (*Shewanella* species) do not appear to be involved in the spoilage of whole uncooked crab, *Shewanella* does appear to be involved with the production of off odours in refrigerated whole cooked Blue Swimmer Crabs.

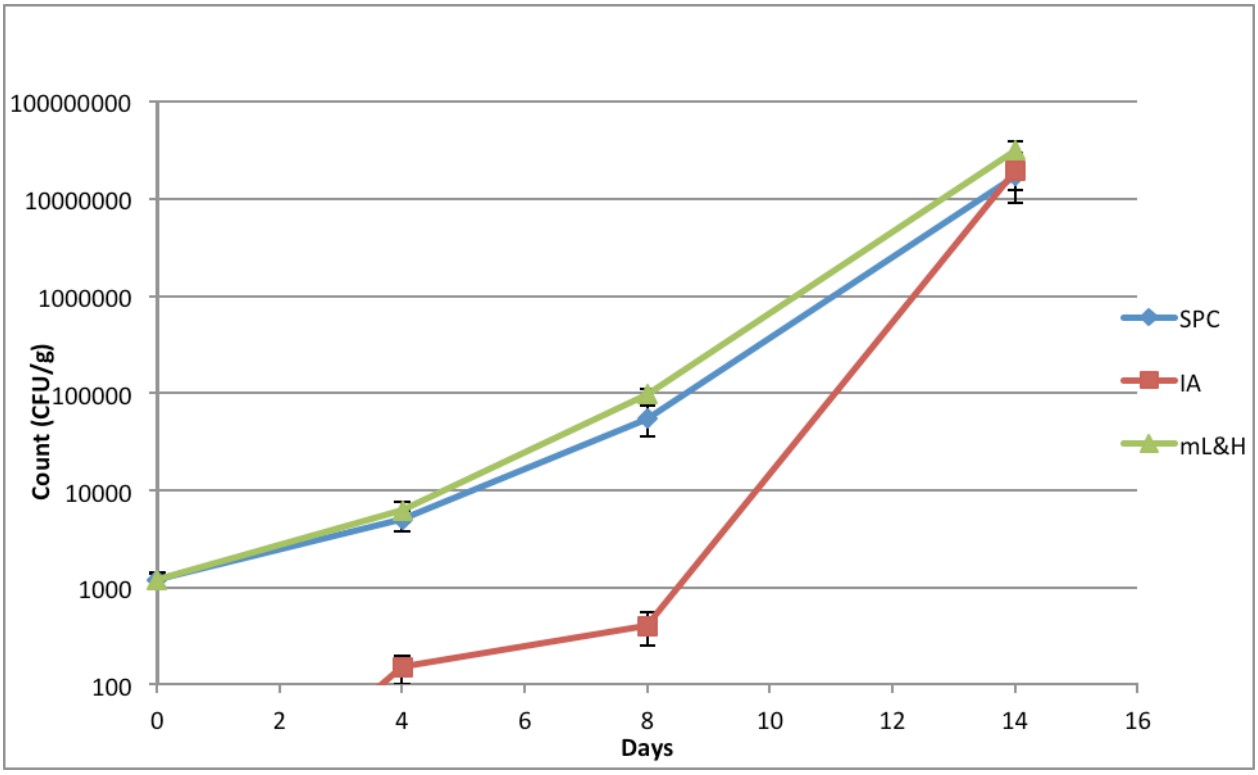


Figure 5.4.1 Effect of storage on bacterial levels in cooked Blue Swimmer Crabs.

Experiments were performed to investigate the impact of immersing cooked crabs in an ice slurry with chlorine dioxide sanitiser was investigated. The crabs were placed into an ice slurry with un-activated and activated chlorine dioxide. The ice slurry was prepared and cooked crabs treated as described in 5.3 above. No significant difference was found in the

bacterial counts of the treated and untreated crabs (Figures 5.4.2 to 5.4.4). Treatment also had no effect on the onset of detectable off odours.

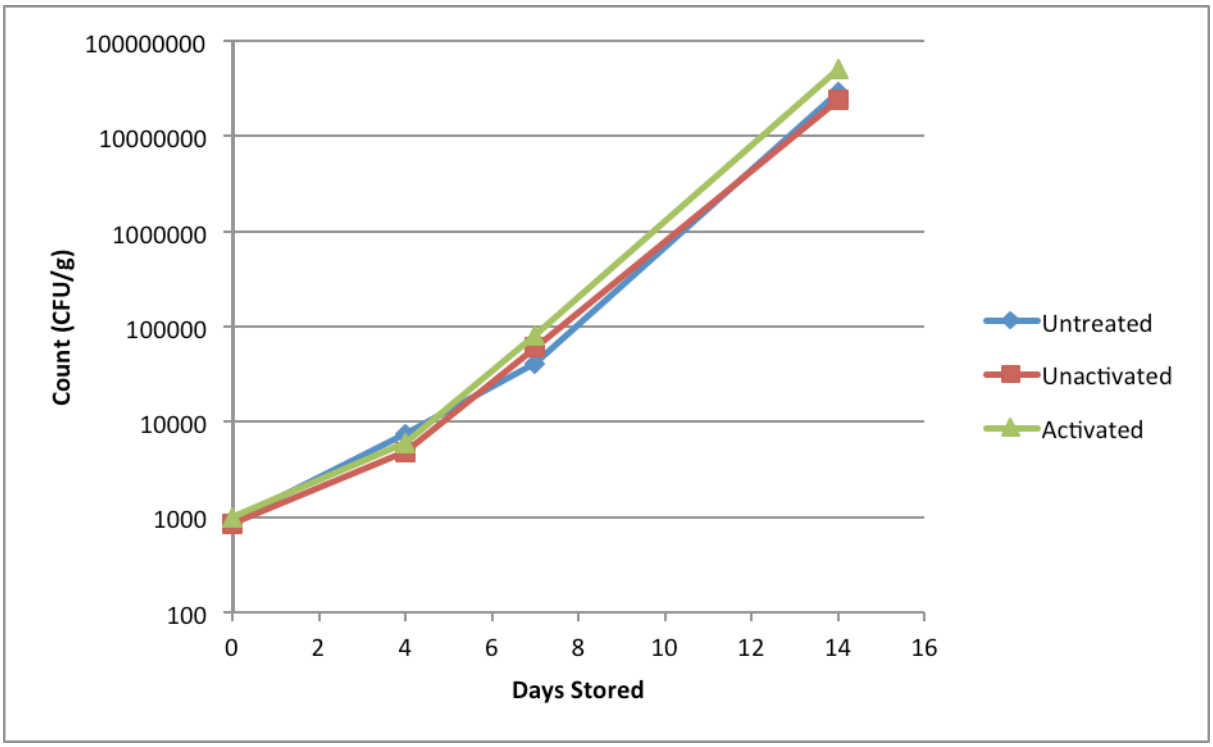


Figure 5.4.2 Effect of treatment of cooked Blue Swimmer Crabs with activated and un-activated chlorine dioxide on aerobic mesophilic bacteria (Standard Plate Count).

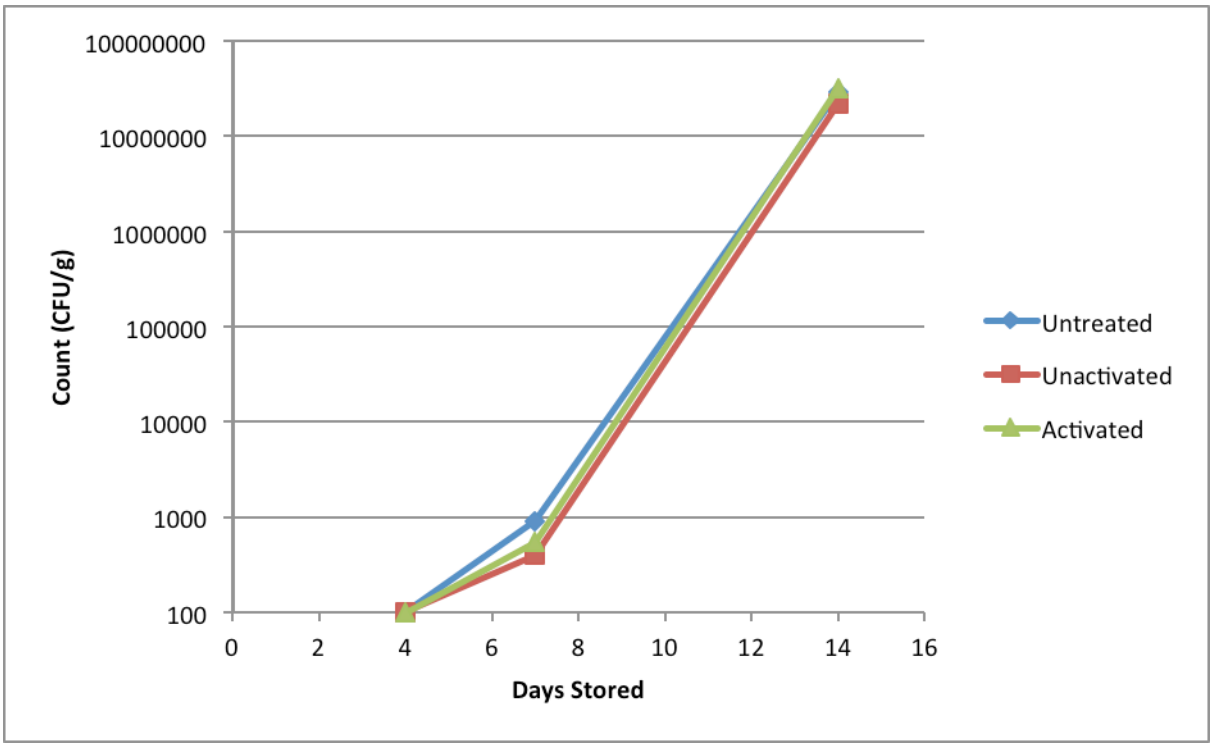


Figure 5.4.3 Effect of treatment of cooked Blue Swimmer Crabs with activated and un-activated chlorine dioxide on specific spoilage bacteria (Iron Agar Count).

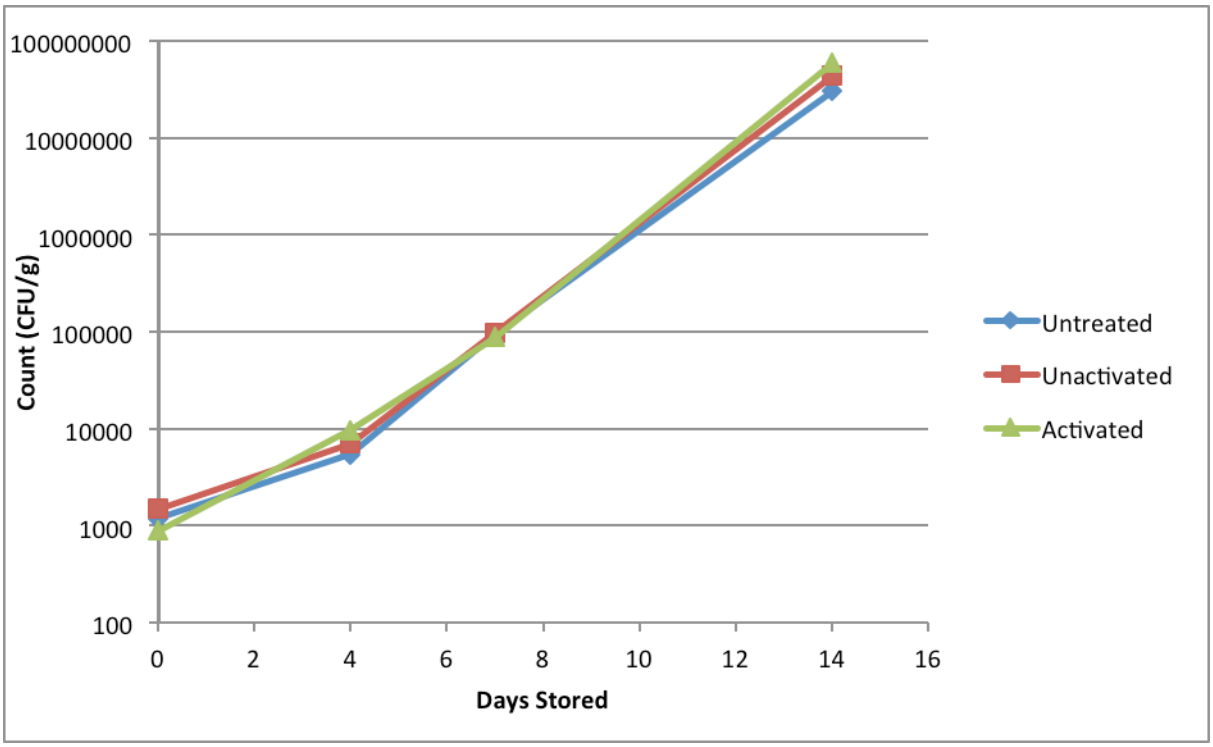


Figure 5.4.4 Effect of treatment of cooked Blue Swimmer Crabs with activated and un-activated chlorine dioxide on aerobic psychrotrophic bacteria (modified Long & Hammer Count).

