School of Public Health

An investigation of the microbiology and biochemical properties leading to extended shelf-life in goldband snapper (*Pristipomoides multidens*)

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This thesis is presented for the Degree of
Doctor of Philosophy
of
Curtin University

September 2012

Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: …….

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Acknowledgements

I'd like to start by thanking my wonderful supervisor Associate Professor Hannah Williams. Her guidance and support has helped me get this thesis finished. It was wonderful to always leave a meeting feeling re-enthused about my project, she was just plain wonderful! Thankyou Hannah!

Secondly I would like to thank Professor Tom Riley who had the patience and time to allow me to get my thesis into shape! His expertise, knowledge and overall easy going attitude was wonderful to have throughout my entire project, thanks Tom!

Thirdly, I would like to thank Steven Munyard who was just plain awesome at helping me in the lab. His patience and extensive knowledge was valuable in helping me gain the skills and knowledge I needed to get through this project. I couldn't have done it without you Steve!

Also a big thankyou to Vicky Solah for helping me with submission. Your guidance was invaluable!!

A big thankyou to Jim Pummell who supplied me with all the media and equipment a girl could ask for! Thanks Jim.

Also a big shout out to all those in the Food Science labs in Building 400! It was fantastic to have such a wonderful group of people around me to learn from their knowledge and expertise and help me when I had problems. Thanks everyone.

To my wonderful family and friends who have helped me get through the last 4 years, still smiling. I'm sure you're just as glad it's over as I am... the party is on me.

Finally, to my wonderful boyfriend Mark, who has put up with my whining and whinging for the last 4 years. It has been a wonderful life experience but it wasn't easy all the time and I'm glad I had someone to help me get through it....

Abstract

Introduction

Goldband snapper (*Pristipomoides multidens*) has an unusually long shelf life compared to many other fish species. Previous studies have indicated that the microflora present on the fish skin influences shelf life, however, no extensive studies have been performed. A comprehensive investigation into the shelf life of goldband snapper was undertaken in this study using microbiological, biochemical, sensory and physical analyses. Saddletail snapper (*Lutjanus malabaricus*) was used as a control species because it is genetically similar to goldband snapper, found in a similar geographic location but has a shorter shelf life. The implications of discovering the reasons for an increased shelf life are significant for the Australian fishing industry, in particular Western Australia, where transit times are extensive. It may be possible to use the knowledge gained by investigating goldband snapper to extend the shelf life of other tropical fish species.

Methods

Bacterial viable counts were done from fish flesh using plate count agar (total plate count), iron agar (spoilage count) and long and hammer agar (psychrotrophic count). Various biochemical tests, MALDI-TOF analysis, fatty acid analysis and DNA sequencing were performed to identify each isolate to species level. Biochemical analyses of fish flesh were determined for TMA, employing colorimetry (picric acid method) TVB – N, using steam distillation (Kjeldahl method) and titration and hypoxanthine which employed high performance liquid chromatography. Sensory analyses were performed by trained sensory panellists who were enlisted to assess to the quality of saddletail snapper from different storage days (3, 10, 17, 24 and 31 days) using the QIM scheme. The Torry scheme, which assesses the flavour and odour of cooked fillets, was used to validate the results of the QIM assessment. The colour change seen with goldband and saddletail snapper throughout storage was measured using a Minolta spectrophotometer (CM-500i/CM-500C). The measured colours were expressed as CIE Lab coordinates which display as L^*, a^* and b^*. The texture analysis method was based on measurements taken using a stable micro

systems (SMS) texture analyser (TA ·XT2i). The fish tested were compressed and the relaxation profile measured. Three points were measured along the lateral line of the fish and three samples were taken for every time point.

Results

Overall, saddletail snapper had a higher number of specific spoilage organisms (SSOs) than goldband snapper. On average goldband snapper had a maximum of 1 x 10^8 CFU / g in comparison to saddletail snapper which peaked between 1 x 10^{11} and 1 x 10^{12} CFU / g. Variation within the microflora for both fish species was at its highest within the first 3 days of storage. The spoilage flora isolated from goldband snapper appeared to be more evenly distributed in comparison to saddletail snapper. This correlated with the lower numbers of SSOs present on goldband snapper (22.58%), compared to saddletail snapper (52.83%), immediately post harvest. The proportion of Gram positive organisms as a total of the microbial flora was higher on goldband snapper than saddletail snapper. The number of Gram positive organisms peaked immediately post harvest for goldband snapper and then followed a downward trend. *Shewanella* and *Pseudomonas* species were the significant SSOs for goldband and saddletial snapper. The *Shewanella* population constituted over 80% of the spoilage organisms present on saddletail snapper. Within 12 h the proportions were approximately even between *Shewanella* and *Pseudomonas* species. The *Shewanella* population constituted approximately 70% of the total spoilage organisms present on goldband snapper immediately post harvest. Within 12 h of storage this ratio had reversed with *Pseudomonas* species now constituting 70% of the spoilage organisms present.

TMA concentrations for goldband snapper remained lower at the beginning of storage compared to saddletail snapper, however, at day 17 of storage the levels of TMA for goldband snapper surpassed those of saddletail snapper. Both species remained below the TMA limit for human consumption, 10 – 15 mg / 100 g. The amounts of TVB-N were higher for goldband snapper for most of storage, however, at the end of storage the amount of TVB-N was much higher for saddletail snapper. Hypoxanthine levels for saddletail snapper remained between 20 and 40 mg / 100g. Goldband snapper had higher levels of hypoxanthine, starting at approximately 50

mg / 100g at the beginning of storage to approximately 100 mg / 100g by day 15 of storage.

Saddletail snapper had a shelf life of only 24 days, using data collected from the QIM validation study. Textural analysis of saddletail snapper displayed a strong negative relationship with storage time, indicating that the fish became softer throughout storage. The texture of goldband snapper was soft and remained soft throughout storage, with the exception for a peak at day 17. The change in colour for both fish species was negligible and did not appear useful for determining spoilage, however, both goldband and saddletail snapper did increase in yellowness.

Conclusions

Biochemical and sensory analyses demonstrated a difference existed between the spoilage of goldband and saddletail snapper, however, they did not explain why goldband snapper had a longer shelf life. Results from the microbiological analyses demonstrated that the microflora present on goldband snapper had an impact on its shelf life. The increased levels of *Pseudomonas* species at the beginning of storage appeared to limit the number of *Shewanella* species resulting in an extended shelf life. Investigation into the microflora of other tropical species to confirm these results needs to be undertaken. Testing for genetic differences between each of the *Pseudomonas* species should be also carried out and the spoilage potential needs to be quantified for each of the species isolated. The knowledge obtained from this project will contribute to a wider understanding of fish spoilage and allow treatments and other preventative measures to be developed to increase shelf life across a range of fish species caught in Australia.

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CHAPTER 1: INTRODUCTION

Introduction

Pristomoides multidens, known commonly as goldband snapper is found in the Indo-pacific region of the world, from the Red Sea, Arabian Sea and East Africa over to Samoa and also from the north of Japan down to the north of Australia and can be found from Cape Pasely, WA across the northern coast to Moruya, NSW (Newman, et al., 2008).

Goldband snapper is a commercially popular fish in Australia due to its year round availability. The species is subtropical inhabiting offshore reefs, shoals and areas close to the seabed and is commonly caught at depths of 40 to 200m (Newman, et al., 2008). Due to its geographical location goldband snapper is one of the target species for the Timor Reef fishery, the demersal fishery in the Northern Territory, and the northern demersal scalefish industry in Western Australia.

Timor Reef Fishery

The Timor Reef Fishery operates in the Timor Sea, in a region extending north –west of Darwin to the Western Australian/Northern Territory border and to the outer limit of the Australian Fishing Zone (D McKey, Lloyd, & Errity, 2006). The total catch value for the Timor Reef Fishery in 2006 was $2.77 million with goldband snapper contributing 57% of the catch and saddletail snapper (*Lutjanus malabaricus*) contributing 32%.

Demersal Fishery

The dermersal fishery operates in the Northern Territory covering waters 15 nautical miles from the coastal baseline to the outer limit of the Australian Fishing Zone (AFZ) (D. McKey & Errity, 2007). In 2007, 46 % of the total catch was made up of goldband snapper species and 33% by saddletail snapper. The total catch value for the demersal fishery in 2007 was $1.94 million (D. McKey & Errity, 2007).

Northern Dermersal Fishery

The northern demersal scalefish industry (NDSF) encompasses an area off the West Australian coast, east of longitude 120°E. The NDSF is divided into two fishing areas, offshore and inshore areas. Catch rates for each species in 2006 were 305kg/ standard day for goldband snapper with a total catchment for the year of 336t (Newman, et al., 2008). Although a target species within

these 3 fisheries, goldband snapper has been recorded in other northern Australian fisheries in smaller quantities and mainly as byproduct catch. These fisheries include the Gulf of Carpentaria Line Fishery (GOCLF), the Gulf of Carpentaria developmental fin fish trawl fishery (DFFTF) and the Pilbara demersal finfish fisheries (S. Newman & Skepper, 2004).

Most tropical snappers caught by line and trap methods within Australia are sold fresh as either whole fish or fillets (Clarke & Lloyd, 2002). The majority of the catch is usually conveyed to major cities close by or sold and marketed interstate. Quality, for the purpose of this thesis, are the characteristics of food that make it acceptable to consumers. Maintaining quality for an extended period of time whilst fish are being transported is difficult. Tropical snappers caught in the north of Western Australia are an example of how difficult logistics can be. After being stored in ice slurry or chilled conditions for at least two days on board the fishing vessel, the fish are offloaded and transported via a refrigerated truck, down to Perth which takes approximately two days. The fish are then distributed to retail outlets around the Perth metropolitan area. Maintaining quality of such a perishable food item is difficult with extended travel providing opportunities for increased enzymatic and bacterial spoilage.

The changes in fish which contribute to spoilage commence immediately post mortem. Early stages of fish spoilage are caused by enzymatic processes, once vital for maintaining life, which have become degradative after death. For example, if a fish is caught and killed whilst eating, its digestive enzymes, which are already active, continue and eventually begin to digest the fish itself. Enzymatic spoilage is responsible for the loss of flavour and fresh odour experienced in early stages of spoilage. Enzymes are slowed in colder temperatures (Ghaly, Dave, Budge, & Brooks, 2010) and so to slow enzymatic spoilage, fish are thrown into iced slurries or cold storage rooms. The cold storage conditions are a multipurpose approach to delaying spoilage, with degradative enzymatic processes being slowed as well as the growth of micro-organisms present on fish skin. Microbial growth is a common cause of fish spoilage and is responsible for the unpleasant odours and flavours paired with softening or toughening of flesh experienced in later stages of spoilage.

Maintaining colder storage conditions can be very difficult throughout the supply chain, with opportunities for temperature spikes occurring every time the fish are moved (eg. during unload from the fishing boat to the auction house, from the auction house to retailers and from the retailers to consumers). A spike in temperature impacts negatively on fish quality, increasing spoilage and decreasing shelf life. An example of the effect storage conditions may have on the storage life of fish was exhibited in a study performed by Surti, Taylor, & Ma'ruf (2002). Red snapper (*Lutjanus argentimuculatus*) usually exhibits a shelf life of 16 days, however it was discovered that this may vary greatly on the storage conditions. For example, after 4 h at ambient temperatures, the shelf life for the red snapper was halved from 16 to 8 days (Surti, Taylor, & Ma'ruf, 2002).

Fresh fish is a popular option to consumers and so focus on storage conditions is a major area of research within the industry. Different methods of maintaining characteristics pleasing to the consumer are continuously being investigated along the processing chain from catch to consumer, as maintaining good quality in seafood is the key to an increase in shelf life and therefore an increase in domestic sales and international export.

Background to the present study

Studies have shown that storage conditions are responsible for extending shelf life of seafood by delaying enzymatic and microbial spoilage (Ghaly, et al., 2010). It has been discovered that the microflora initially present on the fish also plays an important role in shelf life. Water temperature significantly impacts on the microflora of fish species with the microflora isolated from warmer waters adapted to living at high ambient temperatures (mesophilic or mesotrophic in nature) and those from cold waters (psychrotophic in nature) easily adaptable to the chilled storage conditions fish are exposed to once caught (Lima dos Santos, 1981).

Comparative shelf life studies (Devaraju & Setty, 1985; Sumner & Magno-Orejana, 1985) have supported the claim that demersal fish, harvested from cold waters, have a shorter shelf life than pelagic fish, predominantly harvested from warm waters. The microflora present on the demersal fish are dominated by psychrotrophic bacteria and

therefore able to immediately adapt to the storage conditions, growing at an exponential rate and decreasing the shelf life of the fish.

Psychrotrophic bacteria are commonly isolated from spoiled fish. *Pseudomonas* species and *Shewanella* species are the psychrotrophic organisms most commonly involved with fish spoilage and are often referred to as specific spoilage organisms (SSOs). Researchers have concluded that although responsible for spoilage, the SSOs are not responsible for the difference in shelf life since all species caught from tropical, sub-tropical and temperate waters experience the same spoilage bacteria when stored in chilled aerobic conditions (Gram & Huss, 1996). To confirm this theory, Gram, Oundo, & Bon (1989) inoculated Nile Perch (*Lates niloticus*), a tropical freshwater fish with a shelf life of 30 days, with two psychrotrophic strains of *Pseudomonas* species Inoculated fish experienced a reduced shelf life of 2 weeks in comparison to the 30 days.

Fish species with an extended shelf life are advantageous in the Australian fishing industry where distance between catch and consumer is an extreme disadvantage. Shelf life studies performed on tropical fish species are limited especially on commercial species caught within Western Australia. However, an early study performed by Ames & Curran (1985) mentioned an unusual result with a commercially caught sub-tropical snapper experiencing an extended shelf life. The study involved ice storage trials on two demersal fish, goldband snapper and ruby snapper (*Etelis carbunculus*) caught in Vanuatu. The appearance of the gills, eyes and condition of the skin, were assessed, as well as texture and odour. Measurements were also made of the acidity, volatile bases and bacteria on both the skin and in the flesh of the fish. Panelists were asked to assess the flavour of the fish using a five point scale (1. Very fresh odour, 2. Fresh odour, 3. Neutral odour, 4. Slightly off odour 5. Strong off odour). Contrary to previous studies indicating pelagic species were the only species to demonstrate an extended shelf life goldband snapper and ruby snapper both had an extended shelf life of 5 weeks and 4 weeks, respectively. Due to its sub-tropical habitat, the shelf life for goldband snapper was considered unexpected and unusual since many cold water fish species have a limited shelf life of up to 2 weeks. Further research of this species may result in an impact on the quality and shelf life of other finfish species. An improvement in the shelf

life would promote an increase in quality, export opportunities and sustainability of the entire Western Australian fishing industry.

Hypothesis

The microbial populations for each fish species, in particular the specific spoilage organisms, differ between goldband and saddletail snapper, causing a difference in shelf life.

Aim

Determine why goldband snapper experiences an extended shelf life using extensive sensory, physical, biochemical and microbiological analyses.

Objectives

1) To validate the Quality Index Method (QIM) Scheme for the control species, saddletail snapper (*Lutjanus malabaricus*) with West Australian caught saddletail snapper. Allowing the investigator to effectively monitor the sensory changes occurring on the fish during the spoilage process and compare it to those of goldband snapper which already has a developed QI Scheme.
2) To investigate and compare the spoilage of goldband and saddletail snapper by comparing biochemical, microbiological physical and sensory analyses. Comparing analyses will identify any other aspects of spoilage which may contribute to a difference in shelf life between the two species.
3) To identify the changes in the microflora present on the flesh & skin of goldband and saddletail snapper throughout the spoilage process. Changes in microflora during storage will influence the type of spoilage that occurs and its rate. Variations in microflora will then cause variations in food safety & fish species shelf life

CHAPTER 2: LITERATURE REVIEW

Background

Australian Fishing Industry

Pristomoides multidens, known commonly as goldband snapper is a member of the Lutjanidae family. It is a tropical snapper which inhabits a demersal environment living at depths of 40 to 200 m. Goldband snapper are found in the Indo-Pacific region of the world. They are the target species for the demersal fishery in the Northern Territory (NT), the Timor Reef fishery, the northern demersal scalefish fishery (NDSF) in WA and are commercially caught from Shark Bay northwards. The control species used for the project, saddletail snapper (*Lutjanus malabaricus*), is also widespread in the Indo-Pacific region. Saddletail snapper are found from the Fiji Islands to the Arabian Sea and from northern Australia to Southern Japan (Elliott, 1996). Specifically in Australia they can be caught from Shark Bay, WA, around the north coast to Sydney, NSW (Newman, et al., 2008). Saddletail snapper is the other key species targeted by the Timor Reef fishery and demersal fishery in the NT (McKey, Lloyd, & Errity, 2006; Newman & Skepper, 2004).

West Australian Fishing Industry

The commercial fisheries in the North Coast Bioregion of Western Australia are responsible for catchment of tropical finfish, in particular various emperor, snapper and cod species. These fisheries are considered the most valuable finfish sector in the state, having an estimated annual value of $12 million and a total catch of approximately 3,000 t (Newman & Skepper, 2004). The NDSF is responsible for the majority of goldband snapper caught and lies in the north-west shelf region (Newman & Skepper, 2004).

The NDSF encompasses an area off the West Australian coast, east of longitude 120°E. The NDSF is divided into two fishing areas, offshore and inshore areas.

Tropical Snappers, emperors and groupers are caught in the offshore division, with goldband snapper and red emperor being the major target species. Catch rates for each species in 2006 were 305 kg / standard day for goldband snapper and 140 kg / standard day for red emperor, with a total catchment for the year of 336 t for goldband snapper (Newman & Skepper, 2004). Saddletail snapper, spangled emperor, cods, groupers and other demersal scalefish are also by-product species retained in the NDSF (Newman, et al., 2008).

Another fishery in Western Australia is the Pilbara Demersal Finfish fisheries, which targets 10 species of fish, including goldband snapper. The total catch is less than the NDSF with 81 t goldband snapper caught for 2006. The Pilbara Demersal Finfish Fisheries target waters lying north of latitude 21° 35' S and between longitudes 114° 9' 36" E and 120° E on the landward side (Newman & Skepper, 2004).

Goldband snapper is a popular fish being available all year round and it is also considered a high value species, paying on average $6 – 9 / kg. Improving the shelf life of this species will result in an enormous impact on its value, in turn positively affecting a majority of the northern Australian fisheries.

Goldband snapper and saddletail snapper

As previously mentioned goldband snapper is a demersal fish, commonly caught at depths of up to 300 m where the temperature is close to 15˚C (Ames & Curran, 1985). They are subtropical, inhabiting offshore reefs, shoals and areas close to the seabed with occasional vertical relief such as canyons (Newman, et al., 2008). Goldband snapper has an unusually extended shelf life similar to tropical fish species harvested from warmer waters. Ames & Curran (1985) speculated that the extended shelf life may be due to the low fat content of the fish and the slow growth of bacteria in the flesh of the fish.

Saddletail snapper is a reef associated fish and can be found in continental shelf waters at depths of at least 140 m (Newman, et al., 2008). Saddletail snapper inhabit coastal and offshore reefs, shoals and areas close to the seabed with occasional vertical relief such as canyons. On the north - west shelf of WA they are often found schooling with red snapper and associated with sponge and gorgonian habitats (Newman, et al., 2008). Saddletail snapper tends to spoil faster than goldband snapper which is why it was employed as the control for this project.

Food spoilage and its effect on shelf life

Food spoilage can be 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). The term “unacceptable” is product specific, with different levels of certain characteristics deemed acceptable. Food spoilage can be defined on different levels, the most acute form being when spoilage becomes a food safety issue. Less severe forms of spoilage encompass loss of flavour, odour or taste of the product, physical damage, insect damage or microbial growth. Nutritional value of a product may also be affected as a result of degradation and so contents may not meet the nutritional value stated on the packaging (Steele, 2004).

Food spoilage may be caused by physical or chemical reactions such as the action of enzymes or by microbial activity (Huis in't Veld, 1996). Many such changes take place in foods during processing and storage, affecting food quality. The factors that influence these changes in the product are able to be categorized and described as intrinsic, extrinsic or implicit factors. Intrinsic factors are properties of the final food product which may be influenced by the quality and type of raw material used initially, as well as the structure and formulation of the product. Examples include water activity, pH value, redox potential, available oxygen, nutrients, natural microflora and surviving microbiological counts, natural biochemistry of the product and use of preservatives in the product. Extrinsic factors are encountered by the product as it moves through the food chain and may include temperature, relative humidity, exposure to light, environmental microbial counts, composition of atmosphere within packaging, subsequent heat treatment and consumer handling (Kilcast & Subramanian, 2000). 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). These intrinsic and extrinsic processes can be

further divided into microbiological, chemical, physical and temperature related categories. It is these processes which are collectively responsible for either reducing shelf life or accelerating processes that limit shelf life (Dalgaard, 2003).

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). The focus on the shelf life of food products has intensified since demand for higher quality food has increased. Strict labelling requirements are adhered to by manufacturers to ensure the products consumers are buying are of the highest quality (Kilcast & Subramanian, 2000). Understanding the shelf life of a product is important to ensure quality but also to guarantee the product is safe for consumption. It also allows processors and retailers to plan how long a product may be kept before being discarded (Doyle, 1989).

In addition to fulfilling quality legislation which includes laws and regulations to be met by food chain operators to ensure food is safe and of adequate quality, the study of shelf life can also have enormous implications for industry. 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. Most 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 a lot of the catch is sold. Extended travel distances are common, especially in W.A. 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 allowing increased opportunity for sale. An additional benefit is that consumers then have the opportunity to experience quality fresh food products not locally available.

Fish quality and, indirectly, shelf life are affected by two main groups of factors; environmental factors before and after harvest, and the initial state of the fish. Environmental factors impact greatly on the quality of the fish. For example, after spawning fish have a thinner, flabbier appearance, and their flesh is generally more watery and contains less proteins and / or fat (Pedrosa-Menabrito & Regenstein,

1990b). Parasites or other organisms may also affect the quality of the fish, as well as contamination from pollutants located in the fishing ground. The method of capture, on-board handling which includes processing, storage conditions, and fishing vessel sanitation may also affect quality (Fraser & Sumar, 1998b). The initial state of the fish post-harvest can also cause variation in fish quality. Larger fish maintain better quality and so size appears to be influential in sustaining quality (Pedrosa-Menabrito & Regenstein, 1990b). Differing spoilage rates have also been found across different species due to different characteristics. For example, fatty fish deteriorate sooner than leaner fish species and fish caught in warmer waters tend to have a longer shelf life when in chilled storage in comparison to those caught from cold waters (Surti, Taylor, & Ma'ruf, 2002). The initial state of the fish cannot be altered and so research has focused on determining the effects of storage and handling on shelf life across many different fish species. Environmental factors after harvest have a huge impact on the quality and shelf life of a product, especially as fish is such a perishable food product compared to other foods (Ames & Curran, 1985; Fraser & Sumar, 1998b; Pedrosa-Menabrito & Regenstein, 1990b; Surti, et al., 2002).

It is believed that the structure and properties of fish muscle cause it to be susceptible to spoilage (Pedrosa-Menabrito & Reichelt, 1988). There are many processes and reactions that are responsible for changes in appearance and texture, or cause the development of off odours and flavour. Sometimes they are obvious, for example, slime formation, however, at other times they can be hard to identify and quantify (Huis in't Veld, 1996). Many of the techniques used to measure spoilage are performed using microbiological and chemical analysis. Sensory analysis is a simpler method that quantifies the degree and progress of spoilage. Unlike the microbiological and chemical analysis it does not determine the causes of spoilage as it is a non-invasive analysis however, it is able to measure the deteriorative processes of a food product and it is these results that relate best to the consumer (Pedrosa-Menabrito & Regenstein, 1990b).

Microbiological spoilage and detection

The shelf life of fresh fish is greatly influenced by autolysis and growth of microbial populations (Jeyasekaran, Maheswari, Ganesan, Jeya Shakila, & Sukumar, 2005). The proliferation of micro-organisms in foods depends on the properties of the

product as well as its processing and storage conditions (Huis in't Veld, 1996). Intrinsic and extrinsic factors can favour an increased growth rate and select for certain types of organisms however, populations of microbes also influence each other through implicit factors. Implicit factors include the properties of the organisms themselves as well as how the organism responds to its environment and interacts with other organisms (Adams & Moss, 2008). The interactions between micro-organisms may have a positive or negative effect on their growth rate, for example, a synergistic interaction occurs when the production of essential nutrients from the growth of one micro-organism allows another group of organisms the opportunity to grow when usually they would be unable to. Antagonistic processes may occur when there is competition from numerous groups of organisms for essential nutrients, changes in pH or production of antimicrobial substances. A good example is the use of bacteriocins produced by some groups of bacteria to affect the survival and growth rate of other nearby groups of organisms (Huis in't Veld, 1996).

Fish are a proteinaceous food, serving as a good substrate for microbial growth due to its high moisture content, neutral to low pH and high nutritional value (Huis in't Veld, 1996). The flesh 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, 19951995).

A high bacterial load, calculated from the total plate count, is used to indicate 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 (SSOs) 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.

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). The well-known spoilage organism for iced marine fish is *Shewanella putrefaciens,* while *Pseudomonas* speciesspecies 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).

The total microbial flora on cold water fish is dominated by psychrotrophic Gram negative bacteria. Common aquatic bacteria include *Acinetobacter*, *Flavobacterium*, *Moraxella*, *Shewanella*, *Pseudomonas*, and members of the *Vibrionaceae* and *Aeromonadaceae* species The microbial flora present on tropical fish appears to be similar to those on cold water fish, with a slightly higher load of Gram positive and enteric bacteria (Fraser & Sumar, 1998b). Gram positive organisms such as *Bacillus*, *Micrococcus*, *Lactobacillus* and coryneforms are found in differing proportions (Bulushi, Poole, Barlow, Deeth, & Dykes, 2010; Huss, 1995) Studies have shown that habitat can influence the microflora isolated from a fish. For example, Doyle (1989) investigated the shelf life of nile perch (*Lates niloticus*), a freshwater tropical fish. Using sensory, chemical and microbial analysis, iced nile perch were shown to keep for approximately 30 days. It was concluded that the microflora found on the tropical fish was adapted to living at high ambient temperatures, i.e. they are mesophilic or mesotrophic in nature (Lima dos Santos, 1981). Storing tropical species in chilled conditions inhibits the growth of mesophilic / mesotrophic microflora, thus contributing to an extended shelf life. This was supported by another tropical fish study which concluded that red snapper had a reduced shelf life if not iced. For example, after 4 h at ambient temperatures, the shelf life for the red snapper was halved from 16 to 8 days (Surti, et al., 2002).

Many different bacterial species are found on the skin of fish. Fish harvested from colder, more temperate waters tend to be densely populated with psychrotrophs (cold tolerant bacteria) and psychrophiles (cold loving bacteria). Psychrotrophs are capable of growth at 0˚C but have an optimum temperature closer to 25˚C. Comparatively psychrophiles have a maximum growth temperature of 20˚C and an optimum temperature of around 15˚C (Morita, 1975). Mesophiles are bacteria that grow best at moderate temperatures, usually 15˚C - 40˚C and are more commonly found on fish harvested from warmer waters (Huss, 19951995).

Table 2.1: Cardinal temperatures for microbial growth.

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

The bacterial species commonly responsible for fish spoilage are psychrotrophic. Although psychrotrophic bacteria can adapt to the cold conditions their growth rate is slowed considerably, with extended lag phases experienced as the temperatures nears 0˚C. Most other bacteria are unable to grow at temperatures less than 10˚C (Huss, 1995), which is why fish are stored in chilled conditions.

The spoilage flora, which are the bacteria present on the fish when it spoils (Huss, 1995), are significantly influenced by the species of fish, the fishing ground and the handling methods conducted on board the fishing vessel (Bulushi, et al., 2010). Most fish have SSOs that are commonly found in large proportions on spoiled fish.

Specific spoilage organisms

Suphide-producing bacteria, such as *Shewanella* species, constitute a small percentage of the initial microflora isolated from freshly caught fish. During iced storage these Gram-negative, pychrotrophic species proliferate and become dominant, reaching levels between 10^7 and 10^9 CFU / g (Vogel, Venkateswaran,

Satomi, & Gram, 2005). *Shewanella* species are Gram negative rods which are non spore forming and are motile by a single flagellum. They are the SSOs found amongst chilled, aerobically stored fresh fish (Gram & Vogel, 1999; Nordic Committee on Food Analysis, 2006).

Shewanella is a heterogenous genus that includes mesophilic halotolerant strains, *Shewanella algae*, and psychrotolerant strains, *Shewanella putrefaciens* and *Shewanella baltica*. Due to their psychrotrophic nature, *S. putrefaciens* and *S. baltica* are the organisms primarily involved in fish spoilage. *S.putrefaciens* is an important organism in the spoilage of fish stored at low temperatures, due to its psychrotrophic nature and ability to reduce trimethylamine oxide (TMAO) to trimethylamine (TMA) (Vogel, et al., 2005). *S. putrefaciens* is also capable of degrading sulfur-containing amino acids and producing volatile sulphides including H_2S. The food spoilage strains of *S. putrefaciens* all grow at 4˚C and are capable of growth at 0˚C (Gram & Vogel, 1999).

Shewanella species caught from the Baltic Sea appeared to have a phenotypic heterogeneity. Vogel et al. (2005) confirmed *Shewanella* species present on newly caught fish in the Baltic Sea were predominantly *S. baltica* when the fish were caught in colder periods. *S. algae* predominated on fish in the warmer months due to the water temperature being higher. *S. putrefaciens* is considered the main spoilage organism of iced marine fish, however *S. baltica* was the only *Shewanella* species isolated from fish caught from the Baltic Sea, stored at 0˚C for greater than seven days (Vogel, et al., 2005). Gram (1996) concluded that *S. putrefaciens* and *Pseudomonas* species make up a large percentage of the spoilage population on all aerobically chilled fish. *Pseudomonas* and *Shewanella* species appear as the predominant genera due to their shorter generation times at chill temperatures, independent of the initial flora found on fresh fish (Morita, 1975; Shewan, 1977). This phenomenon occurs in fish caught from temperate and tropical waters (Huss, 1995). An experiment using sterile flesh from fresh fish, which can be stored up to six weeks in a fridge at 0˚C, showed that when cultures of *Pseudomonas fluorescens*, *S. putrefacians* and *Pseudomonas fragi* were inoculated, the flesh experienced the changes in odour and flavour commonly found to occur in spoilage of a whole fish (Shewan, (1977).

Pseudomonads are Gram negative rods which are obligate aerobes (Levin, 2009). *Pseudomonas* species have been identified as SSOs in aerobically stored fish (Morita, 1975; Shewan, 1977; Surendran, et al., 1989), which can be divided into two groups containing fluorescent and non - fluorescent isolates (Levin, 2009). Testing for fluorescence involves exposing isolates to an ultraviolet light (Bullock, Snieszko, & Dunbar, 1965). *P. fluorescens* and *P. putida* are fluorescent isolates identified from spoiling fish. Non-fluorescent Pseudomonads responsible for off odours include *P. fragi* and *P. perolens* (Levin, 2009). *P. fragi* is responsible for the "sweet" or "fruity" odours commonly associated with early stages of spoilage. The "musty" odour sometimes associated with early stages of spoilage comes from growth of *P. perolens* (Levin, 2009).