5.5 Investigation of a Crab Processing Chain

The entire chain of a crab processing factory was investigated. The process from capture to dispatch was investigated. Samples taken at sea were processed at sea and were plated onto Plate Count Agar, Iron Agar, and modified Long & Hammer agar using the spread plate method. The crabs were processed within 30 min of capture, following stunning in an ice slurry. All samples taken at the factory were processed within 15 min of their completion of their sampling point and were held under 4˚C refrigeration prior to processing. Samples were plated onto agar plates using the spread plate method. Plates were packaged in an esky with ice-bricks and transferred to the laboratory by flight on the same day of testing. Plates were incubated at 30˚C for 48h for Plate Count Agar, 25˚C for 48h for Iron Agar, and 15˚C for 120h for modified Long & Hammer agar.

Bacterial counts on the Blue Swimmer Crabs through the processing chain are summarised in Figure 5.5.1. Following capture and stunning the crabs had an average count of 25,000 CFU on the Standard Plate Count, 30,000 CFU/g on the modified Long & Hammer Count, and 2,000 CFU/g on the Iron Agar Count. The crabs were then processed after they had been unloaded from the boat and transferred to refrigeration prior to processing. There was a slight but insignificant rise in the average number of aerobic psychrotrophic bacteria (38,000 CFU/g), and SSOs (3,400 CFU/g), and there was a slight decrease in the number of aerobic mesophilic bacteria (13,000 CFU/g).

Crabs were then cooked and sorted, with crabs without imperfections (i.e. missing claws, incorrect colouration etc.) were then individually wrapped and frozen. Crabs with imperfections were placed into a "reject slurry tank" containing an ice slurry. They were then packed into stainless steel, crated and refrigerated overnight before being opened and the meat picked from the crab by hand, placed into containers and frozen.

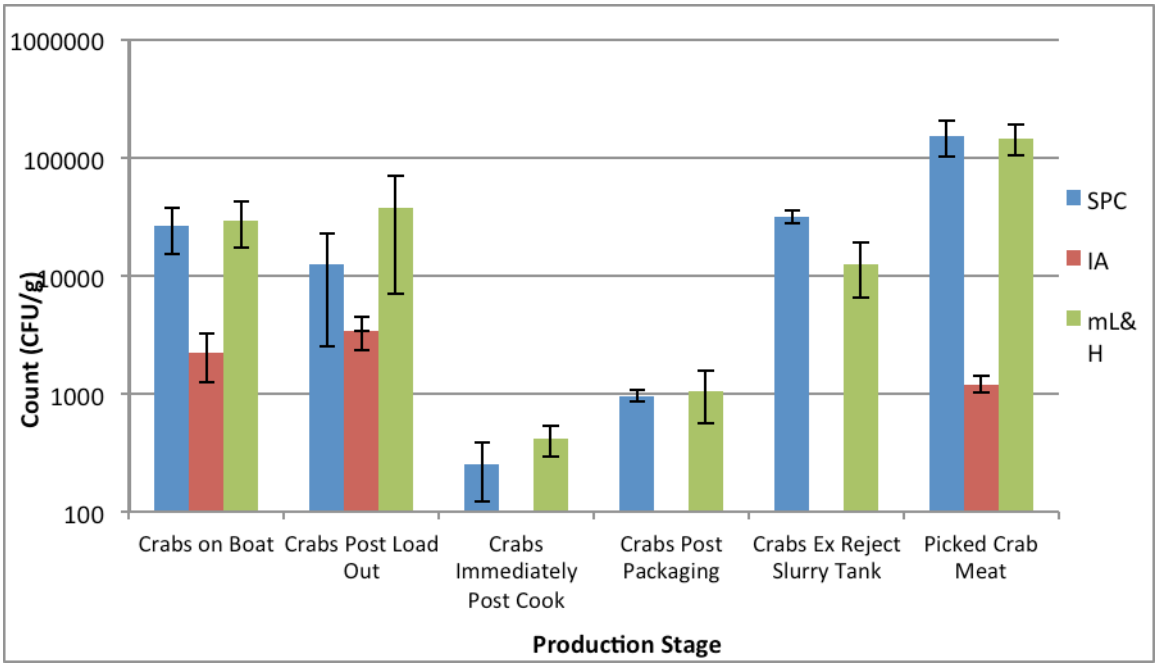


Figure 5.5.1 Levels of aerobic mesophilic bacteria (SPC), specific spoilage organisms (IA) and psychrotrophic bacteria (mL&H) of Blue Swimmer Crabs through a processing chain.

Following immediately cooking, the crabs had an average of around 200 CFU/g on the Standard Plate Count, and 400 CFU/g on the modified Long & Hammer Count. This organism was found to be a *Bacillus* species possible *Bacillus marinus* (Ruger, Fritze et al.

2000). These counts had increased to 950 and 1,000 CFU/g by the time the crabs were packaged and were to be frozen. No SSOs were detected in either of these sample points.

Rejected crabs placed into an ice slurry had a very significant increase in bacterial numbers compared to the product immediately post cooking. The Standard Plate Count and modified Long & Hammer counts increased by around 2 log to an average of 32,000 CFU/g for the SPC and 13,000 CFU/g modified Long & Hammer Count. There were still no SSOs detected at this point. Crab were being held in the ice slurries for extended times until they were transferred into stainless steel crates and placed in refrigeration overnight. Testing of the ice slurry water in the reject slurry tank at the end of the shift showed a standard plate count and modified long and hammer count in excess of 100,000 CFU/mL. The reject crabs were processed the following day to produce picked crabmeat. Following the picking process the crabs were found to have average counts of around 150,000 CFU/g for both the Standard Plate Count and modified Long and Hammer Count, and SSOs were detected for the first time on the cooked product at an average count of 1,200 CFU/g.

Temperature profiling was performed through the processing and is summarised in Figure 5.5.2.

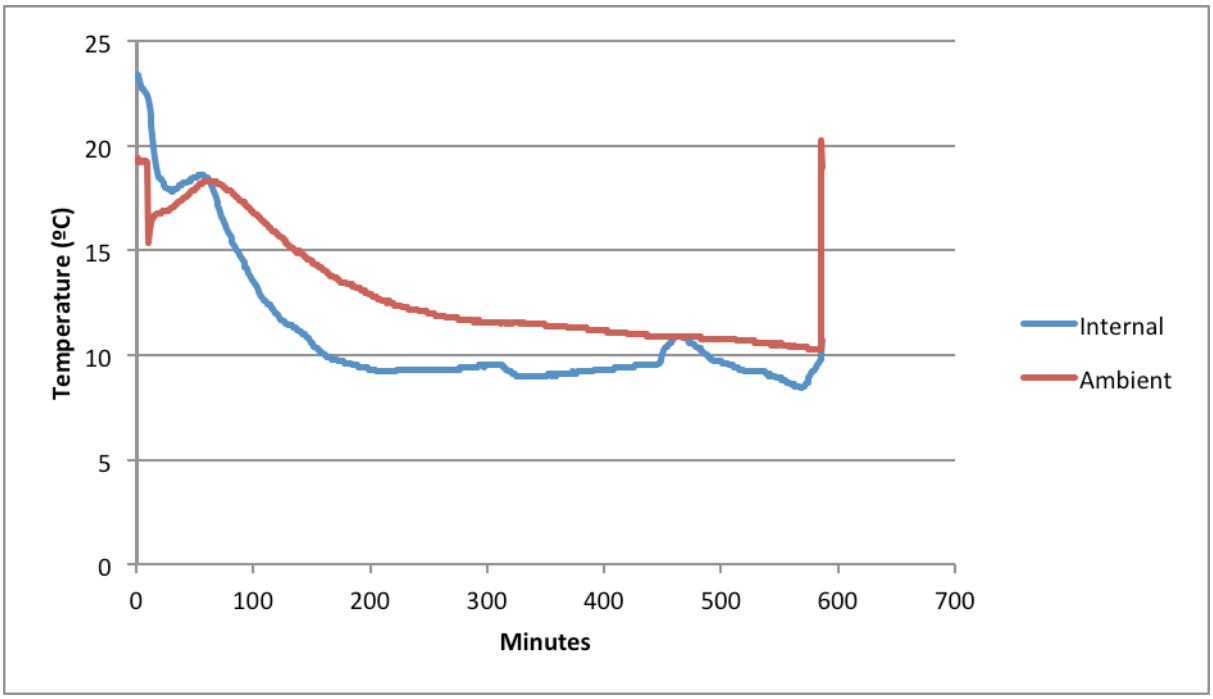


Figure 5.5.2 Internal temperature of Blue Swimmer Crabs and their holding temperature from stunning to offloading from boat.

The temperature profile shown in Figure 5.5.2 shows the temperature of the Blue Swimmer Crabs from post stun sorting to off-loading from the boat. As can be seen the core temperature of the crabs initially started at a temperature of around 23˚C and took approximately 2.5 h to drop below 10˚C. The minimum temperature reached was 8.4˚C. Even though these temperatures are relatively high from a food microbiology perspective, no significant growth of bacteria was recorded during the 10 h from capture to landing. This is due to the fact that the crabs are kept alive, and thus the growth of microorganisms is controlled by the crab's physiology.

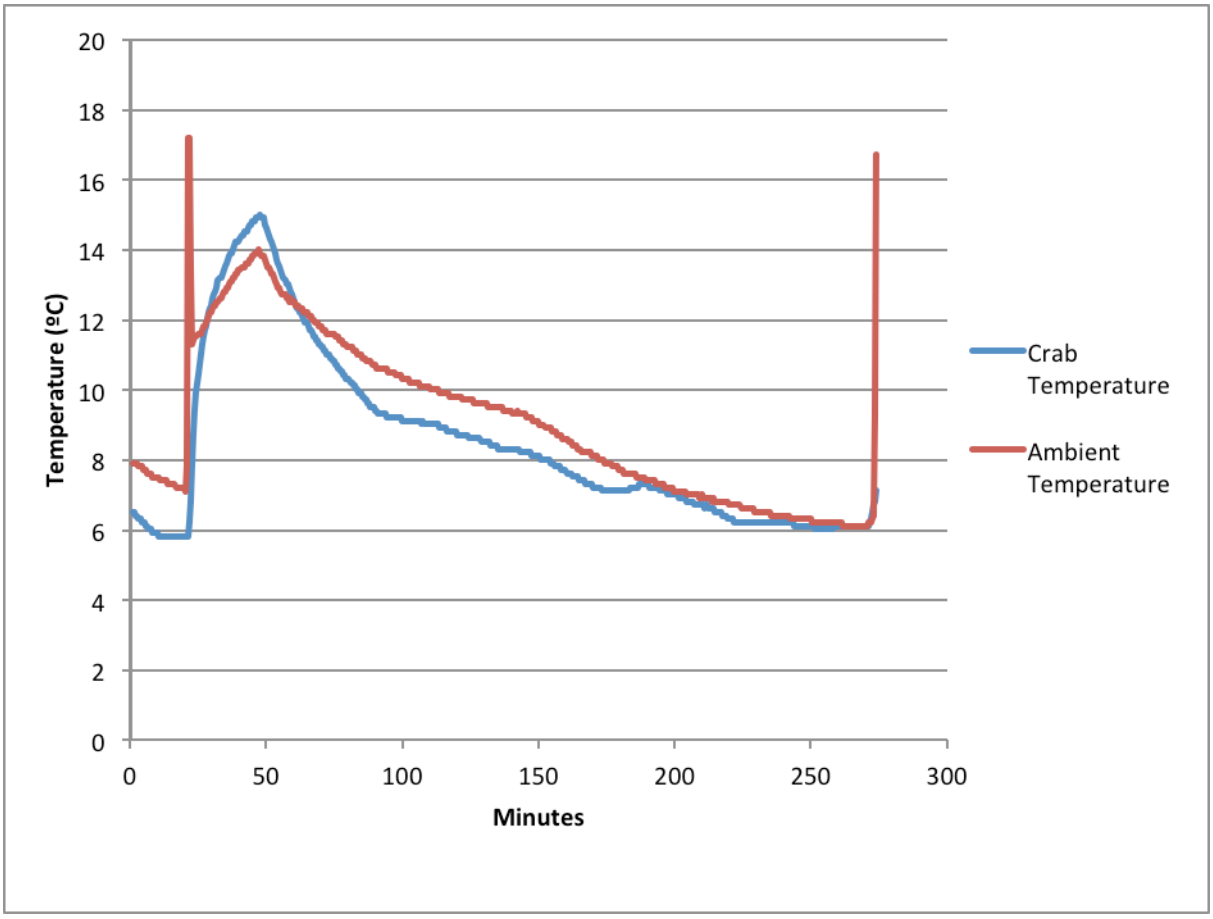


Figure 5.5.3 Temperature of picked crabs from refrigerator to pre-mincing.