Pseudomonads are commonly isolated from fish harvested from warmer waters (Dalgaard, 2000). Surendran et al (1989) investigated the specific flora of tropical marine fish and concluded that the initial flora consisted of *Moraxella*, *Acinetobacter* and *Vibrio* species. These genera accounted for 58 - 76 % of the bacterial flora, however, as the fish were nearing the end of their shelf life, *Pseudomonas* species accounted for 75 – 81 % of the bacterial flora (Surendran, et al., 1989). Adams, Farber, & Lerke (1964) investigated the incidence of bacteria responsible for spoiling fillets of a demersal fish, english sole (*Parophrys vetulus*). The bacterial population at the end of the experiment was dominated by *Pseudomonas* and *Achromobacter* groups. The actual spoilage group was a much smaller percentage of the initial bacterial population. This was also replicated in an experiment conducted by Lee and Harrison (1968) where the initial microflora of pacific hake was compared to the final bacterial population after storage at 5˚C. *Pseudomonas* species were grouped phentypically into Type I, II, III and IV, *Acinetobacter* species, *Moraxella* species. and *Flavobacterium* species were all isolated at day zero. By day 14, *Pseudomonas* species Type I and II dominated the microflora present on the fish (Lee & Harrison, 1968). *Pseudomonas* species produce volatile aldehydes, ketones, esters and sulphides. These volatile compounds produce fruity, rotten, sulphydryl odours and flavours on spoiling iced fish (Gram & Huss, 1996). *Pseudomonas* species can also produce slime polymers, using a disaccharide such as sucrose (Cousin, 1999). Hence

the slimy, wet feeling associated with food once it has spoiled (1996; Huss, 1995; Morita, 1975; Shewan, 1977).

Shewanella and *Pseudomonas* species are the main spoilage organisms isolated from spoiled aerobically chilled fish however, there are other bacterial species commonly isolated from the spoilage flora which may also contribute to spoilage. There are many Gram positive and Gram negative spoilage organisms that are able to reduce the shelf life of a fish. It is important to understand how the microflora interacts and changes over the spoilage process to understand how to delay spoilage. Identification of other organisms isolated as part of the spoilage flora is also important for increasing the database on our knowledge of microbial organisms involved with fish spoilage. Other genera of bacteria commonly isolated from fish include:

Vibrio species

Vibrio species are Gram negative rods which are natural contaminants of raw seafoods (DesmarchelierDesmarchelier, 1999). *Vibrio* species are more actively involved in spoilage in tropical fish species, along with sulphide-producing *Aeromonas* (Fraser & Sumar, 1998b). *Vibrio* species dominate flora at ambient storage temperatures (Gram & Huss, 1996) and are capable of reducing TMAO to TMA producing the "off" smell commonly associated with rotten seafood (Gram & Dalgaard, 2002). An investigation into the specific spoilage bacteria from fish stored at 0˚C and 20˚C, identified *Vibrio* species as spoilage bacteria due to the fish being stored at ambient temperature (Gram, Trolle, & Huss, 1987). Another study performed on Indian oil sardines found that *Vibrio* and *Aeromonas* species were the most active spoilers based on biochemical analysis after being incubated at 28°C (Shetty, Setty, & Ravishankar, 1992).

Psychrobacter species

Psychrobacter species are aerobic, Gram negative spherical to rod shaped bacteria. Due to their genetic similarity *Psychrobacter* species have commonly been misidentified as *Moraxella* species. *Moraxella* species are unable to grow at 4°C and are not halo-tolerant (Bowman, 2006). *Psychrobacter immobilis* is commonly found on fish from different marine

and fresh water environments. It does not appear to have a major role in fish spoilage however (Gennari, Alacqua, Ferri, & Serio, 1989; Gennari, Tomaselli, & Cotrona, 1999). Gennari et al. (1989) discovered that no strain isolated from the microflora of Mediterranean sardines was able to hydrolyse proteins or produce off odours. *Psychrobacter* species, however, may be responsible for contributing to the lipolytic activities which occur during fish storage (Gennari, et al., 1989).

Aeromonas species

Aeromonas species are Gram negative, facultative anaerobic rods that are ubiquitous in the environment and so are readily recovered from most raw foods, including seafood (Blair, McMahon, & McDowell, 1999). *Aeromonas* species become a dominant species in the microflora when the fish are stored at ambient temperatures (Gram, Wedell-Neergaard, & Huss, 1990) and are capable of reducing TMAO to TMA (Gram & Dalgaard, 2002). A study investigating the bacteriology of nile perch when stored at ambient temperature discovered that 15 from 42 isolates cultured were *Aeromonas* species. *Aeromonas hydrophila* is also a documented spoilage organism of trout when it is stored at 37˚C (Gram, et al., 1990). *Aeromonas* species are capable of growth at refrigerated temperatures. These organisms cannot compete effectively with other pyschrotophs, such as *Pseudomonas* species, and so are not commonly referred to as major spoilers of aerobically stored iced fish (Blair, et al., 1999).

Acinetobacter species

Acinetobacter species are Gram negative coccobacilli that are obligate aerobes. They are normally found on many foods and food products, especially those refrigerated. *Acinetobacter* species are ubiquitous, being isolated from soil and water. *Acinetobacter* species have been found on spoiled fish and seafood products, fresh or frozen. It appears that as storage time increases, *Acinetobacter* species decrease in numbers (Kampfer, 1999).

Bacillus

Bacillus species are Gram positive rods once thought to dominate flora on tropical marine fish, however, it has since been discovered that the microbial diversity is quite similar between tropical species and cold water species (Fraser & Sumar, 1998b). *Bacillus* species are spore producers so it is possible that these bacteria may survive a mild heat treatment and continue to grow at chill temperatures ("Fresh and Processed Seafood," 2000). The exclusive presence of *Bacillus* species in fish muscle is associated with low TMA values (Velenkar, 1956) however, so there is no evidence to suggest that *Bacillus* species have a high spoilage potential.

Micrococcus

Micrococcus are Gram positive cocci that are ubiquitous in the environment and so are usually associated with food (Holzapfel, 1992). This group does not appear to play a primary role in food spoilage.

The genera of micro-organisms described above are commonly identified as part of the spoilage flora isolated from tropical fish. A more extensive description of less commonly isolated micro-organisms identified as spoilage flora may be found in Appendix 7.

Isolation Media

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.

Plate Count Agar

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 procedures and conditions (Huss, 1988). Along with a thorough knowledge of the processing system, for example holding temperatures, and handling and packing of the fish, 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

Iron agar is a media used to enumerate hydrogen sulfide producing SSO, such as *Shewanella* species. *Shewanella* species 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).

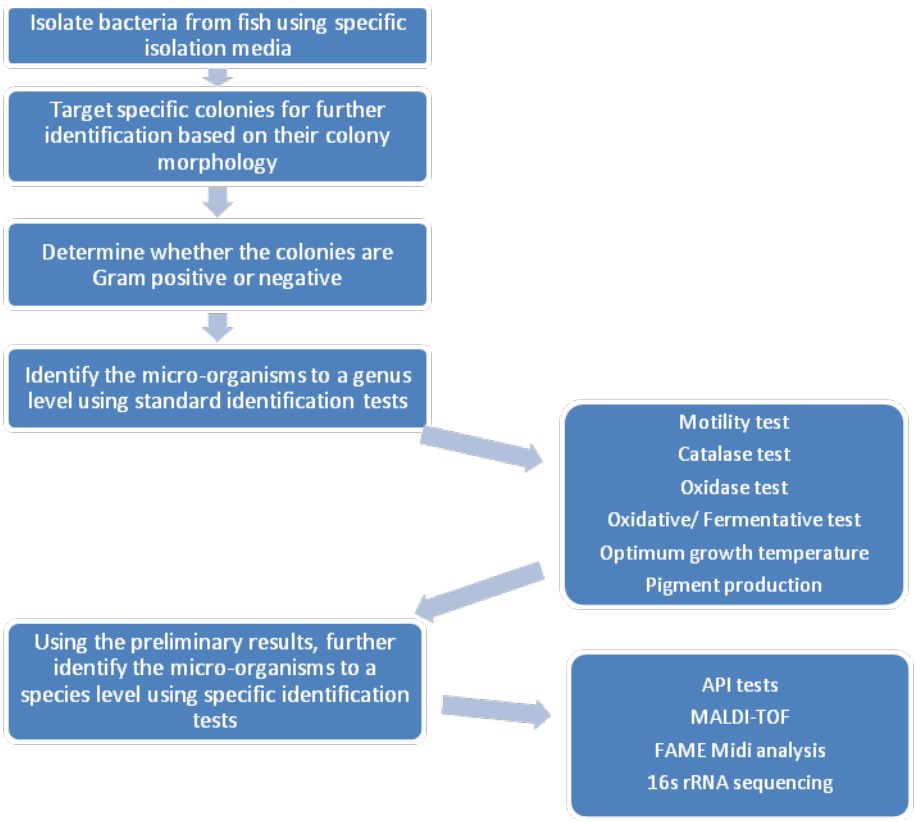
Long and Hammer Agar

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).

Preliminary identification

Preliminary identification of an organism is a quick and simple method of profiling an organism and grouping them into a broad group or into a specific genus. The Gram stain, catalase and oxidase tests, motility tests, oxidative/fermentative tests and optimum temperature tests are preliminary identification techniques often used on bacteria isolated from specialised media as a process of preliminarily identifying spoilage bacteria.



Colony Morphology

The colony morphology of a bacterium is biologically relevant for its ability to survive, if it was not able to change and adapt to its environment it would die. Morphology varies greatly across the different bacterial species however some

organisms are also capable of changing morphology to suit changing environmental conditions (Young, 2007). Although variation exists it is commonly seen that any one particular genus usually exhibits a subset of morphologies that seem to be advantageous for its survival (Young, 2007). Morphology is therefore a useful characteristic commonly used to help preliminarily identify organisms.

Gram stain

The Gram stain is a differential staining technique useful in observing the difference in the cell walls of micro-organisms. The stain was first developed by Christian Gram (Gram, 1884) and is able to divide the bacteria into two groups, Gram positive and Gram negative. Gram variable organisms also exist though these are less common. The cells are treated with crystal violet and then iodine to ensure the dye binds to the cells. The stain is then decolourised using an alcohol or acetone. During this step, a Gram positive organism has a thicker peptidoglycan layer in comparison to Gram negative which is thinner. The final step, counter-staining, is used to differentiate between the two groups. Gram positive organisms appear purple on a Gram stain and Gram negative appear pink (Lovitt & Wright, 2000).

Catalase test

The catalase test is a simple, rapid and cost effective test used to differentiate between different groups of bacteria. The catalase test determines the production of the enzyme catalase by bacteria by measuring the amount of gas produced from 3 % hydrogen peroxide added to a bacterial sample (Mendonca & Juneja, 2000). Most organisms that impact negatively on the food industry are usually catalase positive, these include *Pseudomonas* and *Shewanella* species (Mendonca & Juneja, 2000). The test is useful for the identification of oxidative, Gram negative bacteria (Chester, 1979), which dominate the spoilage flora found on fish.

Oxidase test

The oxidase reaction is a simple fast and effective way of determining whether an organism is oxidase positive or not. The test is based on certain bacteria being able to produce indophenols when the oxidation of dimethyl-p-phenylenediamine and α-naphthol occurs. Kovacs produced a simpler method from the earlier version by Gordon & McLeod (1928). Oxidase positive organisms turn blue after 10 seconds

using Kovac's method. The oxidase reaction relies on the presence of cytochrome oxidase. Organisms from the *Neisseriaceae, Pseudomonadaceae* as well as *Vibrio* and *Aeromonas* species are generally oxidase positive, whereas members of the *Enterobacteriaceae* and most Gram positive organisms are oxidase negative.

Motility test

Motility is the ability of an organism to move by itself (Aygan & Arikan, 2007). Micro-organisms are motile usually by the use of flagella or by the use of fibrils which produce a gliding form of motility (Aygan & Arikan, 2007). The position of the flagella on the bacterial cell is usually characteristic for a genus. It can also help identify to a species level for example, all *Bacilli* are motile except for *B. anthracis*, which is an uncommon characteristic in this genera (Aygan & Arikan, 2007). Motility is best observed in a medium that accommodates a wide range of organisms and is able to grow them more rapidly than other commonly used media. Most organisms grow well at 37°C, however flagella development is increased at a lower temperature and so incubation at room temperature or 25°C is recommended (Yuen & Davis, 2006). Motility is usually detected in a wet mount preparation under a microscope. Electron microscopy and flagella staining is also used (Aygan & Arikan, 2007).

Oxidative/fermentative test

Another technique used to quickly identify bacteria is to assess whether or not they ferment carbohydrate. Triple sugars iron agar (TSIA) is a differential medium used to determine carbohydrate fermentation and hydrogen sulphide production (Lehman, 2005). The TSIA slopes contain three carbohydrates, glucose, sucrose and lactose. The top of the slope is aerobic whilst the bottom or butt of the slope is an anaerobic environment. Fermentation of any carbohydrate results in a change in the pH of the medium and turns the phenol indicator from a reddish orange colour to yellow. Alkalisation of the peptones contained within the medium turn a deep red/pink colour (Lehman, 2005). Production of iron sulphide occurs when the hydrogen sulphide, produced by a hydrogen sulphide producing bacteria, reacts with the ferric ions present within the media. Results are determined based on the colour changes which have occurred within the media (Lehman, 2005).

Pigment Production

Pigment production is characteristic for some bacterial species and is another method used to group isolates during the identification process. Not all bacterial species produce pigment and it is possible that one strain of a bacterial species will produce pigment and another strain may not. Pigment may also be affected by environmental conditions. Using pigment as a means of preliminary identification can be a fast and effective method of separating different species or different strains of bacteria.

Optimum Growth Temperature

The optimum growth temperature is another ideal way of grouping unknown organisms into groups. A range of different bacteria are expected to be isolated from the fish species. By using the optimum growth temperature it will be possible to determine which strains are psychrotrophic (prefer cold temperatures) and which are mesophillic (prefer warm temperatures). This differentiation will help with further specific identification.

Specific Identification

Preliminary identification of the bacterial species will only identify bacteria to the genus level. To identify down to the species level further specific identification methods are needed. Assessing isolates to a species level creates an accurate picture of the spoilage flora present on the fish further contributing to the growing database of bacteria responsible for spoilage. Identification to a species level can be very time consuming. There are simple biochemical tests available which reliably identify the organisms with minimal time and materials required and they also require very little training to operate (Kunitsky, Osterhot, & Sasser).

API System

The API system is a bacterial identification system used in identifying Gram positive bacteria, Gram negative bacteria, anaerobes and yeasts using up to 50 different miniature biochemical tests in a strip form. The Biolog Identification System is another useful kit used in the identification of bacteria. The Biolog Identification System identifies bacteria based on the exchange of electrons during the organism's respiration. A tetrazolium- based colour change results when the panel of 95 carbon tests are oxidized by the organism (Miller & Rhoden, 1991). Simple biochemical

tests are useful as they are a cheap, effective and time efficient in identifying bacteria. A disadvantage they do have is that most are geared for clinical use and so they are limited in identifying environmental species (Kunitsky, et al.).

MALDI-TOF

Matrix-assisted laser-desorption/ionization time of flight (MALDI-TOF) mass spectrometry is another tool used in identifying and characterising microorganisms. MALDI-TOF analyses the proteins from disrupted cells to differentiate between bacteria (Holland et al., 1999). MALDI -TOF works by analysing a small amount of biological material onto a stainless steel target plate. The material is then overlaid with a matrix which helps to disrupt cells. The target plate is then placed in the MALDI-TOF machine which targets a laser onto the cells. The laser results in ionisation and liberation of sample fragments. The time it takes the ions to travel through the vacuum tube of the machine is measured and the mass spectra is produced (Veloo, Welling, & Degener, 2011). This unique fingerprint is then matched by comparing the generated peaks to those already existing in the library spectra (Maier, Klepel, Renner, & Kostrzewa, 2006). MALDI-TOF allows rapid identification of bacteria, although an extended database is still needed as most organisms listed are from a clinical setting not environmental (Veloo, et al., 2011).