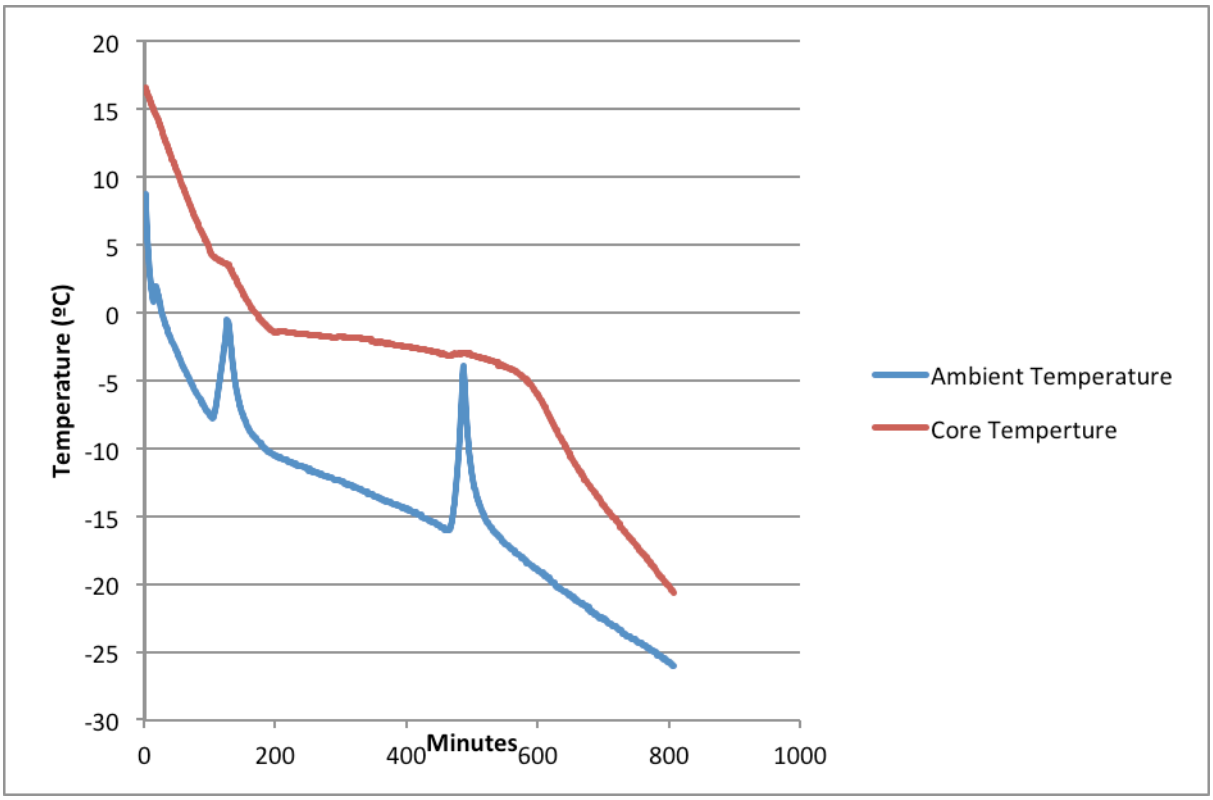


Figure 5.5.4 Temperature of minced crab and ambient temperature from collection of minced crab meat to freezing of packaged product.

An investigation into the effect of processing crabs on the microbiological quality of crab mince was carried out. The microbiological quality of the mince produced from the meat remaining in the shells post hand picking was compared to that of mince produced from whole (unpicked) crabs. Results are show in Figure 5.5.5. There was no significant difference in the counts between the picked and whole crab mince modified Long and Hammer Count but there was a slight (but statistically significant) decrease in the Standard Plate Count of the mince produced from the whole crabs. Very few SSOs were detected in the mince produced from whole crabs (and average of <100 CFU/g), but mince produced from the picked crabs had an average iron agar count of around 1000 CFU/g. There was no significant difference in the levels of bacteria in the picked crabs before and after mincing. Figures 5.5.3 and 5.5.4 above show the temperature profiles of the stages from pre-picking through to freezing of the mince. As there was no significant increase in the counts between picking and mincing, temperature appears to have had no impact. Tests on thawed minced crabmeat following 1 months storage at -20˚C showed up to a 1 log reduction in the Standard Plate Count and modified Long and Hammer Count.

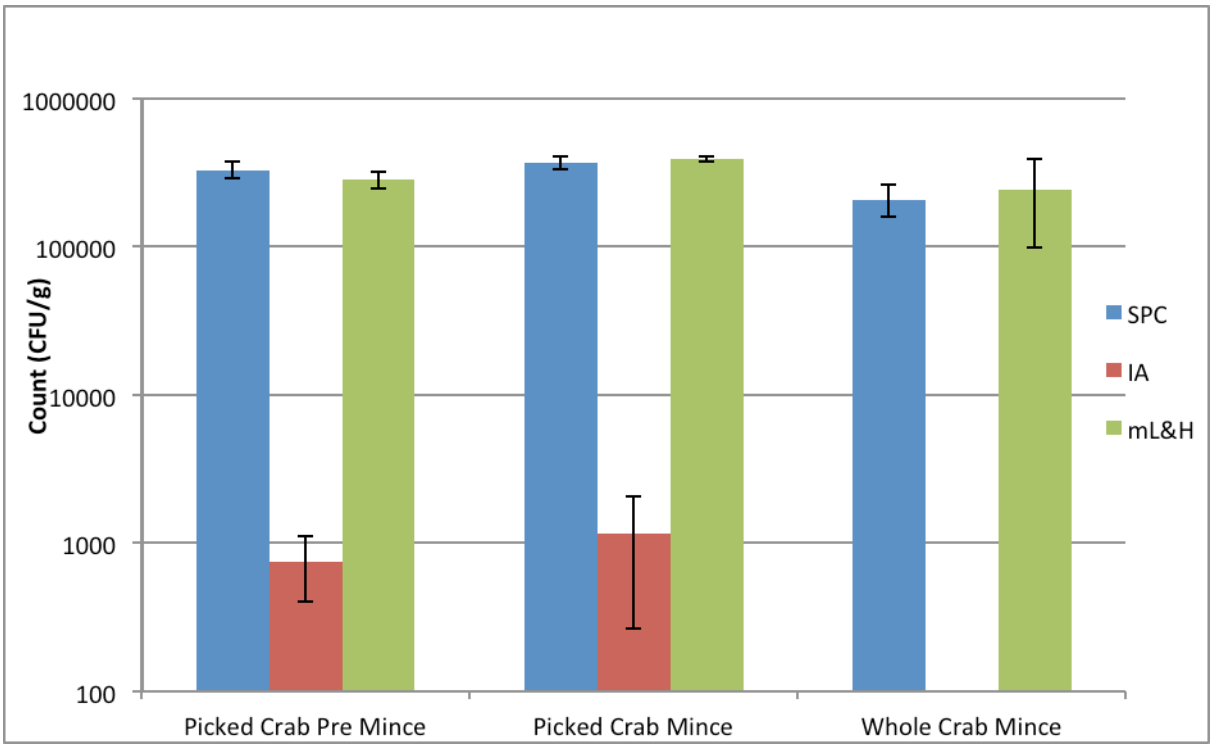


Figure 5.5.5 Aerobic Mesophilic Bacteria (SPC), Specific Spoilage Organisms (IA) and Aerobic Psychrotrophic Bacteria (mL&H) in Crab Mince.

5.6 Major Bacterial Isolates on Blue Swimmer Crabs

Psychrobacter species were the most commonly isolated organisms on fresh Blue Swimmer Crab meat representing a total of 26% of the major isolates (Figure 5.6.1). Of the *Psychrobacter* species *Psychrobacter nivimaris* represented 6% and *Psychrobacter glacincola* represented 4% of the major isolates (other *Psychrobacter* species were 16% of the major isolates), *Vibrio* species, *Psychrobacter* species and *Pseudoalteromonas* species were the next most commonly isolated bacteria representing 22% each of the major isolates. There were a large number of other bacteria making up the remaining major bacterial isolates including *Microbacterium* sp. (6%), *Kocuria* sp. (6%), *Brevundimonas* sp. (4%), *Kytococcus* sp. (3%), *Staphylococcus* sp. (3%), *Chryseobacterium* sp. (2%), *Exiguobacterium* sp. (2%), *Pseudomonas fluorescens* (2%), *Aeromonas salmonicida* (1%), *Bizionia* sp. (1%), *Photobacterium phosphoreum* (1%), and *Shewanella baltica* (1%).

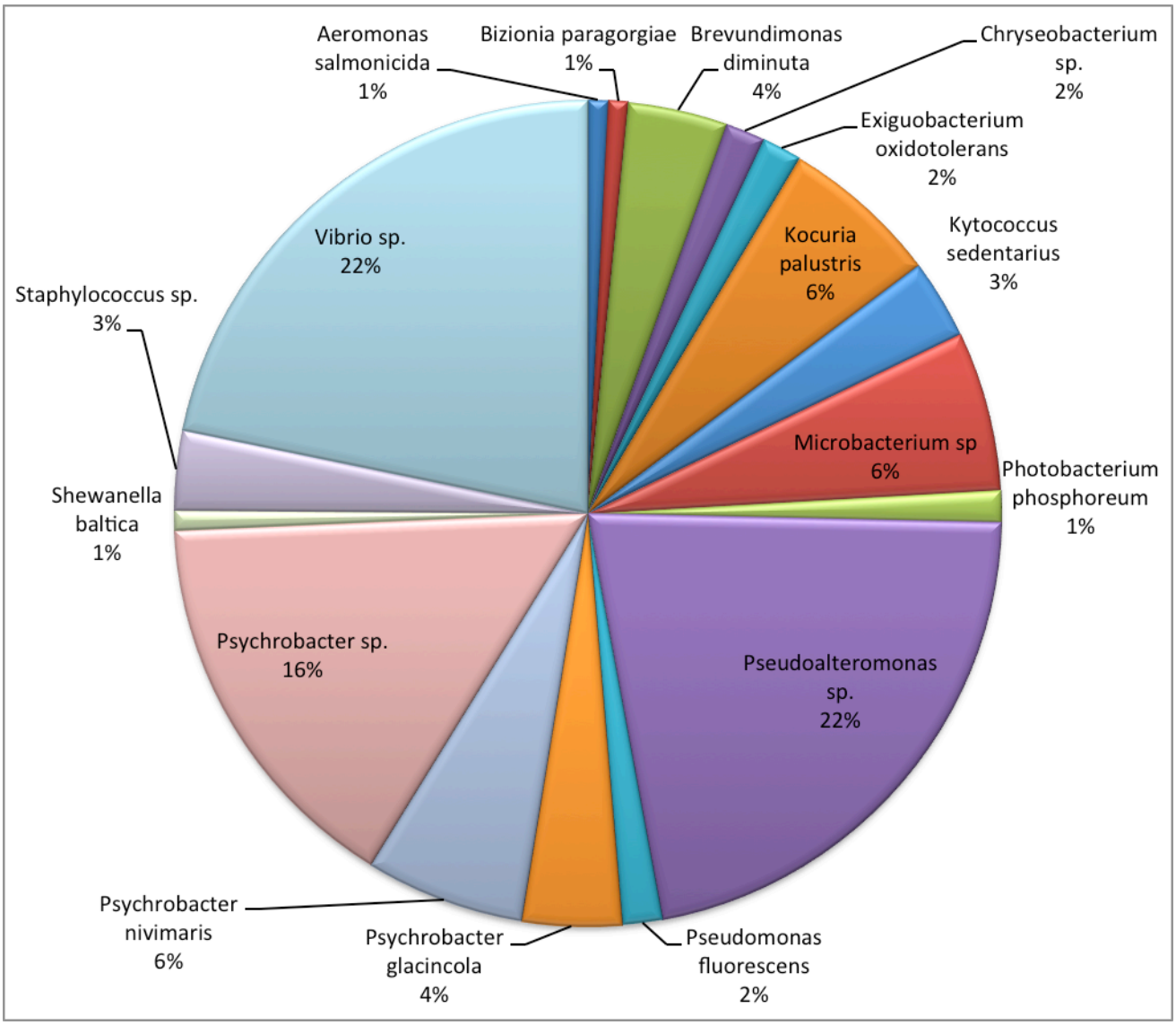


Figure 5.6.1 Major bacterial isolates on freshly caught Blue Swimmer Crabs.

Following spoilage the number of major bacterial isolates lessened quite dramatically (Figure 5.6.2). *Psychrobacter* species were again the most predominant species, but represent 60% of all major isolates. This represented an increase of over two times in the proportion of these organisms. *Pseudoalteromonas haloplanktis* emerged to become the most commonly isolated *Pseudoalteromonas* species, representing 27% of the major isolates. *Staphylococcus hominus* represented 12% of the major isolates while *Shewanella baltica* (0.75%) and *Shewanella marisflavi* (0.25%) were found in relatively small numbers and probably had little influence on the spoilage of the crab.