FAME Midi Analysis

Cellular fatty acids is a universal characteristic found amongst all bacteria yet they are unique to each bacterial species (Kunitsky, et al., 2006). Microbial cells contain fatty acids usually in the form of lipid, which are primarily found in the cell membranes, or the lipid A component of lipopolysaccharide for Gram negative bacteria and lipoteichoic acid for Gram positive bacteria (Welch, 1991).

Bacterial colonies must first be grown in appropriate conditions (28˚C for 24 h) before fatty acid methyl ester (FAME) analysis may be performed. The cells of the sample colonies are removed from the media and the next stage of sample preparation, saponification, is performed. Saponification requires cells to be exposed to substances which will lyse the cells, liberating the fatty acids. Once the cells have been lysed, substances are added to create the formation of fatty acid methyl esters (FAMEs) (Kunitsky, et al., 2006). The FAMEs are then extracted from the aqueous

phase using an organic solvent. Once this has been performed the residing sample is washed in an aqueous solution and analysed by chromatographic analysis (Kunitsky, et al., 2006). The results of the analysis are displayed using a Similarity Index. Each sample is compared to a mean profile of the strains used to compile the database. If a sample is an exact match the result will be displayed as 1.000, if a sample varies greatly from the mean percentage of the organism the SI value will decrease (Kunitsky, et al., 2006). Values lower than 0.300 suggest an organism is not in the library and may perhaps be a new species. The software will still show the closest related species which is why a value is still displayed (Kunitsky, et al., 2006).

FAME analysis is advantageous as it requires uses no subjective testing, no previous testing, such as Gram stains or other biochemical tests (Kunitsky, et al., 2006) are needed and it has a larger database than the simple biochemical methods previously mentioned (Sasser, 1990).

16s rRNA Sequencing

DNA based technology commonly uses the 16S rRNA gene as the basis for identification (Kunitsky, et al.). The 16s rDNA gene more commonly referred to as 16s rRNA gene is a highly conserved gene, existing universally amongst bacteria (Clarridge III, 2004). It appears to be so highly conserved due to its critical component in cell function (Clarridge III, 2004). The 16S rRNA gene is conserved at a species level so it is able to differentiate between species well, however the differentiation between subspecies and strains is not shown (Hazen & Jimenez, 1988; Kunitsky, et al., 2006). The 16s rRNA gene sequencing is a technique widely used in laboratories due to its ability to identify fastidious, or slow growing bacteria (Mignard & Flandrois, 2006). It is also useful in identifying bacteria that do not fit any recognised biochemical profiles (Janda & Abbott, 2007). In this way DNA sequencing is responsible for most of the identifications of new species. Overall, 16S rRNA sequencing is a highly accurate and reliable method for bacterial identification and classification (Clarridge III, 2004; Mignard & Flandrois, 2006).

Chemical spoilage and detection

Chemical analysis is often used alongside sensory and microbial analysis to detect early signs of spoilage. It is the endogenous fish enzymes and bacterial enzymes

which are responsible for degradation of fish muscle immediately after death (Huss, 1995). Enzymatic reactions occurring post mortem are unregulated and so the chemicals being produced begin to reach levels which are detectable by chemical analysis.

Autolysis or 'self digestion' is responsible for early spoilage in fish and begins with the autolytic endogenous enzymes found throughout the fish muscle (Huss, 1995). These enzymes contribute to overall quality loss of fish by causing degradation to fish muscle and skin. Autolysis is very difficult to prevent in fresh fish due to the wide dispersal of enzymes throughout fish muscle. The enzymes involved in autolytic spoilage are distributed in the intracellular and extracellular compartments in fish muscle. Individual concentrations of each enzyme differ depending on the function of each muscle tissue (Mukundan, Antony, & Nair, 1986). These enzymatic systems in post mortem fish muscle are no longer able to be regulated. Nutrient and oxygen supply to the tissue is ceased along with blood circulation also ending all anabolic processes. Catabolic products still accumulate due to the degenerative reactions which are still active within the post mortem tissue (Mukundan, et al., 1986). Chemical enzyme inhibitors have been considered suitable for prevention of autolysis however, they are not used due to their effect on the consumer. Distributing the product through the fish post mortem is also another problem for which a solution has not yet been found (Mukundan, et al., 1986).

Stress endured by fish during capture affects the rate of enzymatic spoilage (Huss, 1995). Chiba et al (1991) showed that with only minutes of pre-capture stress, a decrease of 0.50 pH units in 3 h was noted. In comparison, fish that were not subjected to pre-capture stress only experienced a decrease of 0.10 pH units in the 3 h period. A decrease in pH affects the net surface charge of the fish muscle proteins. The proteins then begin to denature and lose their water holding capacity. A loss in water holding capacity results in the quality of the fish muscle beginning to deteriorate (Huss, 1995). The combined actions of these autolytic enzymes produce a nutrient rich environment for bacteria to multiply. Therefore autolysis acts a precursor to the onset of bacterial spoilage (Mukundan, et al., 1986).

Bacterial invasion into the flesh is limited with most microbial growth taking place on the surface of the fish, however, a large proportion of spoilage is actually due to bacterial enzymes diffusing into the flesh (Huss, 1995). This change in the chemical composition of the flesh is responsible for the changes in flavour which occur in the fish throughout spoilage. For example, inosine monophosphate (IMP) and other 5' nucleotides are considered strong flavour enhancers, and so when they are degraded there is a loss of fish flavour and an increase in off flavours (Huss, 1988). Reliable objective measures regularly used to detect chemical spoilage include measuring the amounts of trimethylamine (TMA), the total volatile bases (TVB), the K-value and hypoxanthine present in the fish muscle.

Trimethylamine (TMA)

Trimethylamine oxide (TMAO) is found in the muscle tissue of all marine species (Huss, 1988). The highest concentration of TMAO appears in the dark muscle of pelagic fish and in the white muscle of demersal fish due to their different physiology (Huss, 1988). TMAO is reduced, usually by bacterial action, to produce trimethylamine (TMA). TMA has an unpleasant "fishy" odour, generally associated with spoilt fish (Huss, 1988). All freshly caught fish contain low concentrations and it accumulates as the fish spoils (Huss, 1988). The average concentration for a freshly caught fish is 2 mg TMA / 100 g wet weight, however, it can range between 1 and 4 mg / 100 g (Oehlenshlager, 1997). The limit for human consumption is considered to be between 10-15 mg TMA / 100 g due to the fish being considered unacceptable to the consumer once this level of TMA is reached (Huss, 1988). Various studies have shown that initial levels of TMA remain until approximately day 10 when microbial action begins (Baixas-Nogueras, Bover-Cid, Veciana-Nogués, & Vidal-Carou, 2002; Oehlenshlager, 1997). A study performed by Rehbein, Martinsdottir, Blomsterberg et. al. (1994) measuring the amounts of TMA in Redfish noted that during the first 12 days of storage TMA amounts gave no information as to the quality changes occurring. The time it takes to measure increased amounts of TMA does not allow TMA analysis to be used as a universal measure of deterioration. The fact that it is only found in marine fish and not all freshwater fish is another disadvantage. However, TMA is still effective in determining the degree of spoilage (Huss, 1988).

Total volatile bases (TVB-N)

The total volatile bases (TVB-N) are an alternative measure of spoilage to TMA estimation. TVB-N is a measure of the total volatile basic compounds produced throughout fish spoilage consisting mainly of the volatile compounds; trimethylamine (TMA), dimethylamine (DMA) and ammonia (Howgate, 2010). TVB-N is commonly used to measure the later stages of spoilage since the components do not accumulate at measurable levels until later (Howgate, 2010). The suggested levels of TVB-N for rejection of fish are 30 mg / 100 g flesh as this is regarded as the limit of acceptability for consumers (Castro, Penedo Padron, Caballero Cansino, Velazquez, & De Larriva, 2006; Oelenschläger, 1992). This range may vary between 10 - 25 mg / 100 g or even 30 - 35 mg TVB-N / 100 g depending on the species and where the fish are caught (Huss, 1988). The whole fillet or at least a representative sample must be used when measuring for TVB-N due to it not being homogenously distributed throughout the fish (Oehlenshlager, 1997).

The total volatile bases of European sea bass stored in ice were investigated during a shelf life study (Castro, et al., 2006). Results from this study showed a delay in the increase of TVB-N values. The shelf life of the European sea bass is 20 days, however, an increase in TVB-N was observed between the 21st and 28th day of storage. Oelenschläger (1992) also investigated the use of TVB-N as a deterioration indicator when studying cod and haddock. In the first 8-12 days the concentration of TVB-N did not increase. Only after 12 days did the TVB-N concentration increase rapidly reaching 30 mg / 100 g between days 15 - 20. Although informative, the large species to species variation in the development of TVB-N prevents it from being a universal deteriorative measure (Huss, 1988). TVB-N levels are however useful in determining whether fish are fit for human consumption (Oelenschläger, 1992).

Hypoxanthine

Hypoxanthine is a normal constituent of fish flesh; in living fish it is found in low concentrations. Post-harvest, deterioration of the fish begins immediately with a series of enzymatic breakdown reactions occurring. The breakdown of ATP over a period of days results in the formation of hypoxanthine, which continues to increase in concentration for the duration of storage (Huss, 1988). Hypoxanthine, the product

of inosine-5´-monophosphate, is considered an index of the autolytic deterioration of several species of fish (van Spreekens, 1977). However, the rate at which hypoxanthine accumulates is not consistent across species and so care must be taken when interpreting results. Hypoxanthine is measured using an enzymatic method which converts the substance into uric acid or by separating the hypoxanthine from remaining volatile compounds using high performance liquid chromatography (HPLC). Hypoxanthine values appear to reach a maximum between days 10 - 18 and negatively correlated with the flavour and odour desirability (Metin, Erkan, & Varlik, 2002). The accumulation of hypoxanthine during storage makes it a useful indicator of the deteriorative processes in marine fish (Beuchat, 1973).

K-value

K-value is an index used to evaluate the initial stage of fish deterioration by measuring the extent of the breakdown of ATP (Huss, 1988). The K-value index is based on nucleotide degradation, expressed as a percentage of the amount of inosine and hypoxanthine to the total amount of inosine monophosphate, inosine and hypoxanthine (Agustini, et al., 2001). Fresh fish have a lower K-value which increases at a species dependent rate (Huss, 1988) and fish of the very best quality should have a K-value of less than 20 percent (Huss, 1988). The K-value is determined by HPLC (Agustini, et al., 2001) and is a useful measure of deteriorative processes as it increases close to linearly in all finfish species studied (Hattula 1997).

Sensory analysis of spoilage

Sensory evaluation is defined as the scientific discipline used to evoke, measure, analyse, and interpret reactions to characteristics of food as perceived through the senses of sight, smell, taste, touch, and hearing (Huss, 1995). Sensory analysis is conducted commonly to assess the shelf life of products due to it being a fast, reliable, non-invasive technique (Ozogul, 2009). Sensory evaluation is very effective in determiningquality, which in relation to seafood reflects the degree to which microbiological spoilage or chemical deterioration has occurred (Botta, 1995; Ozogul, 2009). Sight, for example, is valuable in quickly determining deterioration and imperfections visible on the surface of the fish, whilst touch is effective in determining textural qualities. Assessing the odour and flavour through smell and taste are also important for sensory evaluation of seafood (Pedrosa-Menabrito &

Regenstein, 1990b). Extensive training of assessors allows them to accurately determine the degree of deterioration (Pedrosa-Menabrito & Regenstein, 1990b).

Sensory analysis is best performed by trained assessors in sensory panels. The environment in which sensory analysis is performed should be strictly monitored. Testing areas should have minimum noise, be free from foreign odours, have a low constant temperature, have adequate lighting and must be clean and easy to disinfect (Hyldig, Martinsdottir, Sveinsdottir, Schelvis, & Bremner, 2009). There are two different types of assessments which can be performed, a subjective assessment which is based on the panellists preference and an objective assessment which involves observing the organoleptic changes which occur during storage (Ozogul, 2009). Subjective assessments are commonly used for product development whereas the objective assessments are primarily used to assess fish deterioration. The three main objective sensory techniques used for evaluating fish freshness include the Torry scheme which assesses the freshness of cooked fillets, and the EU (European Union) scheme and QIM (Quality Index Method) Scheme which evaluates the deterioration of raw whole fish.

The Torry scheme is a developed scale which allows trained assessors to evaluate the odour and flavour of cooked fish fillets. The Torry scheme is advantageous because it provides researchers with a point where the fish are considered no longer acceptable for human consumption, regardless of it physical appearance. The European Union Grading Scheme (EU or EC scheme) is a sensory method commonly used in European countries to assess the deterioration of whole fish. Some disadvantages of the EU scheme include its lack of specificity in terms of describing sensory parameters. The scheme condenses the groups of several sensory parameters into general quality grades. This can cause confusion for the assessor if a fish does not fit directly into a particular grade and it also decreases the objectivity of the scheme. The EU scheme is also limited in providing further information regarding shelf life and it does not account for differences between fish species.

The QIM, introduced as an alternative to the EU Scheme, is another grading system used to assess the deterioration of whole fish. In contrast to the EU Scheme, the QIM shares a linear relationship with storage days on ice allowing the remaining shelf life

of the fish to be calculated. The QIM evaluates all relevant attributes needed to evaluate the deterioration of a fish eliminating bias for any particular attribute. This ensures minor differences in judgement will not influence the total sensory score. Unlike the EU scheme the thoroughness of the QIM scheme allows it to be developed specifically for different fish species (Huss, 1988; Ozogul, 2009).

Development of a QI scheme

A fast and simple method for determining the deterioration of seafood is the QIM proposed by the Tasmanian Food Research Unit (Bremner, 1985). This QIM is an easy to follow process that uses a very structured layout along with illustrative material (Hyldig, et al., 2009). Because of this easy to follow structure it can be used by novice and trained panellists alike (Bremner, 1985).

The QIM uses a category scale, measuring significant sensory quality parameters, unique to each fish species. The parameters are chosen after monitoring the fish throughout storage and may include appearance of the skin, odour and texture. Each parameter is subdivided so specific traits of the fish may be monitored and assessed using a score system throughout the spoilage process (Hyldig & Green-Petersen, 2004). No high scores are given for any single parameter and so no undue emphasis is placed on any criterion. All parameters which are to be monitored throughout the QIM are initially graded with a score of 0 to represent the fish at its freshest. As the fish deteriorates and defects become noticeable, the score for each quality parameter increases to a final score of 3, which represents complete spoilage. The sum of the points given for all parameters is added and the total is the quality index score for that time point (Huidobro, Pastor, & Tejada, 2000). The quality index score increases linearly against the decreasing useable life of the fish (Bonilla, Sveinsdottir, & Martinsdottir, 2007). Thus using the known shelf life and the QIM score, the remaining shelf life of a particular species may be estimated even if storage conditions change (Bonilla, et al., 2007; Bremner, 1985). There are other uses for the final QIM score from a business perspective. The QIM score gives buyers the opportunity to assess whether the catch is acceptable and worth buying. It also gives fisherman the freedom to decide how to make the most from each catch, for example, freezing or keeping the product fresh, selling to a domestic market or exporting. If

the quality of fish is not high, it may also lead to changes in fishing grounds or distributors (Bremner, 1985).

QIM is advantageous because it can predict shelf life and can also be developed and adapted for different fish species (Bonilla, et al., 2007). It is cheap, non-destructive, easy to use, is not subject to much variation and has a rapid response (Rahman & Olley, 1984). A disadvantage is that it relies on individual perceptions of each trait (Sakaguchi & Loike, 1992), however this is overcome by using a panel of suitably trained people when developing the QIM scheme.

The development of a QIM involves using a trained panel of ten to twelve people who are responsible for detecting and describing the significant sensory quality parameters. A trained panel of 6 is considered sufficient enough to carry out a QIM trial (Huss, 1988) however, when there are less than ten panellists the variability from their contribution increases. Other logistical problems such as scheduling can become a problem if there are more than twelve panellists (Stone & Sidel, 2004).

Panellists selected for development of a QIM scheme are chosen based on their ability to evaluate appearance, odour, taste and texture. It is important that they possess normal odour and taste sensitivities (Hyldig, et al., 2009). Panel members must pass a standard test to participate in each QIM development study, regardless of prior experience. The ability to work within a group and be able to verbalise their opinions is necessary to participate in the trials. Another requirement essential for participation is that the panel member must be a user or potential user of the product. The reason for this being that during the study the panellists will be exposed to the product frequently and those who do not like or show little interest in the product may be less sensitive to the differences (Stone & Sidel, 2004). If a panel member has had little or no experience with seafood they should be given an introductory course explaining the physical, chemical, microbiological and organoleptic changes which occur during the spoilage process (Shewan, Macintosh, Tucker, & Ehrenberg, 1953).

Panellists experienced with seafood may not have particular experience with the species being evaluated and so training sessions are held so all panellists may familiarize themselves with the species they will evaluate. During these training

sessions panellists are exposed to the species being evaluated at different stages of the spoilage process. They are then asked to comment and discuss each individual quality parameter so as to collect descriptive terms which can be used in the final QIM scheme (Shewan, et al., 1953). The panellists are encouraged to use words they understand and are able to describe to the other panel members. However, words that express a preference, such as the terms good or bad are not used (Stone & Sidel, 2004).

The use of the Torry scheme is generally paired with the development of a Quality Index Method (QIM) scheme as it measures the flavour and odour of cooked fillets. It represents the next step along the food chain and so is useful in validating the sensory score developed in a QIM scheme. The Torry scheme is a 10 point scale developed to evaluate the quality of cooked fillets for lean, medium fat and fatty fish species. The scale ranges from 10 (very fresh in taste and odour) to 3 (spoiled). The scale does not proceed below 3 because the fish is then not considered suitable for human consumption. Panellists generally detect spoilage characteristics and sour tastes at the average score of 5.5, which is why this score is used as the limit for consumption and end of the product's shelf life (Martinsdottir et al., 2001). The Torry scheme is a useful tool when validating a QIM as it provides a reference point for the end of shelf life.

Sensory assessment of fish plays an important role in determining shelf life as it assesses characteristics such as odour, texture and appearance, which are easily detected by the consumer. Sensory methods are very reliable but limitations do exist and so non-sensory methods can be utilised to reduce these limitations and to validate the sensory results (Alasalvar et al., 2001).

Physical analysis of spoilage

Physical tests using scientific instruments have been developed to monitor fish deterioration. Texture and colour are both important aspects in measuring quality which can be easily monitored using instrumental methods over the spoilage process.

Texture

Texture may be defined by common characteristics such as hardness, springiness and chewiness of food. Texture is a valued characteristic for consumers. Also it is very important in a commercial capacity as it determines the value of the meat (Jain, Pathare, & Manikantan, 2007) and is reliable for measuring deterioration in poultry, meat and fish. Enzymatic reactions and microbial growth impact on the texture of fish throughout the spoilage process. The flesh of a fresh fish has a firm texture compared to that of a spoiled fish which may be soft and watery due to autolytic degradation, or tough and dry due to freezer storage (Huss, 1995; Jain, et al., 2007). There are various methods of measuring texture, including the Kramer test, the Warner-Bratzler test, the puncture test, tension analysis, the compression test and texture profile analysis (TPA) (Sanchez-Alonso, Barroso, & Careche, 2009).

- The Kramer test involves the Kramer shear-compression cell which uses multiple blades to measure compression, shear, extrusion and friction as the blades move through the sample (Sanchez-Alonso, et al., 2009).
- The Warner-Bratzler test uses an instrument with two blades cutting at a 60° angle to measure the maximum force used during shearing. It is able to separate the measurements of force used to cut muscle fibres and connective tissue, providing two peaks.
- The puncture test measures the force, slope and energy of the force when a plunger is pushed into a fish sample. Results from the Warner-Braztler and the puncture test may be unreliable if there is insufficient quantity of sample and are altered depending on the orientation of the fish muscle (Sanchez-Alonso, et al., 2009).
- Tension analysis measures the maximum force or tensile strength of the fish sample as it is torn between two parallel clamps at a constant rate.
- The compression test uses two parallel flat surfaces to compress a sample, measuring the overall resistance to deformation. The probe in a true compression test should be much larger than the sample however, in most applications these requirements are not met. Compression tests on fish are usually similar to the well know “finger method” (Sanchez-Alonso, et al., 2009). The “finger method” involves a finger being pressed onto the skin or

fillet of the fish allowing firmness to be evaluated from the force used to press the finger down and also by the mark left. It is a subjective test based upon the evaluation of the person conducting the test (Sigurgisladottir et al., 1999). A variation of the finger test uses a specialized texture analyser and different probes, such as a flat ended cylinder, spherical probe or a blade and investigates the texture by applying a set amount of force and measuring the resistance experienced from the whole fish or fillet (Sigurgisladottir, et al., 1999).

- The texture profile analysis (TPA) can be performed by compressing a bite-size piece of food twice in a reciprocating motion. This test is aimed to imitate the action of the jaw. The height of the force peak on the first compression cycle (first bite) is defined as hardness. The ratio of the force areas under the first and second compressions is defined as cohesiveness (Mai et al., 2009). The distance to which the food recovers between the first and second bite is defined as springiness or elasticity (Mai, et al., 2009). Texture analysis is commonly used in combination with other instrumental techniques in order to evaluate the deterioration of fish (Sanchez-Alonso, et al., 2009).

Texture analysis, as previously described, is able to measure different parameters of texture. These parameters include firmness, elasticity, tensile strength, toughness and the force used to shear a sample. There is no ideal texture method that is universally recommended with tests being selected depending on the desired aspect of texture to be measured. The finger method is the method commonly used within the fishing industry, it is simple and reproducible. As a result the finger test was chosen as the representative method for texture measurement within this body of research (Coppes-Petricorena, 2011).

Colour

Colour is the perception resulting from the detection of light after it has interacted with an object (Schubring, 2003). Similar to texture, the colour of a fish affects the consumer's perceptions of the product. A fresh fish vibrant in colour is appealing to the consumer. A decrease in colour is associated with an increase in deteriorationand so the fish is less appealing to the consumer. Few publications have focused on the

colour changes experienced by whole fish during iced storage and comparison of these changes to the changes in quality.

Instruments used to measure colour are grouped as trichromatic colorimeters or spectrophotometers. Colorimeters imitate the same response a human eye would have in response to light and colour. They contain three filters (red, green and blue) and a light source which is bounced off an object. The reflected rays then pass through the glass filters and a photo detector measures the amount of light which passes through the filters. A spectrophotometer uses a light source to illuminate an object and the light reflected off the object is measured. The reflected light first passes through a grating which breaks it into the full colour spectrum. The spectrum falls onto a diode array which then measures the amount of light at each wavelength which is a more accurate way of measuring colour (Schubring, 2002a). Colour analysis may be performed on the skin of the whole fish (Lapa-Guimarães et al., 2002) or on the fillets (Einen & Thomassen, 1998; Hernández et al., 2009). The measurement of one quality attribute is insufficient, combining colour measurements with other sensory techniques such as texture, establishes a better estimate of fish deterioration (Rehbein & Oelenschläger, 2009).

Conclusion

Current literature concludes that fish harvested from warmer waters have a longer shelf life than those harvested from temperate waters (Surendran, et al., 1989; Surti, et al., 2002). The microflora present on tropical fish are mesophilic in nature and adapted to living at higher temperatures (Lima dos Santos, 1981). Storing tropical species in chilled conditions inhibits the growth of mesophilic microflora, thus contributing to an extended shelf life. Fish harvested from colder, more temperate waters tend to be densely populated with psychrotrophs and psychrophiles. Goldband snapper are a subtropical fish, commonly caught at depths of up to 300 m where the temperature is close to 15˚C and yet they have an extended shelf life of approximately 35 days (Ames & Curran, 1985). It was hypothesised that the difference in shelf life between goldband snapper and other tropical finfish species was due to its low fat content and slow growth of bacteria on the fish flesh (Ames & Curran, 1985).

Shewanella and *Pseudomonas* species are the SSOs commonly responsible for spoilage in aerobic ice storage. The varying amounts and also the particular species of each genus may offer an explanation for the extended shelf life goldband snapper experiences. The interactions existing between the microflora present on goldband snapper, including synergistic or antagonistic, may also provide answers.

The aim of this project is to use extensive sensory, biochemical and microbiological analyses to determine why goldband snapper has an extended shelf life.

CHAPTER 3: MATERIALS AND METHODS

Samples

Logistics made it difficult to obtain all samples of goldband snapper (*Pristipomoides multidens*) and saddletail snapper (*Lutjanus malabaricus*) from the same fisherman and the same location. All samples obtained for this project were caught off the northwest coast of Australia.

Goldband snapper and saddletail snapper samples caught using the longline method were collected from Exmouth during March. The longline method is a commercial fishing technique which employs one main line with baited hooks attached at different intervals to branch lines. The main line can be left to float in the water column or be anchored. Samples caught with traps were collected from Broome during the months March, May, June, November and December and also from Onslow during October. Saddletail snapper samples caught using the trawl method, which involves pulling a net behind one or two boats, were collected during September and October.

All fish were transported by courier to Perth in refrigerated trucks. Samples from Exmouth and Broome were usually in transit for 2 days however, there were instances of the transport trucks breaking down which extended transit time to 3 days. Due to the distance between the testing facilities in Perth and the harbours where the fish were unloaded most of the experiments did not use fish directly after capture. It is important to note that the fish used were approximately 3-4 days post harvest at storage day 0. Fish caught in Exmouth (longline method) were tested immediately after capture and so all results corresponded accurately to days of storage. The age of all other samples were estimated to be 3 days old when received in Perth. To account for the delay, fish affected by lengthy transport were graphed at day 3 for their first data point rather than beginning at day 0.

All fish were treated under the same storage conditions in the laboratory. Fish were stored in plastic tubs, covered in crushed ice and stored at 4˚C. The tubs had a drainage tube attached to limit pooling of liquid at the bottom of the tub and fresh ice was added every 2 days to ensure the fish were fully covered.

The samples were collected over a period of 7 trials. The first trial was a preliminary trial. Two goldband and saddletail snapper were sampled at each time point (days 5, 10, 17, 24, 31 and 37), with a total of 12 fish used for each species. Flesh samples were taken as described in Chapter 3 and bacterial counts enumerated. The validation of the Quality Index for saddletail snapper used a total of 6 fish per storage day tested (0, 7, 14, 21 and 28), with a total of 30 saddletail used. These samples were evaluated using the QI scheme and the texture measured and bacterial counts enumerated, as per Chapter 3. Two trials were performed for goldband snapper using only 1 fish per storage point (0, 7, 14, 21 and 28), with a total of 5 fish used per trial. The colour, QI scheme and bacterial counts were recorded. The sample numbers for this trial did not provide a basis for comparison and so a further two trials were performed with three goldband snapper sampled at every storage point (0, 7, 14, 21 and 28), with a total of 15 fish used per trial. Colour, texture, the QI scheme and bacterial counts were measured for these trials, as described in Chapter 3. A final trial was held using saddletail and goldband snapper samples caught from the same location in Exmouth. For this trial 3 goldband snapper were tested throughout the storage trial and 6 saddletail snapper (3 caught at approximately 100m and 3 caught at approximately 140m). For this trial the same fish were kept for the entire trial and monitored. Swabs were taken for bacterial enumeration instead of flesh samples.

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

Maximum Recovery Diluent (MRD)

Maximum recovery diluent preparation followed the 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 was adjusted so that after sterilisation the pH was 7.0 ± 0.2 at 25˚C.

RODAC plate (Agar contact)

The RODAC plates used for bacterial enumeration were made from a disposable plastic 65 x 15 mm 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 moulten agar was pipetted into each plate, with the meniscus of the agar seen to form a convex surface (Sveum, Moberg, Rude, & Frank, 1992).

Long and Hammer broth

Long and Hammer medium is a medium used for isolating micro-organisms from fresh seafood. Long and Hammer medium was prepared dissolving 20 g of proteose peptone No.2 (Bacto), 40 g of gelatin (Sigma), 10 g of sodium chloride (AnalaR), and 1 g of potassium phosphate (Sigma) mixed into 1 L of distilled water.

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

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 is made by adding 5 g of tryptone, 2.5 g of yeast extract, 1 g of D – glucose and 15 g of agar to 1 L of distilled water. The powder is dissolved and the pH of the mixture is adjusted so that after sterilisation the pH is 7.0 ± 0.1.

The mixture is then autoclaved at 21˚C for 15 min (Anon., 1994; Buchbinder, Boris, & Goldstein, 1953).

Inoculated PCA plates were incubated for 2 days at 30˚C.

Long and Hammer Agar (LHA)

Long and hammer agar was used to enumerate the total aerobic counts in fresh and lightly preserved seafoods. Due to its low incubation temperature and high salt content this media allows for the detection of psychrotolerant bacteria commonly found on seafood. The medium was prepared using 20 g of proteose peptone No.2 (Bacto), 40 g of gelatin (Sigma), 10 g of sodium chloride (AnalaR), 15 g of bacteriological agar (Oxoid) and 1 g of potassium phosphate (Sigma) mixed into 1 L of distilled water.

The ingredients were dissolved and the mixture adjusted to a pH of 7 ± 0.2, using 1 M sodium hydroxide, at a temperature of 20 - 25 ˚C. The media was autoclaved at 121 ˚C for 15 min, and stored in refrigerated conditions for up to 6 months. Just prior to use 0.5 ml 10 % ammonium ferric citrate solution was added per 200 ml of agar (Nordic Committee on Food Analysis, 2006; van Spreekens, 1974).

Organisms plated onto LHA were incubated for 5 days at 15˚C.

Iron Agar (IA)

Iron agar was used to identify hydrogen sulphide producers in fish samples. The hydrogen sulphide producing bacteria are identified by the black pigment they produce on the agar, when thiosulphate and / or L – cysteine is decomposed. The black pigment produced is precipitation of iron sulphide (Nordic Committee on Food Analysis, 2006). The medium was prepared using 20 g of proteose peptone No.2 (Bacto), 6 g of lab lemco powder (Oxoid), 6 g of yeast extract (Oxoid), 0.6 g of ferric citrate (Sigma), 0.6 g of sodium thiosulphate (Sigma), 10 g of sodium chloride (AnalaR) and 24 g of bacteriological agar (Oxoid) mixed into 1 L of distilled water.
The ingredients were dissolved by heating and the mixture adjusted to a pH of 7.4 ± 0.2, using 1 M sodium hydroxide. The media was autoclaved at 121˚C for 15 min and stored in refrigerated conditions for up to 6 months. A 0.04 % L - cysteine

solution is added to the melted agar just before use (Gram, et al., 1987; Nordic Committee on Food Analysis, 2006).

Organisms plated onto IA were incubated for 2 days at 25˚C.

Methods

Bacterial Enumeration

Bacterial counts were performed throughout the project to determine the changes in microbial populations throughout the spoilage process. Various methods for collecting samples for bacterial enumeration were used. The RODAC plates were introduced during the preliminary trials since the bacterial load of goldband and saddletial snapper was unknown. The RODAC pates were an easy method used to determine the initial bacterial counts. The flesh extraction was the technique most commonly used for bacterial enumeration, once the initial bacterial load had been established. Serial dilutions were performed on all laboratory samples and then distributed onto different media using the spiral plater. The swabbing method was introduced when sampling occurred on a commercial fishing boat where normal laboratory equipment was unavailable. The spread plate method was used to dispense dilutions onto media for bacterial enumeration.