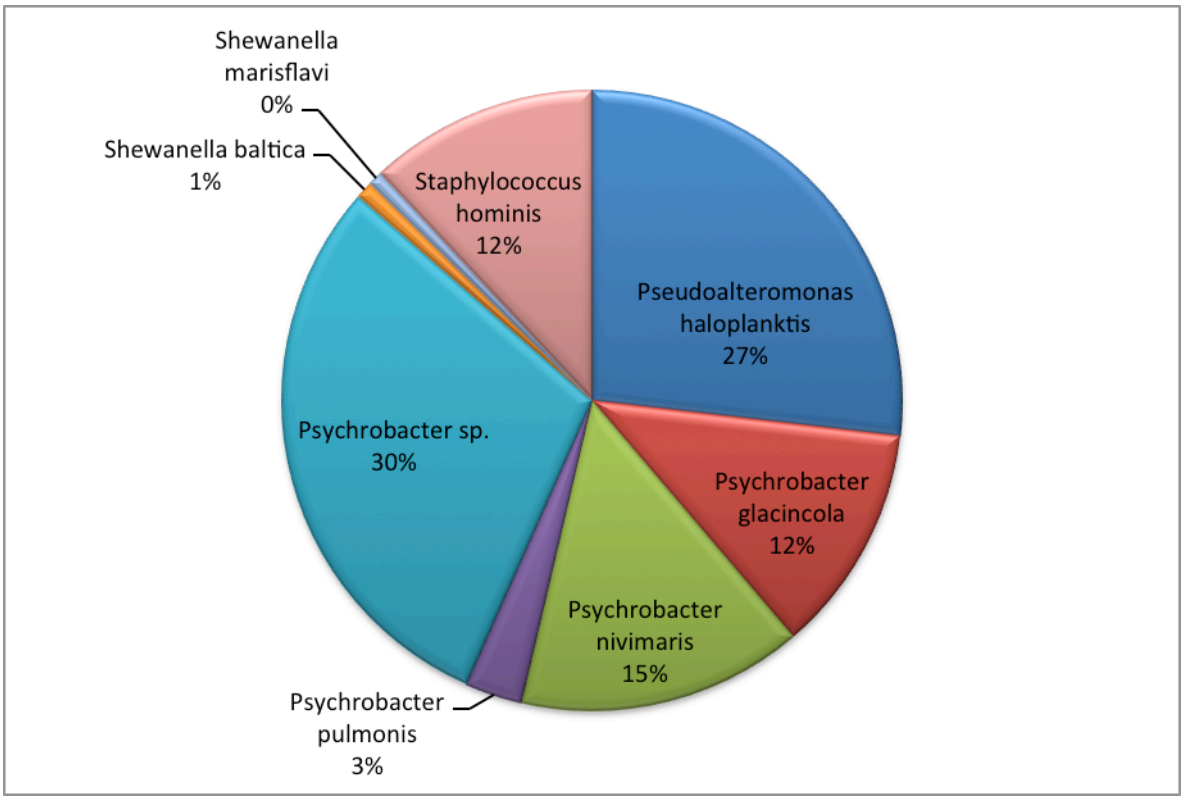


Figure 5.6.2 Major bacterial isolates from whole Blue Swimmer Crabs stored for 8 days under free draining ice in refrigeration.

5.7 Summary of Important Findings

- Aerobic psychrotrophic bacteria show greatest growth in uncooked chilled Blue Swimmer Crabs.
- The bacterial levels are not affected by the sex of the crab.
- Specific spoilage organisms (*Shewanella* species) are not significant to the spoilage of uncooked Blue Swimmer Crabs.
- Storage of Blue Swimmer Crabs in freely draining ice or in a refrigerator with a damp cloth coverage produced slightly less bacterial growth than when crabs were held in undrained ice.
- Treatment of chilled whole crabs with sanitisers did not impact the growth of microorganisms or rate of spoilage.
- *Shewanella* has an impact on the spoilage of cooked Blue Swimmer Crabs, even though their levels are below detection following cooking. Diverse micro-flora were seen on fresh Blue Swimmer Crabs.

- *Psychrobacter* sp. and *Pseudoalteromonas* sp. most likely to cause spoilage of uncooked Blue Swimmer Crabs.
- *Shewanella* sp., *Pseudomonas* sp., *Psychrobacter* sp. and *Pseudoalteromonas* sp. most likely to cause spoilage of uncooked Blue Swimmer Crabs.
- Treatment of whole cooked Blue Swimmer Crabs with sanitisers did not significantly lower the rate of bacterial growth.
- No significant growth of bacteria occurs between capture and transport to shore, even though core temperatures of crabs remain relatively high for many hours.
- The use of an ice slurry post cooking had a detrimental effect on the microbiological quality of cooked crab.
- Splitting of crabs for manual meat picking had a significant detrimental impact on the microbiological quality of the crab meat.
- Treatment of shelled and de-gilled crabs with sanitisers prior to picking saw a reduction in bacterial numbers.
- The level of impact of the treatment was affected by the amount of crabs processed, but crabs processed last still had lower bacterial counts than untreated meat.
- Some detrimental impact on the organoleptic quality of the final picked product resulted in the discontinuing of the treatment.
- Quality of crab mince investigated. The use of whole (unpicked) crabs gave slightly better microbiological counts than picked crabs.
- Crab mince now being used commercially to produce crab cakes.

6. SPOILAGE OF AUSTRALIAN SALMON

6.1 Development of a QI for Western Australian Salmon

6.1.1 Development of Preliminary QI Scheme for Western Australian Salmon

The preliminary trial was carried out with fish obtained on the day of harvest. Following harvest fish were left head on but gutted and bled and stored in flaked ice. Typical changes in attributes over time were documented by two trained observers daily for three weeks. Changes observed included skin appearance, odour, scales, eye colour and shape, gill odour, colour and mucus and abdomen changes such as stretch marks, odour and swelling. A score sheet was written from the observed results. From these observations specific descriptors were determined and agreed as describing the particular attributes at any point of storage. A draft scheme was then developed and presented to an industry based panel. Parameters of the QI scheme were added and removed at the discretion of the panel.

6.1.2 Validation Trials

An industry panel of at least six in each sitting were expected to rate four to six fish on a biweekly basis according to the finalised scheme (Table 6.1). In total, four separate trials were carried out to provide the quality index assessment data to validate the model.

Four validation trials using the finalised scheme were done in which fish stored for different periods of time were evaluated. A linear relationship between QI and equivalent days on ice was found (Figure 6.1.1). The QI can be calculated from days on ice:

$$\text{Quality Index} = 1.0435 \times \text{days on ice} + 2.2025 \; (R^2 = 0.9322)$$

Table 6.1 Quality Index scheme for Western Australian Salmon.

	Parameter	Description	Score
Skin	Colour / appearance	Grey-Green, bright	0
		Blood patches/lines appearing	1
		Dull grey/bronze	2
	Stiffness of the Whole Fish	Very stiff	0
		Slight flexibility	1
		Very flexible	2
Eyes	Shape/Form	Convex	0
		Flat	1
		Completely Sunken	2
	Pupils	Clear and Black	0
		Milky	1
		Grey	2
	Iris	Bright Green	0
		Yellow	1
		Pale/bloodshot	2
Gills	Colour	Bright red/pink/orange	0
		Dull red	1
		Brown	2
	Mucus	Transparent/Slightly cloudy	0
		Bloody / Opaque	1
		Brown	2
	Odour	Mild ocean/Fresh sea weed/iron	0
		Varnish	1
		Neutral/flat	2
		Sour/dirty socks	3
Abdomen	Odour	Fresh seaweed	0
		Varnish	1
		Stale/flat	2
		Sour	3
Quality Index			0-20

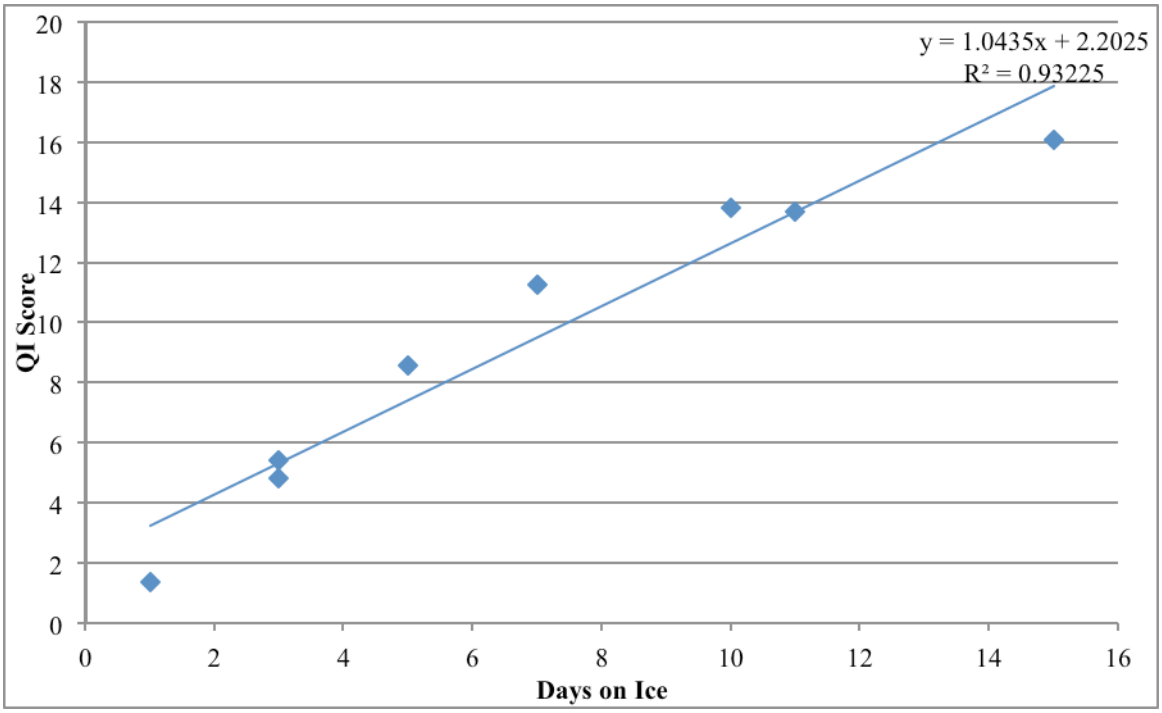


Figure 6.1.1 Comparison of Quality Index score with days stored on ice.

6.1.3 Evaluation of Cooked Fish Flesh using the Torry Scoring Scheme and Comparison with the QI scheme

The panel also assessed cooked samples of the fish at each sitting. A negative linear relationship was evident between days on ice and flavour of the fish (Figure 6.1.2) as judged using the Torry scheme ($R^2 = 0.7375$). The flavour can be calculated by:

Torry flavour = -0.2156 x days on ice + 8.8178

There was a good relationship between the QI and the Torry scores although there was greater variation in the Torry results.

The clarity of the information collected can be summarised well when the data collected from one sitting is charted (Figure 6.1.3). The agreement between panellists can be seen as the Torry scheme and days on ice had a very high R^2 value of 0.9737 compared with the R^2 of 0.7375 measured over the four sessions.

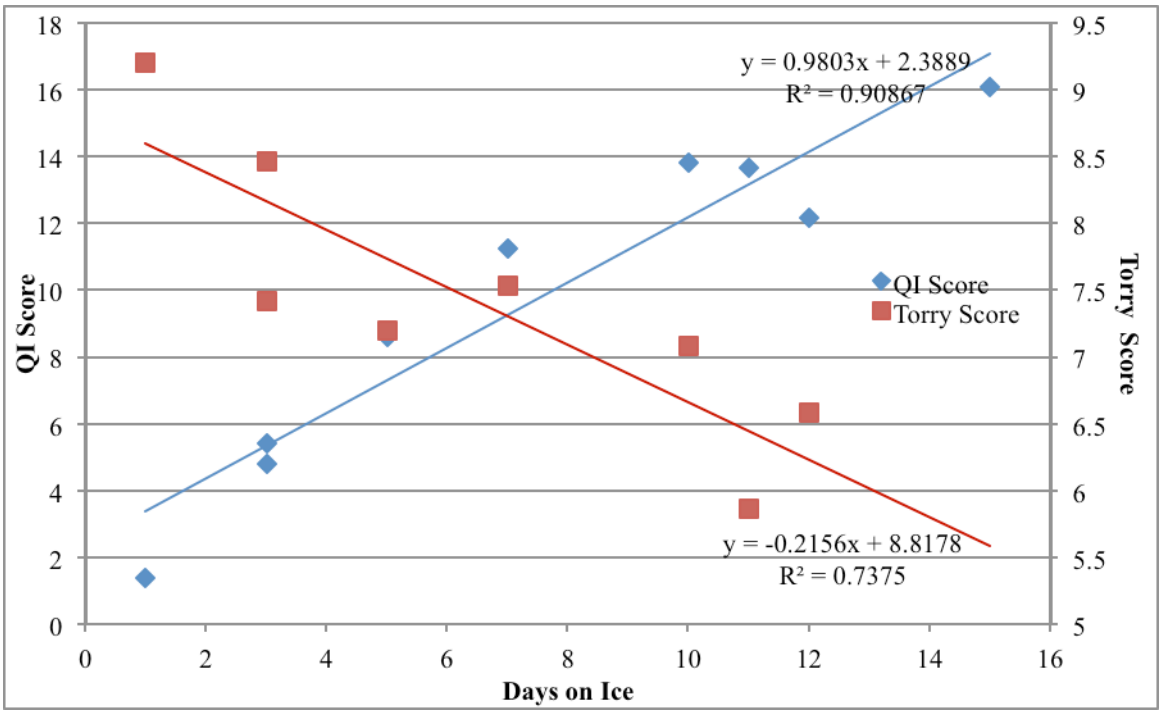


Figure 6.1.2 QI scores versus flavour scores for cooked samples of Western Australian Salmon flesh.