RODAC (Replicate Organism Detection and Counting) plates

The principle behind RODAC plates is that they are simple, transportable and reliable media which may be used in industrial environments to test bacterial counts on surfaces. RODAC plates are an easy to use agar dish that possesses a convex surface allowing total surface contact to the area being sampled. To sample the bacterial load of goldband and saddletail snapper the RODAC plates were placed against the surface of each fish, just above the dorsal fin. Three sample points were taken at different points along the dorsal fin. Three RODAC plates, containing different growth media (PCA, IA and LH agar), were used per fish. Each plate was then incubated according to the growth media requirements. RODAC plates were used for the first sampling point of the preliminary trial to obtain an initial count since no previous studies have been performed on the bacterial load of goldband and saddletail snapper.

Flesh extraction

The subsequent sampling sessions involved cutting a 2 cm^2 square of flesh and skin from the top left hand corner of the fish. This dissection was only used on two fish, whilst the third was used for sensory analysis. The microbiological sample was placed in a stomacher bag and weighed on an A & D EW 300B digital scale. A 1 in 10 dilution was prepared using maximum recovery diluent (MRD) and the sample was placed in the stomacher for processing. 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 PCA (incubated at 30 °C), IA (incubated at 25°C) and LHA (incubated at 15°C) using the spiral plate method.

Swabbing Method

It was not feasible to use the flesh extraction method for all experiments and so another sampling method was introduced. Samples were collected by rubbing a sterile swab in a back and forth motion whilst turning the swab over a measured area of 2 cm^2 on the fish above the dorsal fin. Once the area had been sampled the swab was placed in 10 ml of peptone water (0.01 %) + 0.85 % saline. Further serial dilutions were performed to obtain accurate counts as the total counts began to increase with storage time. Dilutions ranged from 10^{-1} through to 10^{-7}, and two dilutions were plated every sample point based on a probable count for each sample point. This method is quick and simple, requires less equipment and was useful during the Exmouth field trip where conditions were unpredictable and the laboratory setup on board the boat was primitive.

Spiral Plater

The Whitley Automated Spiral Plater (WASP) was used to enumerate the number of viable bacteria present in the sample. The 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

darkfield colony counter and a counting template. 50 µl of dilution was pipette onto the PCA, IA and LHA plates and incubated as previously described.

Spread Plate

The spread plate method involves a set volume of inoculum (typically 100 µl per 90 mm petri dish) being dispensed onto an agar plate. Once dispensed, the inoculum is spread across the agar, commonly with an L shaped spreading stick, until a thin film covers the surface. Once the inoculum absorbs into the media, the petri dish is incubated at specific temperatures for a defined period of time, depending on the media used and the bacteria being isolated (see above) (McCLure, 2008).

Preliminary Identification

Preliminary identification was used to group the organisms isolated from goldband and saddletail snapper. By grouping the organisms based on phenotypic characteristics such as Gram stains, colony morphology, pigment, optimum growth temperature, positive and negative reactions for motility, oxidase and catalase reactions, and fermentation we were able to determine the specific tests needed to identify the organisms to a genus and species level.

Gram stain

Gram stains were performed based on the method introduced by Jensen (1912). This method involves using crystal violet solution, Lugol's iodine solution, acetone and dilute carbol fuchsin stain.

A thin smear was prepared by spreading a loopful of bacterial suspension on a clean glass slide. The suspension was air dried before being passed through a flame 3 - 4 times to ensure the suspension was fixed to the slide. Once cooled the smear was flooded with crystal violet solution for 30 sec. The slide was then washed with distilled water. The smear was then flooded with Lugol's iodine solution for 30 sec, after which time the slide is again washed with water. The slide was decolourised by placing it on an angle and sending drops of acetone onto the smear until only colourless solvent flowed from the slide. The smear was immediately washed with water and then flooded with dilute carbol fuchsin stain for 30 sec. The slide was then

washed with water and blotted dry (Conn, Bartholomew, & Jennison, 1957; Harrigan, 1998).

The Gram stains were examined under a 100 x magnification using immersion oil.

Colony Morphology

The colony morphology was observed for each isolate. Approximate size, colour, elevation (domed, convex, flat), texture (shiny, dull, mucoid etc), edge (entire, filamentous, irregular) were determined and recorded (Yuen & Davis, 2006).

Pigment

The presence of pigment is useful in species identification. Pigment is not always obvious and so to ensure correct colours were recorded using a sterile white cotton swab. The swab was used to sweep some of the bacterial growth onto, after which the pigment colour, if present, was observed. Some pigments were obvious and so were recorded immediately (Yuen & Davis, 2006).

Motility

To determine the motility of each bacterial isolate a 9 ml tube of brain heart infusion broth (BHIB) was inoculated with a loopful of each colony. After 4 h incubation, a wet mount of each inoculation was prepared by placing one to two drops of culture on a slide covered with a coverslip. The wet mount was examined under a microscope to determine if the isolates were motile. All non-motile isolates were re-evaluated after 24 h. If after 24 h there was no movement the isolate was considered non-motile. If an organism did not grow, long and hammer medium was substituted for BHIB, since it contains a higher salt content aiding the growth of isolates extracted from a marine environment. The process for examining for motility was then repeated (Conn, Jennison, & Weeks, 1957).

Oxidase test

Oxidase reagent (In vitro Diagnostic) was used to identify if the organisms were cytochrome c oxidase positive or negative. A vial of the reagent was emptied onto a filter paper and organisms were then smeared onto the paper. A blue colour change within 10 sec indicated a positive result because the indicator has been oxidised, an

unchanged smear indicated a negative result as the indicator has been reduced (Harrigan, 1998).

Catalase test

This test determines the production of the enzyme catalase by bacteria. The catalase reagent is 3 % hydrogen peroxide (AnalaR) solution. Part of a colony is collected on a sterile loop and placed onto a drop of the catalase reagent. A positive test is indicated by the production of oxygen bubbles. Bacteria without the catalase enzyme will not a produce bubbles. It is important to isolate the colony on the loop without any residual agar as this may interfere with the results of the catalase test by producing a false positive (Harrigan, 1998).

Oxidative/Fermentative metabolism

To determine if the isolates possessed an oxidative or fermentative metabolism, triple sugar iron agar (TSIA) slopes were smeared and stabbed with a bacterial inoculum. TSIA slopes were used to identify bacteria based on hydrogen sulphide production and fermentation of lactose, sucrose and glucose. A colour change from orange - red to yellow (acid reaction) in the butt of the TSIA slope indicates glucose fermentation has occurred. A colour change in the slant from orange - red to yellow indicates sucrose and / or lactose fermentation has occurred. A colour change to red (alkaline reaction) occurs when organisms are forced to catabolise peptones and amino acids for their energy supply. If the slope turns black it means that the organisms are hydrogen sulphide producers. The slopes were incubated in the optimum growth temperature already established for each isolate. After 24 h each slope was assessed, if no growth or colour change had occurred the slopes were left for another 48 hours. If no change had occurred after this time, a “no change” was recorded (Harrigan, 1998).

Optimum Temperature

Determining the optimum growth temperature is a phenotypic characteristic that helps preliminarily identify bacteria as a psychrophile, mesophile or thermophile. Each isolate was plated onto its isolation media (PCA, IA, LHA) and incubated at 30°C, 25°C and 15°C respectively. The optimum growth temperature was established

for each isolate by assessing the amount of growth on each plate and also the size of the colonies.

Specific Identification

Preliminary identification of all isolates allowed them to be sorted into groups, based on their phenotypic characteristics. Further identification was needed using more specific identification tests including biochemical reactions, fatty acid analysis, protein fingerprinting and DNA analysis to identify the organisms to a species level. All of the tests described below were used based on the results obtained from preliminary identification.

API

There are different types of API test strips that are used to identify different groups of bacteria including *Staphylococcus, Streptococcus, Bacillus, Enterobacteriaceae* and *Vibrionaceae* species. The API strips described below were used in this project.

To interpret the results from each API strip an extensive database has been setup on the BioMerieux website https://apiweb.biomerieux.com. Results from each test strip were entered into the Apiweb which then compares each biochemical test against the other profiles in the database. Results are displayed as an ID percentage and Typicity index which allows users to assess how closely related the organism is to the species listed on the report.

1. API 20NE

   This simple biochemical test strip is used to identify non-fastidious, non-enteric Gram negative rods (i.e. not belonging to the Family *Enterobacteriacea*). The test strip was inoculated and incubated using instructions from the API 20NE instruction manual. All results were interpreted using the API 20NE instruction manual. The conventional tests were determined by a colour change and the assimilation tests determined by turbidity in the cupule (BioMerieux). Final results were entered into the API web whereby a numerical profile was established. The analytical profile index was then entered into the database and potential IDs were reported.

2. API 20E

The API 20E test strip is a standardised system used to identify *Enterobacteriaceae* and other non-fastidious (mainly enteric) Gram negative rods. The test strip was inoculated and incubated using instructions from the API 20E instruction manual (BioMerieux). All results were interpreted using the API 20E instruction manual. The results were all displayed as colour changes (BioMerieux). Final results were entered into the API web whereby a numerical profile was established. The analytical profile index was then entered into the database and potential IDs were reported.

3. ID 32 STAPH

The ID 32 STAPH is a simple standardised strip used to identify *Staphylococcus*, *Micrococcus* and related genera. The test strip was inoculated and incubated using instructions from the ID 32 STAPH instruction manual. All results were interpreted using the ID 32 STAPH instruction manual. The results were all displayed as colour changes (BioMerieux). Final results were entered into the API web whereby a numerical profile was established. The analytical profile index was then entered into the database and potential IDs were reported.

4. Rapid ID 32 STREP

The rapid ID 32 STREP test strip is a standardised test for the identification of streptococci and enterococci and closely related organisms. The test strip was inoculated and incubated using instructions from the Rapid ID 32 STREP instruction manual. All results were interpreted using the API Rapid ID 32 STREP instruction manual. Final results were entered into the API web whereby a numerical profile was established. The analytical profile index was then entered into the database and potential IDs were reported.

5. 50CHB/E Medium

The API 50 CHB/E Medium is used for the identification of *Bacillus* species as well as *Enterobacteriaceae* and *Vibrionaceae* families. The test strip was inoculated and incubated using instructions from the 50 CHB/E instruction manual. An API 20E test strip is prepared every time an organism is tested

using the API 50 CHB/E test strip (bioMerieux). All results were interpreted using the API 50CHB/E Medium and 20E instruction manuals. Final results were entered into the API web whereby a numerical profile was established. The analytical profile index was then entered into the database and potential IDs were reported.

MALDI-TOF

MALDI-TOF mass spectrophotometry is a rapid and accurate technique for identifying bacteria. All bacteria identified using this method were fish isolates, grown at the growth conditions which they were isolated from. Colonies chosen for identification were spotted on the FlexiMass targets and then overlaid with 1 µl of matrix solution and mixed (Hsieh et al., 2008). Gram positive organisms or isolates with a mucoid morphology were first treated with 0.5 µl of 25 % formic acid. The colony was mixed and then left to dry. The 1 µl of matrix was then applied over the dried sample. The standard used for each Fleximass target slides was the *Escherichia coli* strain NCTC 12923. The protein mass fingerprints were obtained using a MALDI-TOF mass spectrometry Axima Assurance machine (Shimadzu-Biotech Corp., Kyoto, Japan). The laser used for detection had a frequency of 50 Hz and operated between a mass range of 2000 - 20000 Da. A minimum of 5 laser shots were taken to generate each ion spectrum and 100 protein mass fingerprints were averaged and processed using Launchpad (Shimadzu-Biotech Corp., Kyoto, Japan). Spectral Archiving And Microbial Identification System (SARAMIS) (Shimadzu-Biotech Corp., Kyoto, Japan), was the data processing software used for identifying the organisms.

Fatty Acid analysis- MIDI system

Microbial fatty acid profiles are unique to every bacterial species. A method currently being used in research and clinical laboratories is the identification of micro organisms based on the MIDI Sherlock system which uses gas chromatography to analyse extracted microbial fatty acid methyl esters (FAMEs) (Kunitsky, et al., 2006).

The fatty acid analysis was performed by the Clinical Bacteriology Department at PathWest Laboratories. The method followed for this analysis originated from the

Manual of Bacteriology Identification Procedures at PathWest (Aravena-Roman, 2011). The MIDI Sherlock system uses a standard preparation technique for all aerobic samples. Environmental samples were prepared by inoculating the organism onto Trypticase Soy Broth Agar and incubating at 28 ℃ for 24 h. 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. Once the bacteria were harvested 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 (25.0 m, 200 µ) 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, & Kroppenstedt, 1998).

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

DNA 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 (Sanchez-Alonso, 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) to 100℃ for 15 min. The suspensions were centrifuged at 9,000 x g and the supernatant was used for further analysis. Eight micro litres of the supernatant was added to duplicate PCR mix tubes (Appendix 5). 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℃. After 0.4µl of first round product was

inoculated into the second-round tubes, cycling was carried out under the same conditions as for the first round but with a 50°C annealing temperature.

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. Sequencing was carried out using the previously mentioned first-round product and primers bp1 and bp4. The PCR products were treated with presequencing clean-up enzyme (ExoSap-It USB Corp., Cleveland, Ohio) and then used as the template in a sequencing mix (Applied Biosystems, BigDye terminator v3.1). The now labeled products of the sequencing reaction were then filter purified using Microcon PCR filters (Amicon Millipore, North Ryde, Australia) and sequenced in the Applied Biosystems xl 3130 Sequencer.

Sequences were analysed using the chromatogram plot using Chromas Lite 2.0 version Applied Biosystems. The 16S rDNA gene sequence was analysed using BLAST with nrdatabase of NCBI GenBank database to find closely related bacterial 16S rDNA sequences.

Biochemical analysis

Trimethylamine (TMA)

TMA analysis was performed using the method developed by Baixas-Nogueras, Bover-Cid et al. (2001) based on the Dyer (1959) method. To begin, 25 g of fish muscle was added to 50 ml of 7.5 % aq trichloroacetic acid. The fish was homogenised using a mortar and pestle and the mixture was then filtered using Whatman paper No. 1. Once the samples were prepared 1 ml of sample extract was added to 3 ml of deionised water. Added to every sample tube was 1 ml of 20 % FA, 10 ml of anhydrous toluene and 3 ml of potassium carbonate solution. The tubes were stoppered and shaken vigorously and the toluene phase was transferred into tubes containing 0.2 g of anhydrous sodium sulphate. The tubes were then shaken to obtain a dehydrated extract. Five millimeters of the water-free toluene extract was mixed with 5 ml of 0.2 % picric acid solution in another tube. Absorbance was measured using a spectrophotometer (CM-500i/CM-500C) at 410 nm wavelength. The TMA was calculated from the optical densities using the standard curve. The calibration curve used for this method involved dispensing 1, 2, 3 and 4 ml of a

0.01mg/ml TMA standard solution along with 3, 2, 1 and 0 ml of deionised water, respectively.

Total Volatile Bases (TVB - N)

GC Analysis

Sample extraction was performed with the modified version used in the TMA analysis. The total volatile basic nitrogen determination was based on the method developed by Nogues, Hurtado et al. (1996). After extraction, 0.6 ml of toluene was added to 1 ml of sample. The tubes were stoppered and incubated in a water bath (10 min at 60°C). After incubation they were shaken for 2 min. One microlitre of the separated toluene layer was then manually injected into the GC machine.

A Perkin Elmer Autosystem XL gas chromatograph with a Zebron ZB-Wax column, L= 30 m x ID = 0.53 mm x df = 1.00 µm, temp 20°C (Phenomenex) was used for TVB analysis. The chromatograph used a flame ionization detector which ran at 190°C. The initial loading temperature of the machine was 32°C with the oven temperature ranging from 20°C – 150°C. The run time was for 11.9 min per sample. The carrier gas was helium and the detection gases were hydrogen and air.

Before samples were injected into the system the sensitivity of the GC machine was measured using spiked samples of saddletail snapper. To test the sensitivity of the machine 3 different concentrations (1000 ppm, 500 ppm and 300 ppm) of DMA and TMA were inoculated onto 1 g of fish. The samples were then extracted per the method outlined above.

Kjeldahl Method

Another method used to determine the amount of TVB - N present in the fish flesh was based on a distillation and titration method (Baixas-Nogueras, et al., 2001). All samples were prepared using the same method for TMA analysis. Ten millilitres of the extract was transferred into a distillation tube and 3 drops of phenolphthalein was added. Sodium hydroxide (40 % W/V) was then added to the tube until the extract had alkalised and a pink colour appeared. The mixture was then distilled until 125 ml of distillate was collected into an erlenmyer flask containing 20 ml of 4 % boric acid containing 3 to 4 drops of indicator. The distillate was then titrated using 0.01 N

hydrogen chloride, with and end point of green to red. The control for this test was a sucrose solution.

All TVB - N values were calculated by multiplying the titration (less the value from the blank) by 2.8. All answers were expressed in mg N per 100 g sample.

Hypoxanthine

The method used to determine hypoxanthine was a modified version of another earlier method used on canned sardines (Vazquez-Ortiz, Pacheco-Aguilar, Lugo-Sanchez, & Villegas-Ozuna, 1997). The extraction process was based on methods by Ryder (1985) and Veciana-Nogues, Albala-Hurtado (1996). Extracts for analyses were prepared by homogenising 1 g of fish muscle in 5 ml of 0.6 M perchloric acid over a vortex mixer. The sample was then stored in a – 80 °C freezer overnight. After defrosting the samples they were centrifuged at 3000 g for 10 min in a refrigerated centrifuge at 0 – 2°C (Eppendorf Centrifuge 5810R) (Ryder, 1985). The supernatant was removed by filtration through a Whatman No. 1 filter at 0 °C. The solid residue was re-homogenised and the previous process repeated. The filtrates were combined and made up to 10 ml with 0.6 M perchloric acid (Veciana-Nogues, et al., 1996). Six millilitres of the filtrate was neutralised to pH 6.5 - 6.8 with 1 M potassium hydroxide (Vazquez-Ortiz, et al., 1997). After standing at 0°C for 30 min the potassium perchlorate formed was removed by filtration through Whatman paper No. 1. The filtrate was then frozen at – 80°C for further analysis.

A Hewlett Packard Agilent 1100 series High Performance Liquid Chromatography (HPLC) machine was used for the determination of TMA and other amines. Separations were achieved on an Alltech 25 cm x 4.6 mm Apollo C18 5 µ stainless steel column. The mobile phase of 0.04 M potassium dihydrogen orthophosphate (pH 7.5) and 0.06 M dipotassium hydrogen orthophosphate (pH 7.5) dissolved in deionised water was used at a flow rate of 1ml / min. Fifty microlitres of each sample was injected into the column and was read by a Variable Wavelength Detector (VWD) at an absorbance of 254 nm.

The identification of the nucleotides and nucleosides was performed using retention time comparisons of individually prepared standards. A standard mixture with a

concentration of 20 mM for each different standard compound (ATP, ADP, AMP, IMP, HxR and HX) was prepared. The standard mixture was used to calibrate the machine before any sample testing.

All results were presented as (mg / 100 g) and graphed against the number of storage days.

K value

Extraction of all samples used for K value analysis were prepared using the same method used Hypoxanthine analysis (Ryder, 1985; Veciana-Nogues, et al., 1996). K values were calculated using the equation below.

$$\text{K value (\%)} = \frac{(\text{HxR} + \text{Hx}) \times 100}{\text{ATP} + \text{ADP} + \text{AMP} + \text{IMP} + \text{HxR} + \text{Hx}}$$

Physical methods

Texture

Instruments may be used to measure changes in physical properties to determine fish spoilage. Measuring the texture of a fish over its storage life is one method of measuring deterioration. The method used was based on a paper written by Macagnano et al. (2005) which also highlighted other instruments which could be used to mimic human senses and determine deterioration.

The measurements were taken using a stable micro systems (SMS) texture analyser (TA ·XT2i). The fish tested were compressed by 5 % with an aluminium cylinder probe (diameter 10mm) with a cross speed of 0.8 mm / sec. The pressure was kept constant for 60 sec and the relaxation profile was measured. Three points along the lateral line of the fish were measured (near the gill plate, above the pectoral fin and near the caudal fin) on the same side for each fish. There were 3 samples tested per data point for storage days 0, 7, 14, 21 and 28.

The relaxation profile was measured by dividing each data point by the maximum force exerted.

The calculations were as follows:
Force 1- Force 2 = Force 3

Force 1 being the initial force, force 2 being the final decaying force and force 3 being the difference between the two. The changes in force 3 over time enabled measurement of the difference in texture as the fish deteriorate.

Colour

The method for measuring the changes in colour was based on the method used by Macagnano et al. (2005). A Minolta spectrophotometer (CM-500i/CM-500C) was used to measure the colour changes experienced by goldband and saddletail snapper during storage. The measured colours were expressed as CIE Lab coordinates which displays as L, *a** and *b**. L measures lightness on a 0-100 scale from black to white, *a** measures the red (+) to green (-) spectrum and *b** measures the yellow (+) to blue (-) spectrum. Measurements were taken above the pectoral fin and on the caudal fin by leaning the instrument against the fish. Three individual samples were measured for each time point of shelf life.

Data analysis

Data and results were statistically computed, graphed and tabulated using normal Microsoft Office Excel 2007 and the statistical package SPSS 16.0.

Statistical analysis of the sensory data measuring all QIM scores, Torry scores and the individual QIM attributes obtained for Goldband and Saddetail Snapper were averaged and graphed as a function of time using Microsoft Office Excel 2007. The individual QIM and Torry scores for each panellist were collated and graphed as a function of time.

A Friedman Two-Way ANOVA was performed to compare the panellists and determine if there was any variation existing between them. To identify the source of the significance the Wilcoxon Signed Rank Test with a Bonferroni adjustment was performed using SPSS 16.0. Statistical analysis of the data collected from the physical analyses, colour and texture, were averaged and graphed as a function of time using Microsoft Office Excel 2007.

All data collected for the bacterial counts were averaged and graphed as a function of time using Microsoft Office Excel 2007. The percentage of the different bacterial species isolated from goldband and saddletail snapper were presented in a pivot table as a function of time in Microsoft Office Excel 2007. All data collected for TMA - N, TVB - N and hypoxanthine analyses were averaged and graphed as a function of time in Microsoft Office Excel 2007.

CHAPTER 4: VALIDATION OF THE QUALITY INDEX METHOD OF SADDLETAIL SNAPPER

Introduction

The Quality Index Method (QIM) is a fast simple sensory method used to determine the deterioration of seafood (Bremner, 1985). The QIM is a species specific sensory analysis which allows specific traits of a fish species to be monitored and assessed throughout storage (Hyldig & Green-Petersen, 2004). The Quality Index score increases linearly in relation to storage days on ice and so the remaining shelf life is able to be calculated by determining the Quality Index score during any point of storage. QIM is a fast accurate method that provides producers, buyers, sellers and retailers with a standard method of measuring quality (Hyldig, et al., 2009).

A QIM for goldband snapper has been published in the Australian Quality Index Manual (Boulter, Poole, & Bremner, 2006). The QIM for saddletail snapper has only recently been developed and is currently being introduced to the industry by the Sydney Fish Market (Boulter, et al., 2006). Since the QIM for saddletail snapper has not been widely distributed throughout the Australian fishing industry, validation sessions were needed to ensure that the developed QIM would apply to saddletail snapper caught in Western Australian waters.

Genetic variation exists amongst species from different locations (Ovenden, Salini, O'Connor, & Street, 2004). If genetic variation exists between east and west coast saddletail snapper it is possible that this may have an effect on the shelf life and/or the criteria used to assess the QIM for saddletail snapper. A validation of the established QIM using West Australian caught saddletail snapper was performed to determine if any adjustments were necessary before it was used in this project. The shelf life of a fish may also be calculated by assessment of cooked fillets, using the Torry scheme. This was also undertaken.

Methods

Samples

Saddletail snapper samples were sourced from Exmouth. One company supplied samples during the months of September and October which had been harvested using the trawl method, all samples were assumed to be 3 days old on receipt in Perth. Six fish were used for each storage day.

Panellists

Six sensory panellists were recruited for this study. They were all volunteers; five were Curtin University students and the sixth was a post-doctoral researcher working in the seafood industry. All panellists were trained in how to use the QIM scheme over three sessions however, only one training session was performed for the Torry scheme.

Before participating in the study, all panellists were given a consent form indicating that they would be expected to attend three validation sessions and a training session. All were asked to sign the consent form to acknowledge that none of them had any food allergies or was pregnant, and that they all understood the limited risks involved with the study.

QIM setup

The procedure for validating the QIM closely followed the procedure outlined by Martinsdottir, Sveinsdottir et al (2001). Since the QIM for saddletail snapper had already been established, one training session was needed for panellists to become familiar with the general appearance of saddletail snapper and also to demonstrate how to perform sensory evaluation using the QIM. To help panellists determine the difference between fresh and spoilt, they were taken through the QIM using a fresh saddletail snapper and one stored for 28 days. Each attribute was observed and any questions the panellists had were discussed and resolved.

Three validation sessions were held to confirm the QIM established in Queensland accurately measured the deterioration of West Australian caught saddletail snapper. The validation sessions took place within the sensory laboratories at the School of Public Health at Curtin University. This was due to the lack of the availability of appropriate facilities and panellists in Exmouth, WA, where the fish had been caught. Refrigerated transport from Exmouth to Perth takes at least 2 days; the freshest fish used in the validation study were therefore assumed to be 3 days old. Fish were assessed weekly by the panellists. Three different storage days were assessed at every session. The fish were placed randomly on sterile, neutral coloured desks all facing the same direction. Each fish was labelled with a random corresponding

number. Panellists were asked to assess each fish using the QIM scheme shown in Appendix 1.

Photo setup

Photographs were taken at the time of Quality Index assessment to document and compare the visible spoilage changes against the QIM score. They were used as reference images in the final QIM scheme. The fish were laid flat inside a black box illuminated by several lamps. Constant illumination of the sample precludes any variation between photographs and the black box prevented any variation due to outside light sources. The camera was positioned above the fish at a height to include the whole fish in the photograph. The fish varied in size considerably and so it was necessary at times to adjust the height of the tripod to which the camera was attached.

Torry analysis

Panellists assess the flavour and odour of cooked fillets, determining their acceptability by grading them on a scale from 10 (fresh sample) to 3 (spoiled sample). The Torry scheme is commonly used to validate the QIM's ability to assess the maximum storage life of a fish species. The procedure used in this experiment closely followed that of Martinsdottir, et al. (2001). Since an established scheme was being used, only one training session was organised to allow panellists to become familiar with saddletail snapper and to demonstrate how to perform sensory evaluation using the Torry scheme. Panellists were given the Torry scoresheet, "*Freshness evaluation of cooked lean fish*", to assess the saddletail snapper fillets (Appendix 3). The samples being assessed were fillets from the whole fish assessed in the preceding QIM session. All fillets were a standardised 2cm x 2cm and were cooked in a microwave oven (1800 Watts, for 20 sec) in the kitchen behind the assessing area. Each panellist was seated in a separate sensory booth (all white) and access to the kitchen was supplied by a small window which could be opened from the kitchen side. All samples were placed on white plates and labelled with a separate set of random numbers. Samples were passed through this window to each participant where they were assessed for odour and flavour using the Torry scoresheet provided. Water and plain crackers were provided to allow the panellists to assess each sample with a fresh palette.

Results and Discussion

Quality Index Method

As shown in Figure 4.1 the Quality Index (QI) calculated from multiple sample points formed a linear relationship with storage days on ice. The linear relationship observed, y = 0.3524x + 3.4095 (x = days on ice, y = QI) had a correlation (R^2 = 0.9299) slightly lower than those obtained from the initial QIM studies performed on saddletail snapper (R^2 = 0.9398, R^2 = 0.9832, R^2 = 0.9851) (Boulter, Poole, & Bremner, 2006).

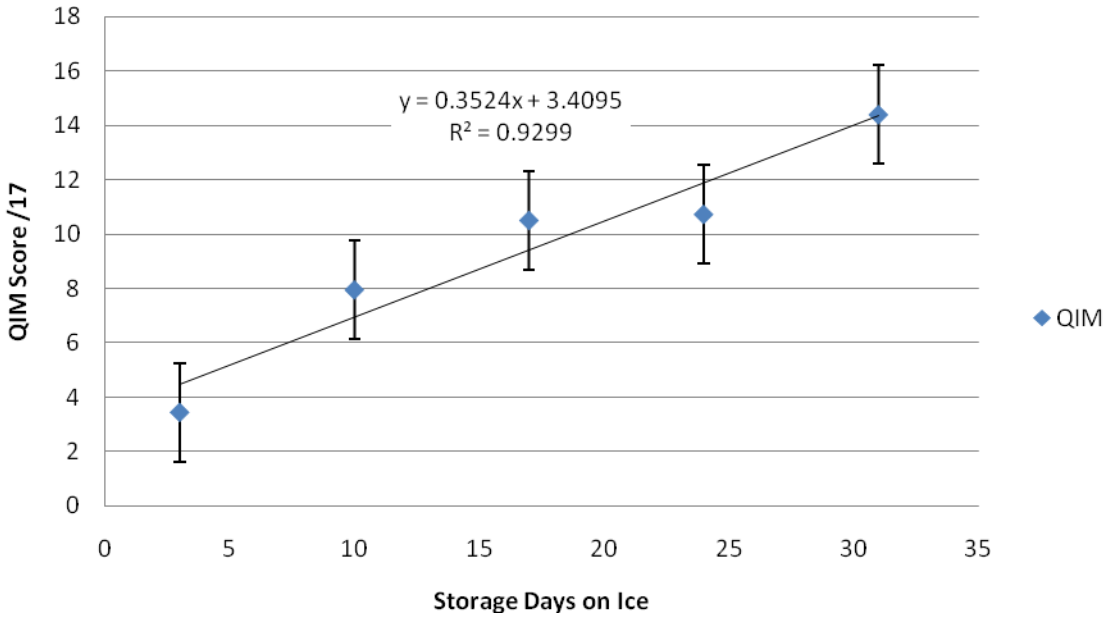


Figure 4.1: Comparison of the Quality Index (QI) and storage time of saddletail snapper

A perfect correlation was unlikely to be attained due to the experimental setup. Insufficient storage space limited the number of experiments that could be performed at any one time. All replicate experiments were run weeks apart and the fish used for each replicate experiment were not guaranteed to be caught from the same fishing ground. The variation in fish was not only limited to catchment area and time of harvest but also to the size of the fish. The fish used throughout all experiments varied in size from small, juvenile fish to larger, more mature fish. These differences may have affected the QIM score; for example, larger fish have shown to maintain quality better than smaller fish (Pedrosa-Menabrito & Regenstein, 1990). Although all fish were supplied by the same company, storage and handling conditions on the

boat and during transport to Perth would not have been identical. All of these factors affect the quality of the fish which in turn affects the quality of the correlation between the QIM score and storage days.

Although the QI score increased with storage time (Figure 4.1) it did not reach the maximum QI score, even when the fish were considered spoiled, at 24 days.