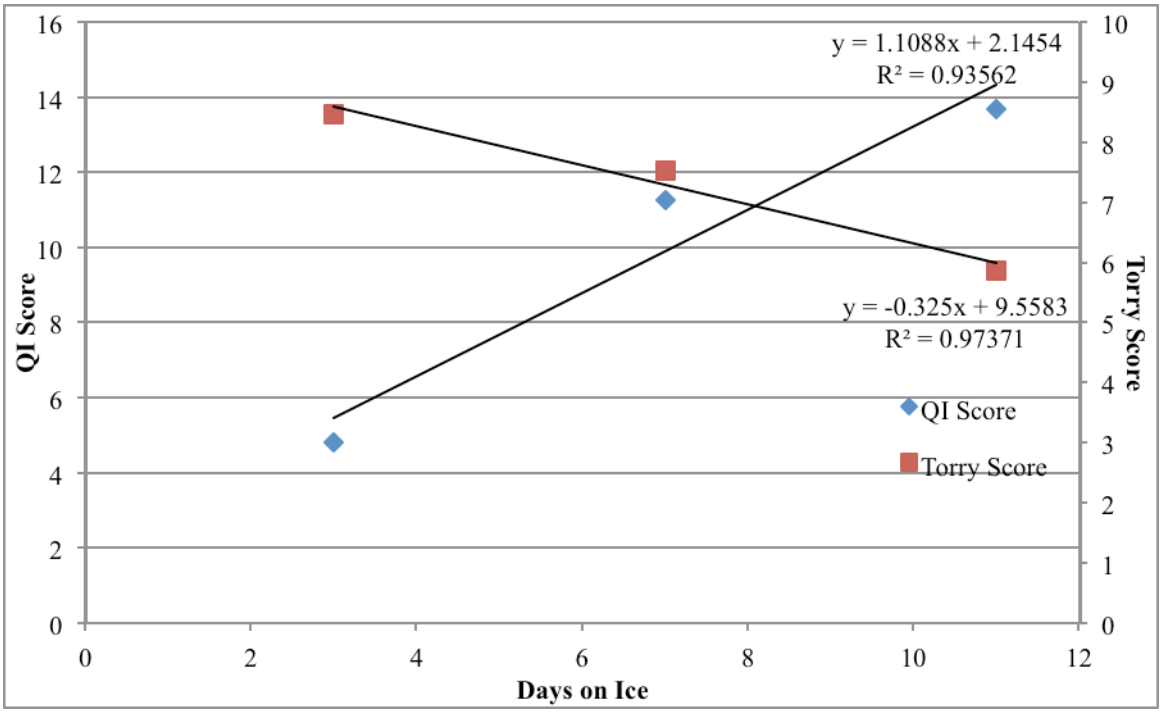


Figure 6.1.3 QI scores versus flavour scores for cooked samples of Western Australian Salmon flesh taken on one panel sitting.

6.1.3 Other Analyses

6.1.3.1 Photographs of the Fish

Photographs of the whole fish, as well as close ups of the eyes and gills were taken. Photographs were used as a benchmark for the validated scheme and may be inserted into the QI Manual (see Figure 6.1.4).

6.1.3.2 Microbiology

From the microbial tests taken, relationships between the QI and microbiological tests were found. A positive linear relationship exists between QI and log of Standard Plate Count (R^2 = 0.733) (Figure 6.1.5) and a positive linear relationship exists between the quality index and the log of Long and Hammer count (R^2 = 0.750) (Figure 6.1.6). Therefore, as the fish ages and the QI increases, the microbial activity on the fish also increases.

WEST AUSTRALIAN SALMON

(Arripis truttaceus) Head on gills on gutted (HOGOG)

Day 3

SKIN: grey-green, bright
EYES: convex, clear, bright green iris
GILLS: bright red, transparent/slightly cloudy mucus
QI SCORE: 3-6

Day 7

SKIN: blood patches, lines appearing
EYES: flat, metallic/yellow iris
GILLS: dark red, bloody/opaque mucus
QI SCORE: 8-10

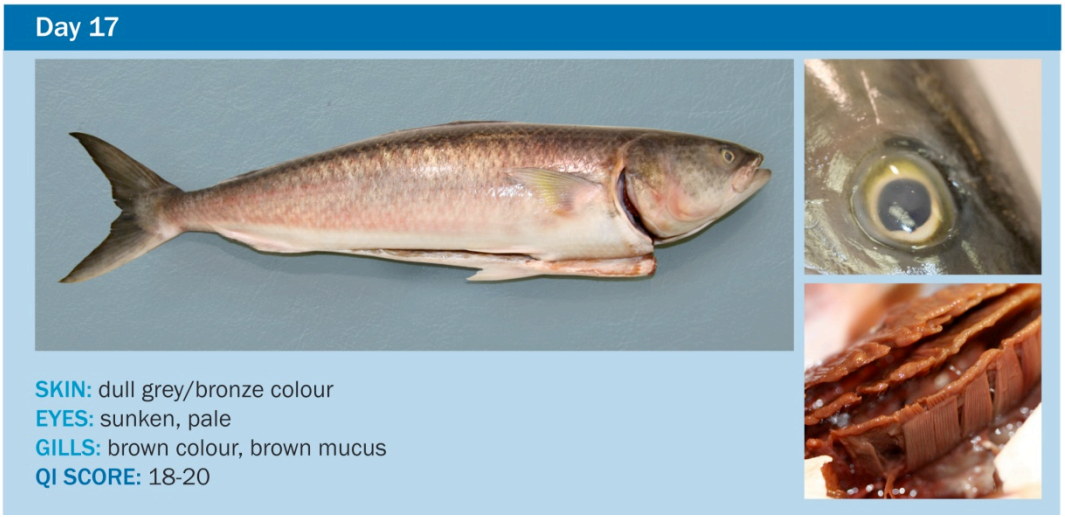


Figure 6.1.4 Photographs illustrating observed changes to Australian Salmon during ice storage.

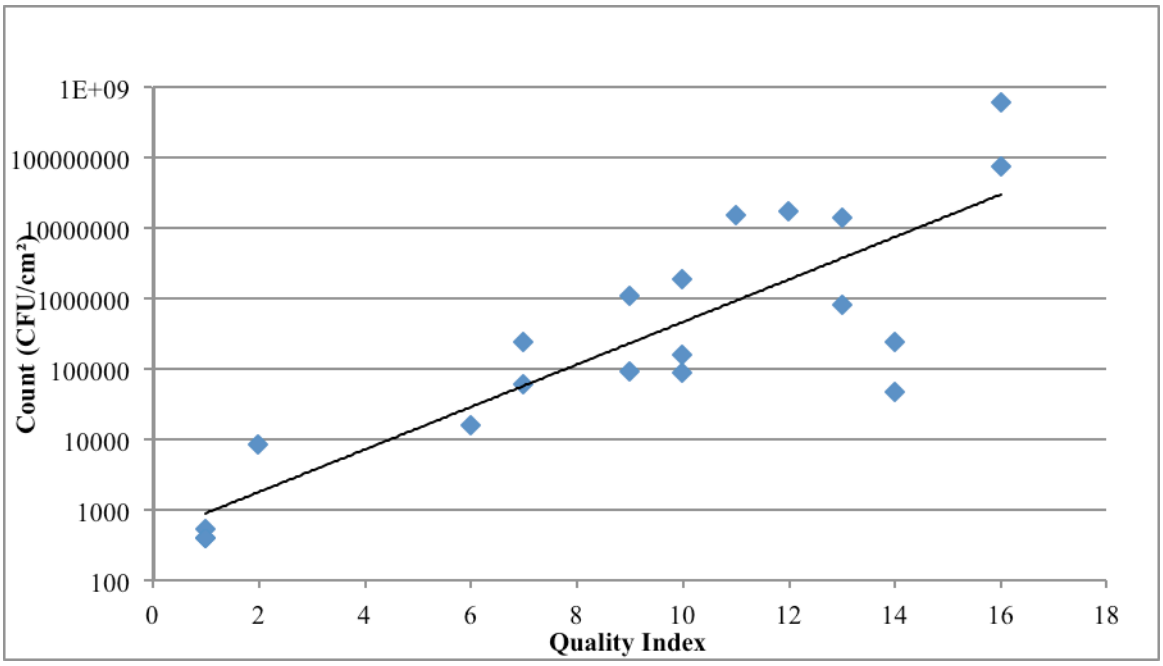


Figure 6.1.5 QI scores versus Standard Plate Count for Western Australian Salmon.

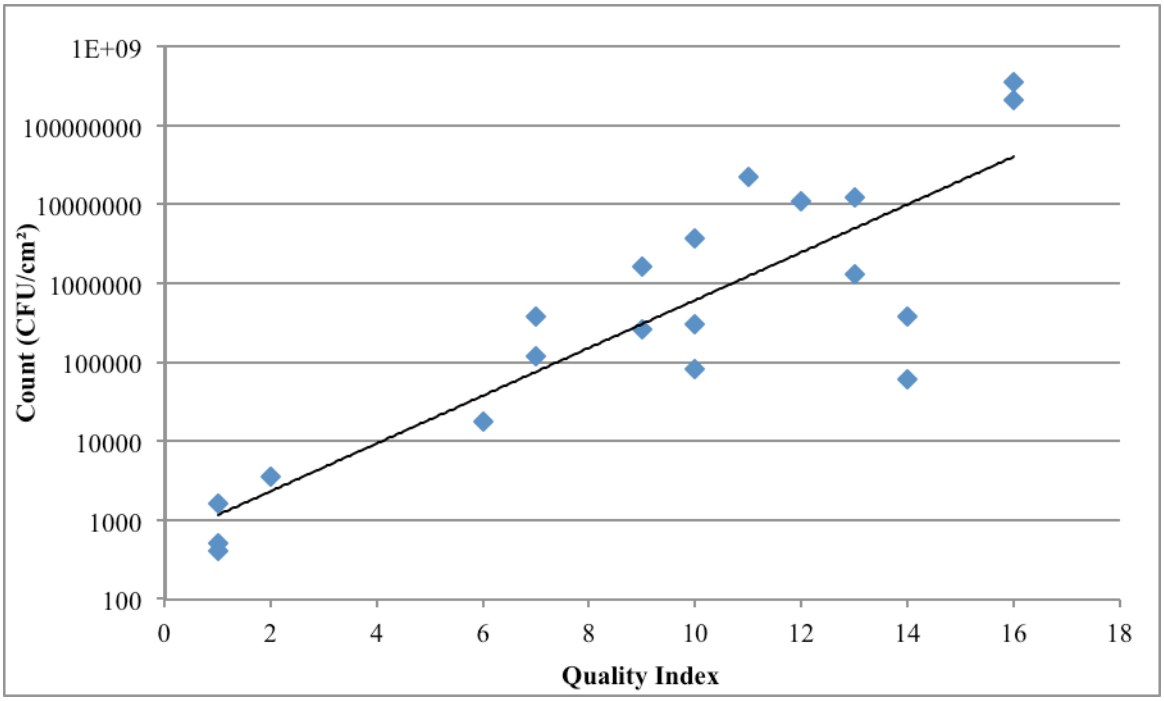
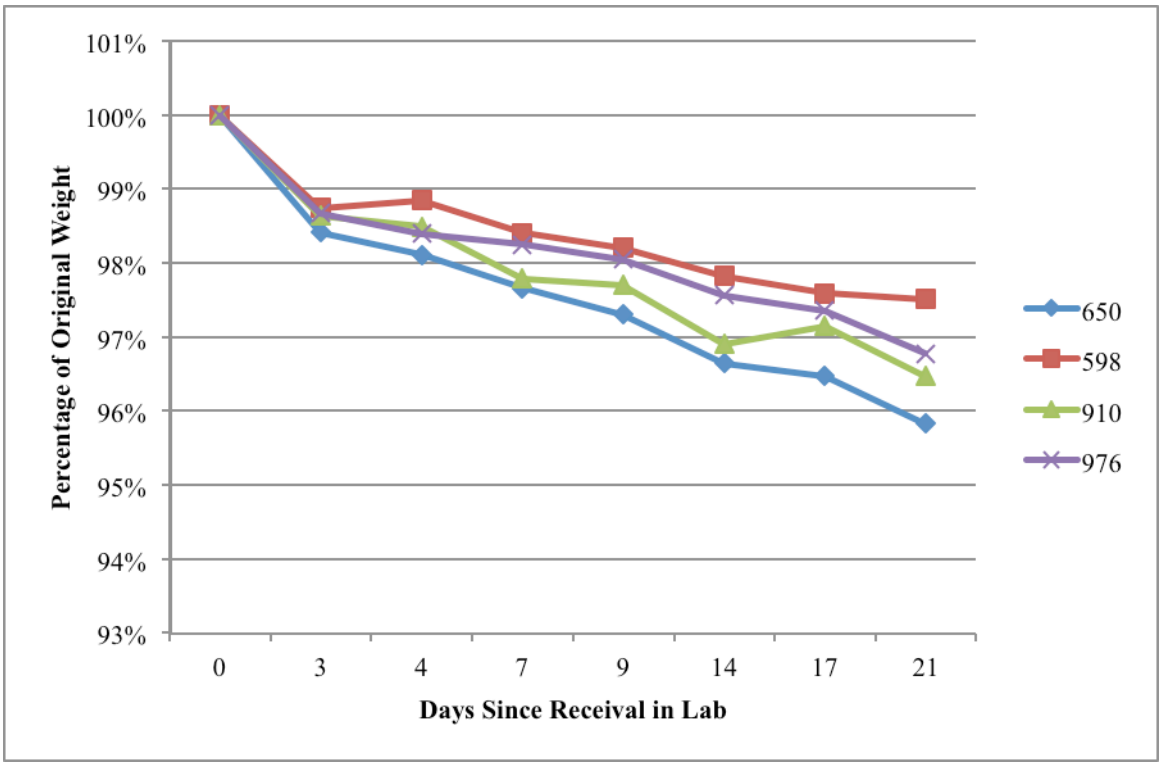


Figure 6.1.6 QI scores versus Long and Hammer Count for Western Australian Salmon.

6.1.3.3 Drip Loss

Western Australian Salmon had a very small percentage of drip loss (Figure 6.1.7).



6.1.7 Percentage weight loss of whole Western Australian Salmon.

6.2 Microbiological Spoilage of Australian Salmon

The Australian Salmon were harvested from Bremer Bay, Albany and Hamlin Bay as per commercial operation. Fish were gutted and bled, iced, packed in eskies and transported to the laboratories on the day of capture. In the laboratory, fish were stored in a refrigerated container, in drained tubs containing ice. Fish were checked daily and re-iced as needed. Sampling was performed using the swab method and quality index assessed using the QI scheme in detailed in Table 6.1.

A comparison of the average growth of aerobic mesophilic bacteria, specific spoilage organisms, aerobic psychrotrophic bacteria, and quality index over storage time is summarised in Figure 6.2.1. There was no significant difference observed between the counts for the aerobic mesophilic bacteria and aerobic psychrotrophic bacteria over 15 days of

storage on ice. The average count for both these parameters started at around 2000 CFU/cm^2 rising to an average count of around 3x10^8 CFU/cm^2.

The counts for specific spoilage organisms were consistently lower than those of the above parameters. On day 1 of storage there was no specific spoilage organisms (SSOs) detected on iron agar. By day 3 the number of SSOs increased to an average of around 1,000 CFU/cm^2 and by day 15 the average counts were around 6 x 10^6 CFU/g. These levels were significantly lower than the counts of aerobic mesophilic bacteria and aerobic psychrotrophic bacteria. A reduction in numbers of SSOs was observed between 8 and 10 days of storage. High levels of background flora were observed on the iron agar plates.

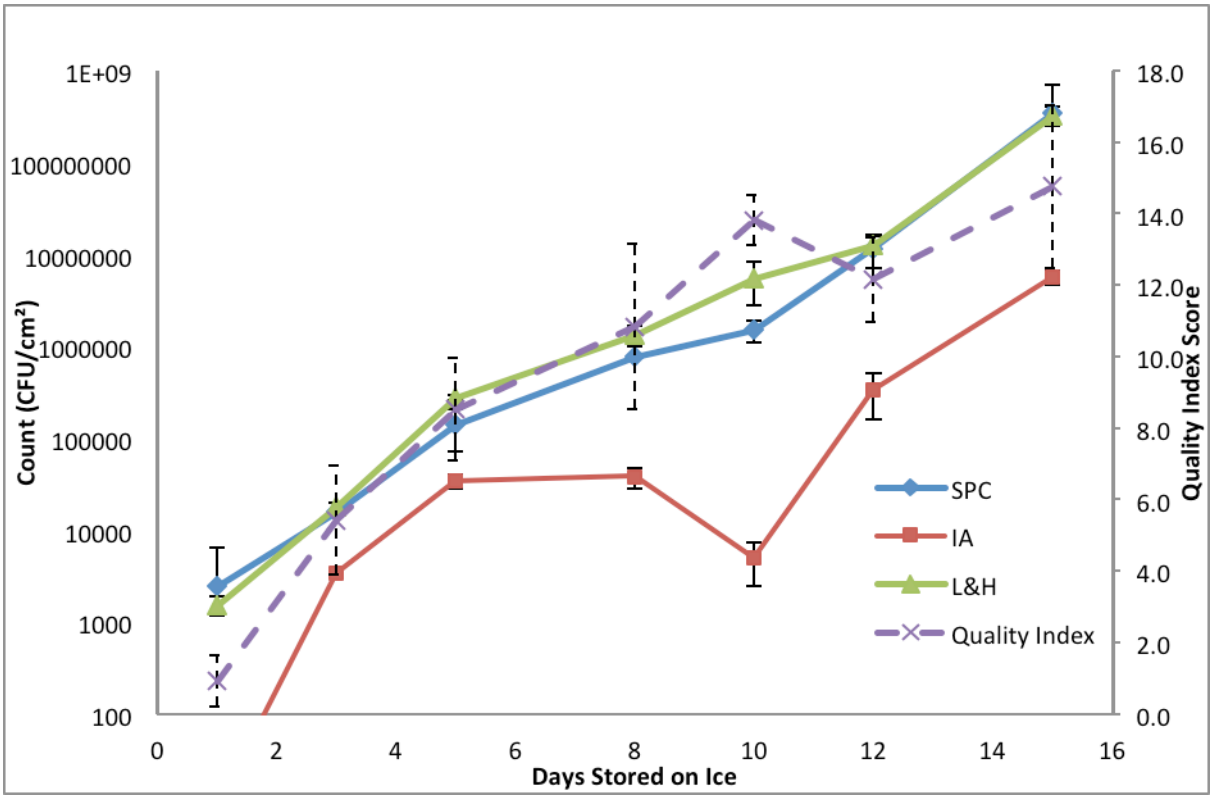


Figure 6.2.1 Microbiological quality and quality index of Australian Salmon stored on ice.

6.3 Bacterial Isolates From Australian Salmon

The major colony types from each agar plate (i.e. Plate Count Agar, Iron Agar & modified Long & Hammer Agar) were selected and tested as described in the Method section (up to 10 colonies per plate). Figure 6.3.1 shows the proportion of major genera and species present on fresh Australian Salmon.

Pseudomonas species account for a total of 23% of all major bacterial isolates on fresh Australian Salmon. *Pseudomonas putida* represented 14% of all major isolates. *Pseudomonas fluorescens* was the next most common single species of *Pseudomonas* found on fresh Australian *Salmon* representing 6% of all major isolates with other *Pseudomonas* species making up 13% of major isolates. *Microbacterium* (14%) and *Acinetobacter johnsonii* (13%) were the next most commonly isolated bacteria on Australian Salmon. The remaining major isolates included *Psychrobacter cryohalolentis* (11%), *Staphylococcus auricularis* (10%), *Chryseobacterium* sp. (9%), and *Acidovorax* sp. (1%).

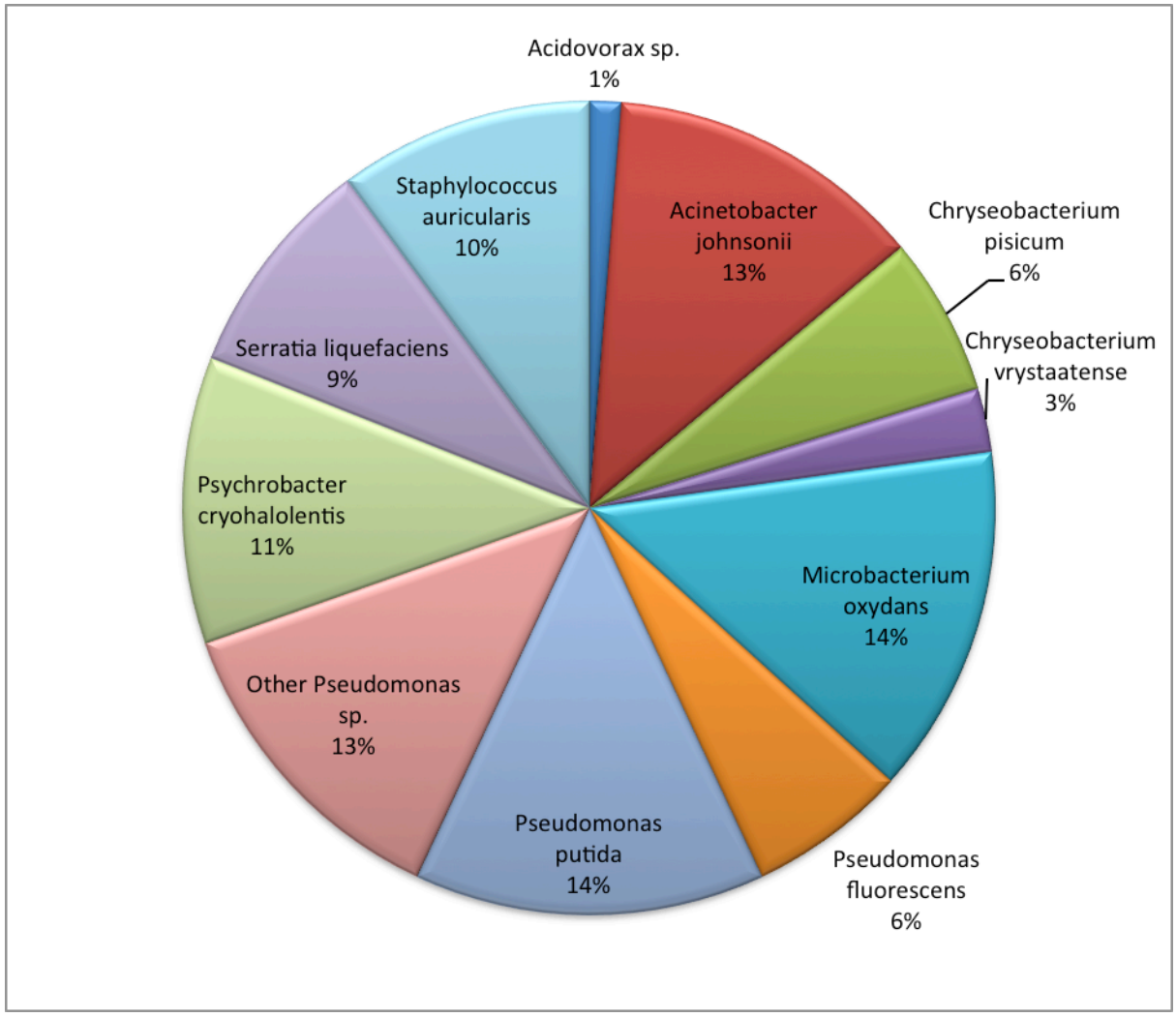


Figure 6.3.1 Major bacterial isolates from freshly caught Australian Salmon.

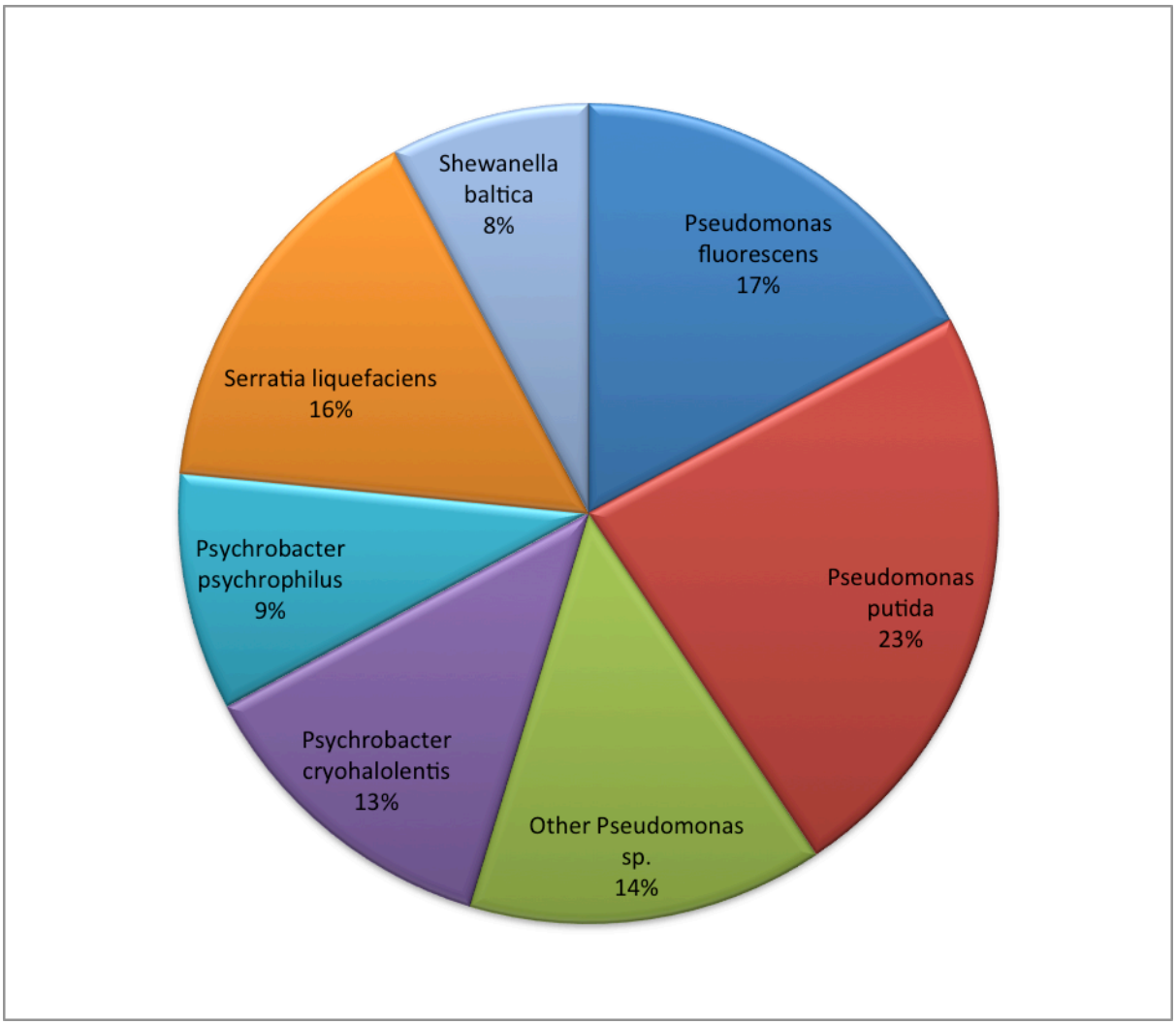


Figure 6.3.2 Major bacterial isolates from spoilt Australian Salmon.

Following refrigeration for 15 days, Australian Salmon's micro-flora showed a significant shift in the proportion of bacterial species present. *Pseudomonas* species represented 54% of all major isolates on spoilt Australian Salmon (Figure 6.3.2). *Pseudomonas putida* was still the major species of *Pseudomonas* isolated with 23% of all major isolates. *Pseudomonas fluorescens* was found to represent 17% of all major bacterial isolates and other *Pseudomonas* species representing 14% of major isolates. *Psychrobacter* species made up 22% of major isolates with *Psychrobacter cryohalolentis* being 13% of isolates and *Psychrobacter psychrophilus* being 9% of the isolates. Significantly 16% of major isolates were *Serratia liquefaciens*. This is significant as *Serratia liquefaciens* has been found to be involved in histamine production in scromboid fish (Rodriguez-Jerez, Mora-Ventura et al. 1994). *Shewanella baltica* made up 8% of the major isolates on spoilt Australian Salmon.

6.4 Summary of Important Findings

- A fully validated Quality Index Method for Australian Salmon was developed.
- QI scheme scores correlated well to Standard Plate Count and modified Long & Hammer counts.
- No significant difference was found between the standard plate count and modified Long & Hammer count on chilled Australian Salmon.
- The presence of *Serratia liquefaciens* on fresh and spoilt Australian Salmon significant as this species has been noted to produce biogenic amines (including histamine).
- *Pseudomons* sp., *Psychrobacter* sp., *Serratia liquefaciens,* and *Shewanella baltica* involved in the spoilage of chilled Australian Salmon.

BENEFITS AND ADOPTION

Fishers of all species tested in this project will benefit from a better understanding of the causes of spoilage for their product, improved handling and storage techniques, and the importance of temperature control for each of their target species.

The potential advantages of the use of sanitisers on the shelf-life of each tested species has been presented, allowing producers of each species to assess the potential costs and advantages to an increased shelf-life. Fishers of Sea Mullet and other netted species will benefit from the adoption of the use of sanitisers in ice slurries, as this had been shown to significantly reduce the rate of spoilage, and thus improving the shelf life of Sea Mullet. Anecdotal evidence suggests the use of sanitisers had a positive effect on the shelf life of netted whiting also.

The development of a Quality Index method for Australian Salmon will assist the industry to get a better penetration of their product into the human food market by assisting in the assessment of high quality produce. Likewise the development of the Float Test to assess the freshness of mussels could help the mussel industry ensure only quality product is sold.

FURTHER DEVELOPMENT

Dissemination of the results of this study should be carried out by seminar series to appropriate fisher groups to ensure producers are aware of the advantages of changes to their handling practices. Further research on the use of sanitisers on the quality of tropical fish species other than Goldband Snapper should also be investigated, as many species have a much shorter shelf-life than Goldband Snapper.

PLANNED OUTCOMES

The following planned outcomes have been or will be met as detailed.

1. Short document for each selected seafood line with results and recommendations to improve product quality and shelf-life and/or develop new products.
   These documents will be produced at the conclusion of the writing of the final report.
2. A final report for Seafood Directions.
   A report to be written for an upcoming issue of Seafood Directions
3. Additional information on effectiveness of sanitising in decreasing bacterial numbers and/or spoilage time in selected finfish species.
   The effectiveness of sanitiser application on a variety of finfish, and other seafood such as crabs and octopus, has been completed. The use of sanitisers proved effective for some seafood species (e.g. sea mullet), but not for others (e.g. octopus and crabs).
4. A better understanding of any correlation between bacterial numbers and shelf-life (as measured by the QI).
   The correlation between spoilage and bacterial numbers has been elucidated for all six seafood species investigated. For some there was a good correlation (e.g. Sea Mullet), for others there was no correlation (e.g. octopus), and for others the bacterial levels in the environment it is stored in had as large an impact on the perception of spoilage as the bacterial levels in the flesh (e.g. mussels).
5. Optimisation of sanitising and storage regimes to decrease numbers of spoilage bacteria and enhance shelf-life in selected finfish species.
   Storage regimes for all species were investigated and where appropriate changes were made.
6. Publication (websites, SQMI pamphlet) of information on the effective use of sanitisers

in the fishing industry.

Pamphlets have been published (see Appendix 5)

7. Further communication and possible application/adaptation of results to other seafood supply chains.

   Communication with producers was carried out by participation with a number of seminars for the seafood industry. Further communications will be carried out in the future to make the industry aware of the final results.

8. Enhancement of a culture collection of fish spoilage bacteria for future study and characterization.

   This project has resulted in a culture collection of over 5,000 organisms many of which have been identified. Different techniques for the identification of bacteria have been developed including biochemical, cell wall fatty acid, and sequencing based identification which have already been used by other researchers.

CONCLUSIONS

The spoilage of six different seafood species was investigated and means to overcome spoilage investigated. Each species had unique microbial populations and the impact of microorganisms on spoilage varied greatly between species. The use of sanitisers had a significant impact on the rate of spoilage of some species, and in some cases they had no impact. A one size fits all approach to controlling spoilage on seafood does not appear to applicable based on the findings of this study. Variation was not only found between major groups (e.g. finfish, crustaceans, molluscs, and cephalopods), but also within groups.

Spoilage of Sea Mullet occurred rapidly, with low bacterial levels increasing rapidly between 5 and 20 days of storage on ice. The rate of spoilage of Sea Mullet was reduced when the mullet was held iced in containers which allow the melted ice to freely drain away, or when sanitisers are added to the ice slurry which the mullet are placed into when caught. The use of sanitisers in the ice slurry has a significant impact on both the bacterial counts and quality index of sea mullet. Un-activated chlorine dioxide was found to be the best sanitiser to use in ice slurries. The bacterial species most likely to be in involved in spoilage are *Pseudomonas fluorescens, Psychrobacter nivimaris, Carnobacterium maltaromaticum,* and *Shewanella baltica*. There was no impact seen on the rate of spoilage or in the microbiological

populations on fish caught in different geographical locations. The treatment of Sea Mullet with sanitisers results in an overall reduction in all bacterial levels and does not appear to target particular bacterial groups or species.

The growth rate of bacteria and the rate of spoilage (as determined by the Quality Index Score) was slower for Goldband Snapper than for Sea Mullet. The microbial population on fresh Goldband Snapper appears to be more diverse than that found on Sea Mullet. *Shewanella baltica, Pseudomonas fluorescens,* and *Pseudoalteromonas* species appear to have the greatest impact on spoilage of Goldband Snapper. The use of chlorine dioxide based sanitisers results in a marginal improvement in quality index score and bacterial levels. This marginal increase would possibly not be financially viable for Goldband Snapper, but may be of use for other tropical snappers with a shorter shelf-life. A PhD project funded by the CRC for Seafood Quality was commenced following preliminary results of this project. A thesis by Rachel Tonkin (Curtin University) has been submitted.

The spoilage of refrigerated octopus does not appear to be related to microbiological growth, and the growth of "Specific Spoilage Organisms" (*Shewanella*) on octopus was insignificant. The growth of aerobic mesophilic and aerobic psychrotrophic bacteria occurred rapidly between 15 and 21 days of refrigerated storage. Storage of octopus in drained iced under refrigeration resulted in the dehydration of the tips of tentacles at and the formation of gelatinous blebs. The storage of octopus in an ice slurry stopped the dehydration of the tentacle tips, but worsened the rate of bleb formation. The formation of blebs was not due to the presence of high levels of bacteria.

Bacterial growth was found to be higher in the liquor expressed by Blue Mussels than in the mussel meat, and the bacterial growth in the liquor appeared to be the cause of a large proportion of the off odours detected during spoilage. The psychrotrophic bacteria counts were significantly higher mussel meat than the counts for mesophilic bacteria (Standard Plate Count) and "Specific Spoilage Organisms". The growth of Specific Spoilage Organisms (*Shewanella*) was much higher in expressed liquor that in mussel meat. Refrigeration of mussels in freely draining conditions and washing mussels frequently reduced overall bacterial levels. Unspoilt Blue Mussels had a diverse micro-flora dominated by *Vibrio* species. *Shewanella colwelliana* was the most commonly isolated specific spoilage organism.

This organism has been found to have positive impacts on the setting of larval stages of oysters in the United States. A test to assess the freshness of mussels was developed. The float test was found to have a good correlation with days stored, and microbiological counts.

The spoilage of Blue Swimmer Crabs was found to be significantly different depending on whether they were cooked or uncooked. Aerobic psychrotrophic bacteria showed the greatest growth in uncooked refrigerated Blue Swimmer Crabs, although the bacterial levels was not affected by the sex of the crab. *Psychrobacter* sp. and *Pseudoalteromonas* sp. most likely to cause spoilage of uncooked Blue Swimmer Crabs. Specific spoilage organisms (*Shewanella* species) are not significant to the spoilage of uncooked Blue Swimmer Crabs. It was found that the storage of Blue Swimmer Crabs in freely draining ice or in a refrigerator with a damp cloth coverage produced slightly less bacterial growth than when crabs were held in non-drained ice. *Shewanella* sp., *Pseudomonas* sp., *Psychrobacter* sp. and *Pseudoalteromonas* sp. most likely to cause spoilage of cooked Blue Swimmer Crabs. *Shewanella* levels were below detectable levels in cooked crabs immediately post cooking, but tended to grow rapidly after cooking. Treatment of crabs with sanitisers had no impact on the rate of spoilage of both cooked and uncooked crabs.

A Quality Index method for Australian Salmon was produced. The QI scores correlated well with the days of storage, plus the Standard Plate Count and modified Long & Hammer counts. When investigating the bacterial counts on Australian Salmon there was no significant difference between the standard plate count and modified Long & Hammer count. *Pseudomons* sp., *Psychrobacter* sp., *Serratia liquefaciens,* and *Shewanella baltica* involved in the spoilage of chilled Australian Salmon. The presence of *Serratia liquefaciens* on both the unspoilt and spoilt Australian Salmon is of great significance as this species has previously been found to produce biogenic amines (including histamine).

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APPENDIX 1: INTELLECTUAL PROPERTY

This research is for the public domain. The report and any resulting manuscripts are intended for wide dissemination and promotion.

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APPENDIX 5 SANITATION BROCHURE

Why use a sanitiser?

A) To prevent food poisoning from your product

The use of sanitisers will kill bacteria which may contaminate seafood, causing illness in consumers. Contaminants may be from pathogens naturally occurring in low numbers on fish, or present in the general aquatic environment and passed onto fish. Alternatively, there are other pathogenic bacteria which can be passed onto seafood product from handlers in processing plants.

B) To increase the shelf-life and maintain the quality of your product

Fish also harbour non-pathogenic, spoilage organisms on their surfaces that, once the seafood is harvested, may continue to thrive at low temperatures, resulting in rapid spoilage of the product. Fish deteriorate rapidly when contaminated by such species and the resultant odours and flavours prevent contaminated fish from being sold to the public. Addition of sanitiser (either to storage water, by dipping product in sanitiser or by addition to ice) has been shown to increase the shelf-life of the product.

Type of sanitisers

Sanitisers are identified by their active ingredient. The active ingredient will be labelled on the product. There will be different trade names of products, even those that have the same active ingredient. A list of commercial products is available at www.wafic.org.au.

The following table summarises some of the different types of sanitising agents that may be useful for the seafood industry.

Sanitiser type	Hypochlorites (K, Ca, and Na)	Chlorine Dioxide	Peracetic Acid Solutions (peracetic acid, acetic acid and hydrogen perioxide)	Quaternary Ammonium Compounds	Bitter orange extract
Properties of concentrated sanitiser					
- Form	Concentrated solution or powder	Stabilised Solution	Stabilised solution	Stabilised solution	Stabilised solution
- Stability	Good as powder, fair as liquid	Good	Good	Good	Good
Ease of preparation	Easy	Easy	Easy	Easy	Easy
Properties of working solution					
- Stability	Good	Moderate	Good	Good	Good
- Toxicity	Low	Moderate	Low	Moderate	Low
Conditions affecting effectiveness					
- pH	Ineffective>pH 8	Effective at broad pH range	Effective over broad pH range	Effective over broad pH range	Effective over broad pH range
- Hardwater	Ineffective in hard water	No effect	Limited effect	Not known	Not known
- Organic matter	Reacts to form chloramines	No effect	No effect	Not known	Not known
Food/safe	Must rinse unless <200ppm	Rinsing not necessary	Must rinse off	Must rinse off	Rinsing not necessary

More information

The Department of Fisheries Seafood Quality Management Initiative (SQMI) and the Western Australian Fishing Industry Council (WAFIC) have produced a number of publications that give greater detail on product handling and seafood safety. This includes Codes of Practice for lobster, marron, fish trapping, fish trawling, demersal gillnet and longline fishing and seafood retailers. These are available for free download at www.fish.wa.gov.au/seafoodquality or hard copies can be ordered by email from sqmimanager@fish.wa.gov.au or by contacting SQMI (9482 7333) or WAFIC (9492 8888).

Seafood Services Australia has many publications designed to help the fishing industry increase quality and comply with food safety legislation.You can find more information from the website www.seafoodservices.com.au or by phoning 1300 013 321.

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THE UNIVERSITY OF WESTERN AUSTRALIA

SEAFOOD SERVICES AUSTRALIA

Fish for the future

WAFIC
Western Australian Fishing Industry Council Inc.

WA SEAFOOD QUALITY SQMI MANAGEMENT INITIATIVE

JUN 2006 CR269

Effective sanitation for the seafood industry

Which is the most effective sanitiser for my operation?

Firstly, ask yourself these questions.

What is being sanitised?

Processing equipment and surfaces: Sanitisers should be used on equipment and surfaces to maintain a clean storage and processing environment for the fish.

Seafood product: There are some sanitisers that cannot be used on contact surfaces due to the effect they have on fish. Some compounds alter the odour of fish and may affect the taste. Others may leave residues that will require rinsing to meet food safety standards.

What is the environment being sanitised?

A sanitiser must work well in the conditions where it will most frequently be used. Fishing boats may be out at sea long enough that chilling alone will not prevent deterioration of the fish so sanitisers are commonly used, either in refrigerated seawater (brine) ice slurries or when making ice itself. Since the sanitiser will be diluted with sea water, compounds which work well with seawater of a pH of 8.5 at ambient temperatures of about 25-30°C are the best to use. The criteria for which the sanitiser is effective are crucial. For example, chlorine products are not effective when used in Australian coastal waters as the pH is 8.0 - 8.5 and the optimum pH for chlorine compounds such as those used in swimming pools is only approximately 7.2-7.6.

What price am I prepared to pay?

Obviously a sanitiser must add value to the catch, by both preventing spoilage and maintaining quality. It is generally a small cost in relation to the overall fishing operation, but one that can provide considerable benefit. Buyers should particularly look at the dilution rates at which the sanitiser remains effective and calculate the cost in terms of the quantity of product handled.

Does the sanitiser need to meet FSANZ//AQIS approvals?

Food Standards Australia New Zealand (FSANZ) does not regulate sanitisers, but does have maximum residue limits for fish products and does require that materials in contact with food are not 'otherwise likely to cause bodily harm, distress or discomfort' to a consumer. If a fish handler has any doubt about whether a sanitiser may make the seafood unsafe they should contact the local Environmental Health Officer or FSANZ for advice.

AQIS has a list of approved sanitisers for various products applications. Check the AQIS website or contact your local AQIS office if you are ensure of the suitability of your chosen sanitiser.

Are there any environmental considerations?

The industry has a responsibility for protecting the environment. Sanitisers that are likely to harm the environment should not be used in situations where effluent will reach the natural environment (e.g. on boats). Empty containers should be disposed of in a responsible manner. Also consider any reaction that the sanitiser may have with the container that it is to be used in.

Are there any occupational health and safety considerations?

Some concentrated forms of sanitiser may be harmful and difficult to store safely in a confined environment (such as, a boat). The sanitiser may require mixing or preparatory steps which may carry some risk. Users should always read the label, noting warnings and ensuring the sanitiser is used in accordance with the manufacturers instructions.

The most effective sanitisers for the seafood industry

Effective sanitation for fishing/processing equipment and surfaces

In experiments completed on used cutting boards and knives, the quaternary ammonia compound product was the most effective sanitiser. Slightly less effective were the bitter orange extract sanitiser, and the chlorine dioxide - based sanitiser. Note that in all cases bacterial levels were still significantly reduced when compared with the original microbial load. The dilution of sanitisers in artificial seawater (rather than potable fresh water) did not have a significant impact on the efficacy of the sanitisers.

The effectiveness of sanitisers is related to the cleanliness of the surface to be sanitised. If organic material such as blood, slime and gut is not removed by scrubbing and using detergents, it rapidly combines with and neutralises the disinfecting ability of any sanitiser solution. Simply soaking baskets, crates, knives and processing equipment in a sanitiser is ineffective.

Effective sanitation for seafood product

Sanitation experiments were completed using Australian herring, tailor and spangled emperor. The effectiveness of the sanitisers was assessed using draft quality index (QI) scores. QI is a scheme which allows handlers to visually examine a number of characteristics of the fish, assign a QI score and hence predict shelf-life. For more information on the quality index scheme please contact Mark Boulter, Sydney Fish Markets, markb@sydneyfishmarket.com.au.

When sanitisers were applied to the fish as a dip prior to storage, the application of sanitisers to all of the tested species consistently resulted in the reduction of the QI score when compare the QI score of untreated fish. In the market this would result in an increase in shelf-life. Chlorine dioxide based sanitisers were the most effective, giving slightly better results than the bitter orange extract based sanitiser.

Sanitisers were also added to ice prepared from artificial seawater, and stored with herring. Storage of herring in treated ice resulted in an increase in the shelf life of the herring as determined by QI scores. Again, chlorine dioxide was the most effective sanitising agent.

APPENDIX 4 SHEWANELLA CELLULAR FATTY ACID COMPOSITION (%)

FA	S. profunda	S.putrefacien	S. baltica	S.oneidensis	S.denitrific-ans	S.fridiidima-rina	S.marisflavi
14:0	4.0	2.3	2.2	2.6	3.9	3.7	1.4
15:0	4.3	3.2	7.8	4.7	4.4	2.5	4.2
16:0	13.3	19.1	4.3	14.8	13.3	11.8	13.0
17:0	0.8	1.5	0.6	2.8	0.8	1.2	1.8
18:0	ND	2.1	ND	1.1	0.3	0.1	ND
13:0-iso	6.4	2.5	12.4	2.5	9.3	6.3	6.7
14:0-iso	ND	0.3	1.6	2.3	0.6	0.6	0.8
15:0-iso	15.1	21.1	14.3	25.4	11.3	9.0	25.2
16:0-iso	ND	0.1	0.2	1.4	ND	ND	0.3
17:0-iso	ND	1.7	0.5	1.7	0.7	1.2	1.8
15:1ω6*c*	0.7	0.2	2.2	0.3	1.0	1.2	ND
16:1ω7*c*	30.4	29.6	24.1	23.3	31.3	51.1	ND
16:1ω9c	2.9	3.5	1.6	2.1	1.7	2.2	0.7
17:1ω6*c*	ND	0.9	1.4	1.5	ND	ND	0.7
17:1ω8*c*	5.9	6.7	11.0	8.0	4.8	3.0	6.0
18:1ω7*c*	1.8	6.0	0.8	5.7	1.7	5.3	3.8
18:1ω9*c*	1.5	3.8	0.8	2.9	1.0	1.7	1.6

ND= No Data

APPENDIX 3 BIOCHEMICAL REACTIONS OF *SHEWANELLA* SPECIES

	Cellobiose	Citrate	Galactose	Gluconate	Glucose	Gentobiose	Lactate	Maltose	Sucrose	α Cyclodextrin	N-Acteyl Glucosmine	4˚C	25˚C	30˚C	35˚C	37˚C	42˚C	$Na^{=}$ Requires	6% NaCl	10% NaCl	Gelatinase	DNase	Amylase	NO_3 - NO_2	NO_2 - N_2	Glycogen	TMAO	Tween 80	Haemolysis	Ornathine
S. baltica	+	v+	-	+	+	+	+	+	v+	+	+	+	+	+	+	d	-	-	+	-	+	+	-	+	+	+	+	v+	ND	+
S. putrifaciens	-	-	+	-	-	-	+	d	d	-	v	+	+	+	+	+	-	-	+	-	-	+	-	+	-	-	+	+	ND	+
S. figidimarina	+	v+	-	d	+	ND	+	+	+	ND	+	+	+	-	-	-	-	-	+	-	+	+	-	+	-	ND	+	ND	ND	d
S. oneidensis	ND	-	v+	-	-	ND	+	ND	-	ND	-	d	+	+	+	+	-	ND	d	-	+	+	-	v+	-	ND	+	ND	ND	+
S. colwelliana	-	-	-	v*	-	ND	-	-	-	ND	-	+	+	+	-	-	-	+	-	-	+	+	+	+	ND	ND	ND	+	ND	v*
S. marisflavi	+	-	-	ND	+	ND	+	+	-	ND	+	+	+	+	+	+	+	-	+	+	ND	ND	-	+	-	ND	ND	+	+	ND
S. aquamarinia	-	-	+	ND	-	ND	+	+	-	ND	-	+	+	+	+	+	+	+	+	-	ND	ND	+	+	-	ND	ND	+	+	ND
S. affinis	ND	-	-	-	+	ND	-	ND	w-	ND	ND	-	+	+	-	-	-	+	+	-	+	+	-	+	ND	ND	ND	+	+	+
S. denitrificans	-	-	-	ND	ND	ND	ND	+	-	ND	+	+	+	+	-	-	-	-	+	-	+	ND	+	+	+	+	-	+	ND	ND
S. japonica	+	-	+	-	+	ND	ND	+	-	ND	+	-	+	+	+	+	-	-	-	-	+	+	+	+	ND	ND	ND	ND	+	ND
S. olleyana	ND	-	+	+	+	ND	ND	-	+	ND	-	+	+	+	-	-	-	+	+	-	+	-	+	d	ND	ND	ND	+	ND	-
S. hafniensis	ND	+	ND	+	+	ND	+	+	-	ND	+	+	+	+	+	-	-	-	+	-	+	+	ND	+	ND	ND	+	ND	ND	+
S. profunda	-	+	-	-	-		+	w+	-	ND	ND	+	+	+	+	+	-	-	+	+	+	ND	+	+	ND	ND	ND	ND	ND	+

ND = No data
w = Weak reaction
v+ = Variable reaction - Majority of strains positive
v- = Variable reaction - Majority of strains negative