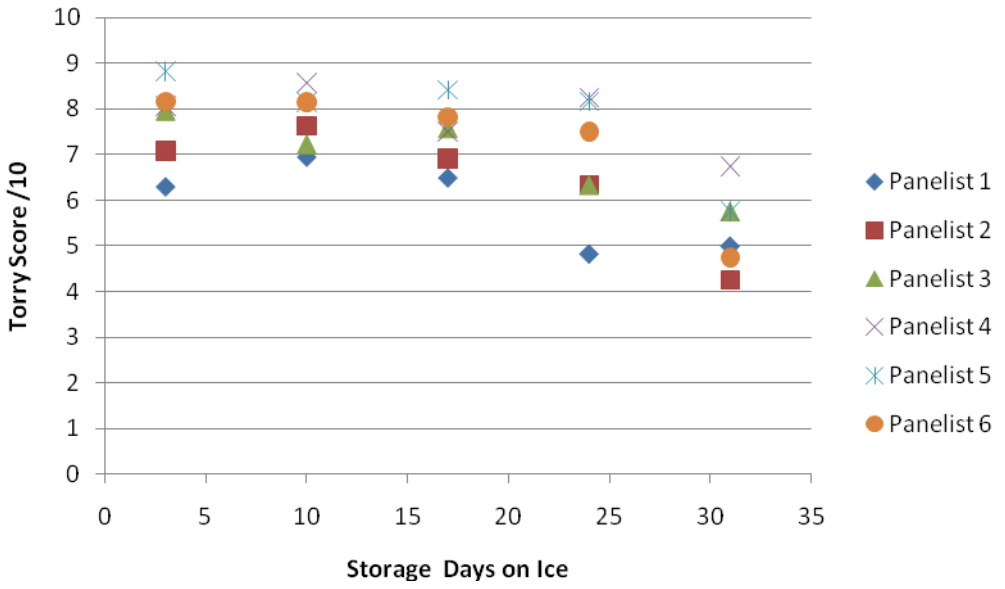


Figure 4.2: Average QI of saddletail snapper given by each panellist.

Figure 4.2 shows the individual panellist rankings. A Friedman two way ANOVA indicated that the rankings from each panellist varied significantly when it came to assessing the deterioration of each fish, $\chi^2_F = 14.059$, $df = 5$, $p = 0.015$. Follow-up pairwise comparisons with the Wilcoxon Signed Rank test indicated that Panellist 1 was significantly different to the other panellists. Panellist 1 had a high mean rank of 5.9, in comparison to Panellists 2 - 6 who had mean ranks of 4.4, 3.1, 2.6, 2.4 and 2.6, respectively. There was a significant difference ($p = 0.043$) between the scoring of Panellist 1 and all other panellists except Panellist 3. Although Panellist 1 was significantly different, the rankings followed the same trend as the other panellists, on a higher scale.

The panel used was a trained sensory panel; however, the training was not as extensive as professional training sessions due to limited resources. Hence, personal experiences and cultural differences may still have affected the way the panellists viewed the fish throughout the spoilage process. Figure 4.3 shows the score of each component of the QIM scheme, measured throughout the spoilage process.

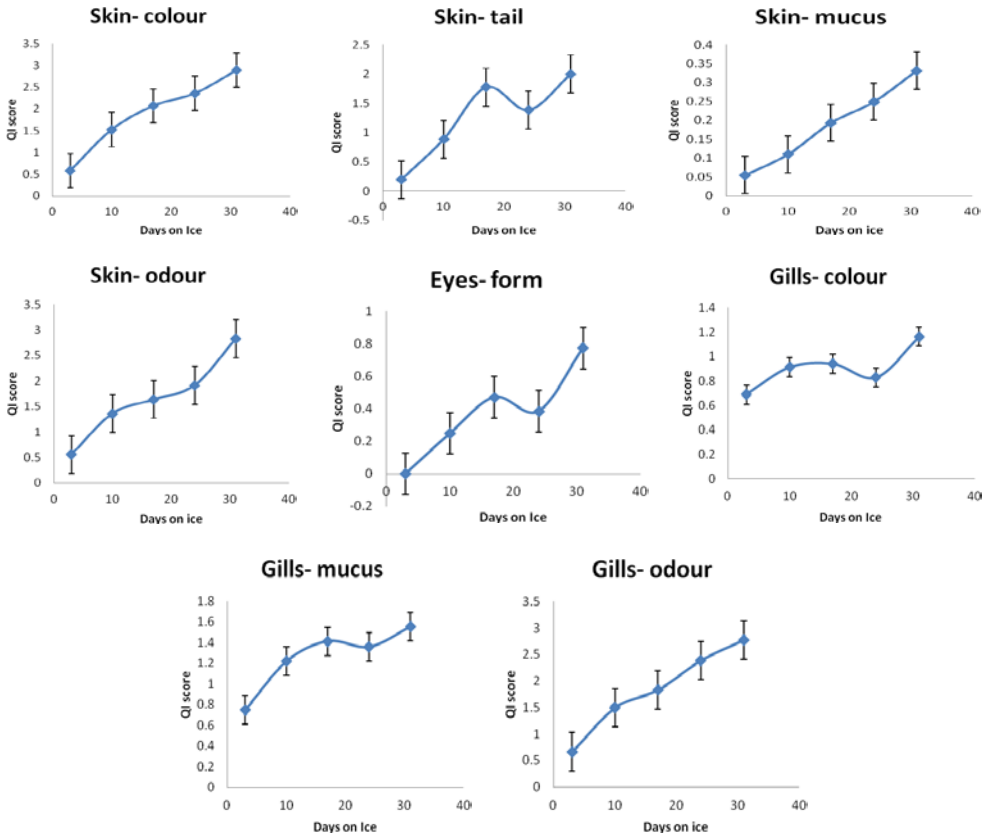


Figure 4.3: Average scores of each quality attribute assessed with the QIM for saddletail snapper stored in ice.

The scores for the QIM attributes increased with storage time on ice, as observed in Figure 4.3. The average scores for skin colour, skin mucus, skin odour and gills odour increased consistently throughout storage. There was a slight decrease in the scores for the gills colour, gills mucus, form of the eyes and the appearance of the tail at day 24, before an increase at day 31. Overall, all QIM attributes increased and all contributed to the overall QIM score equally.

Evaluation of cooked fish

A negative linear relationship was observed between the odour and flavour components of the Torry scheme when compared to the storage days on ice (Figure 4.4). There was a high linear correlation between the Torry score for flavour and storage time, R^2= 0.873 (Figure 4.44). The linear relationship observed for the flavour component of the Torry scheme was y = -0.090x + 8.478. The Torry score for flavour began at 7.8 and decreased 0.5 by day 17. By day 24 it had decreased to 6.5 and by day 31, the end of shelf life, it had reached 5.25, indicating slight sourness and traces of "off" flavours. A score of 6 was obtained by day 27; this is usually the cut off point for sale as it is the stage before "off" odours and flavours are detected.

There was a good correlation between the Torry score for odour and storage time, R^2= 0.663 (Figure 4.4). The linear relationship observed for the odour component of the Torry scheme was y = -0.070x + 8.378. The odour score began at 7.69 and decreased to 7.3 by day 24. There was a drop to 5.5 at day 31 of storage.

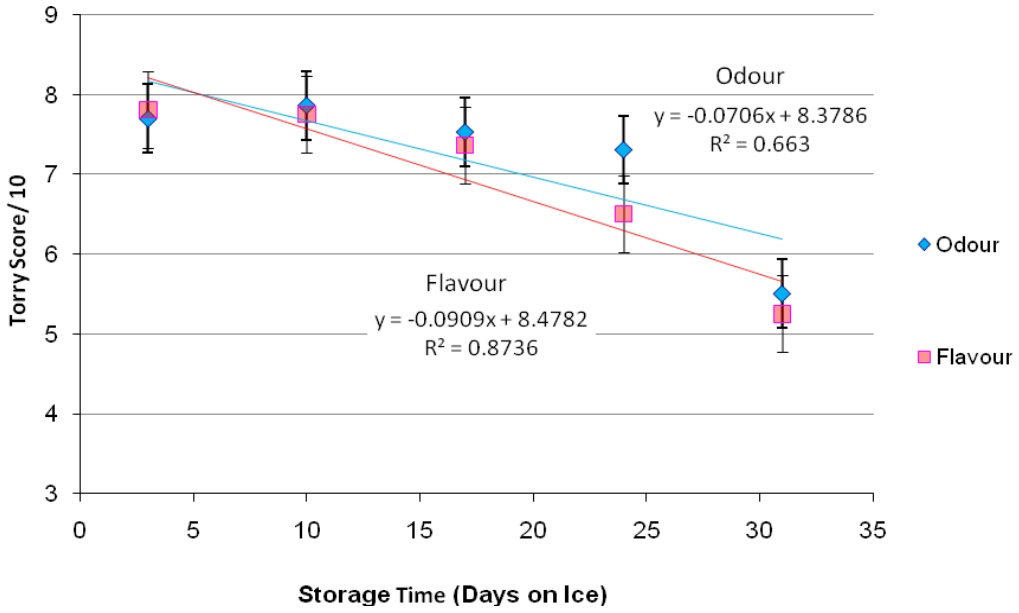


Figure 4.4: Average Torry score for odour and flavour from each panellist.

Previous studies (Bremner, 1985; Huss, 1995) have shown a decrease in the Torry score for odour and flavor as storage time is increased. The loss of the fresh odour

and flavor experienced when the fish is newly harvested is a result of biochemical and microbiological changes occurring on the fish as it spoils.

The individual Torry scores were graphed for each panellist in Figure 4.5. Overall, most panellists were consistent throughout the sensory sessions, with some variation occurring between panellists at day 24.

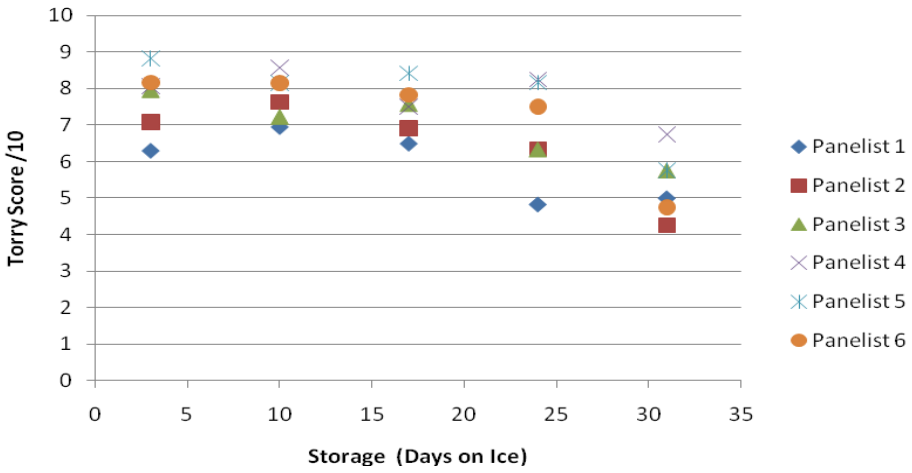


Figure 4.5: Average Torry score for cooked saddletail snapper fillets, given by each panellist.

Overall the consistency between the panellists was acceptable, however, a more detailed training session or multiple sessions being held instead of the single training session might have prevented any variation. A larger judging group would have also decreased the effects of this variability however, scheduling sessions and other logistics would then have become problematic.

QI score verus Torry score

The average QI and Torry scores for saddletail snapper were compared in Figure 4.6. The Torry score formed a negative linear relationship with storage days on ice. The linear relationship observed was y = -0.0819x + 8.4233 and had a correlation of R^2= 0.8006. The results from this analysis were similar to the results of previous studies (Boulter, et al., 2006; Hyldig, et al., 2009) which observed that as a fish deteriorates the QI score increases to show an increase in deterioration . Alternatively, the Torry score is expected to decrease as a fish spoils, displaying an increase in “off” flavours and odours.

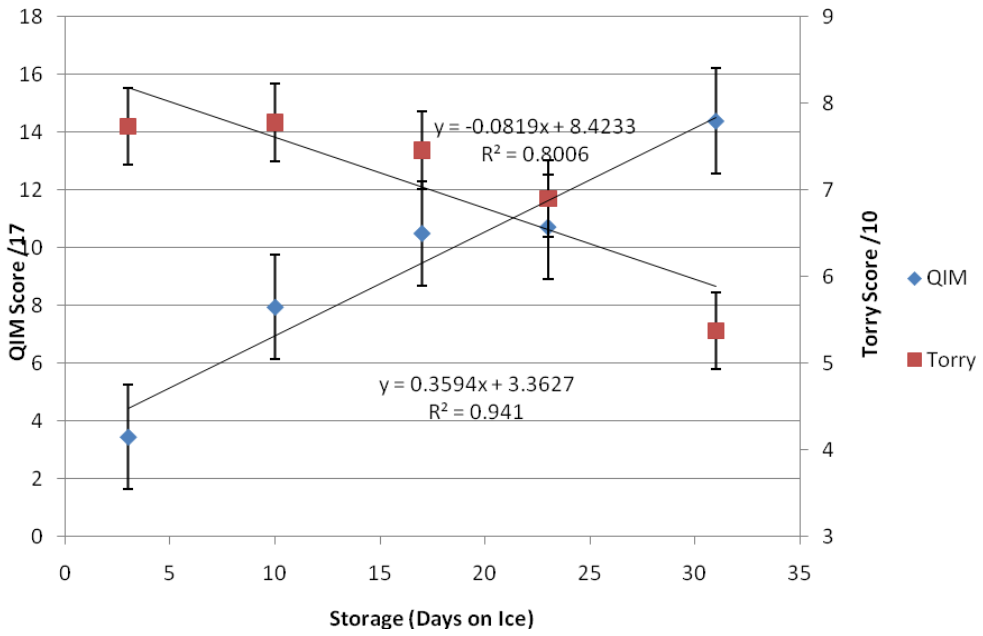


Figure 4.6: Average QI and Torry scores for saddletail snapper during storage on ice.

These analyses show that the QIM for saddletail snapper developed and used with saddletail snapper caught off the east coast of Australia is also able to be used for saddletail snapper caught in Western Australia. The QIM data showed greater variability than the Torry score due to the scoring of panellist 1 with the saddletail snapper QIM.

Colour

To validate the sensory score, other quantitative measures were employed. A Minolta spectrophotometer (CM-500i/CM-500C) was used to measure the colour changes seen with saddletail snapper during storage. This system utilises the LAB colourspace to assess colour. The colour change was measured using three different colour spectrums, the white to black spectrum (L*), green to red spectrum (a*) and blue to yellow spectrum (b*). There was little colour change in L* measurements for saddletail snapper displayed in Figure 4.7.

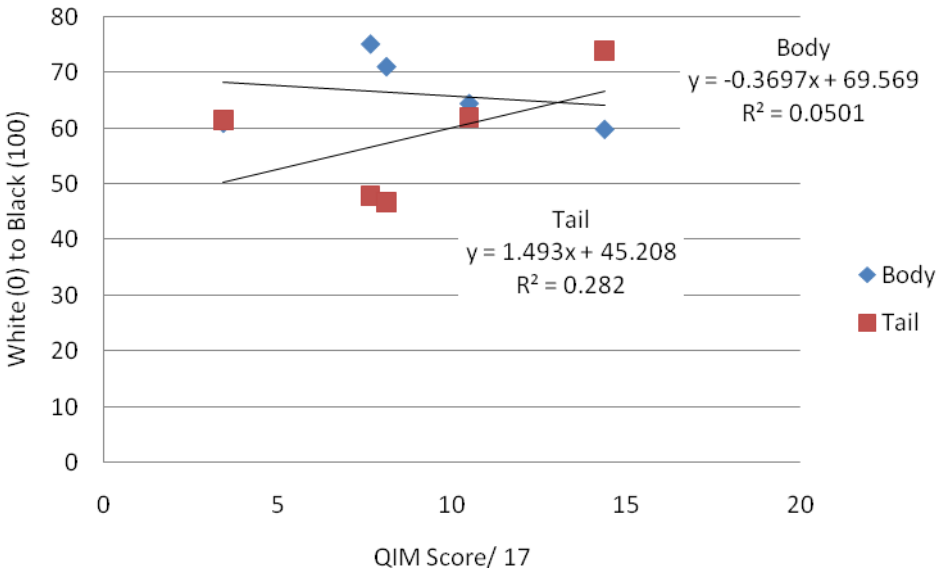


Figure 4.7: Colour change measured using the white to black colour spectrum.

The a* colour measurements displayed in (Figure 4.8) showed that values for red were higher for the body of saddletail snapper as storage increased (y = 1.677x + 4.0728, R^2 = 0.4432) in comparison to a decrease in red values for the tail of saddletail snapper (y = -1.113x + 24.356 , R^2 = 0.7103) as the QI score increased.

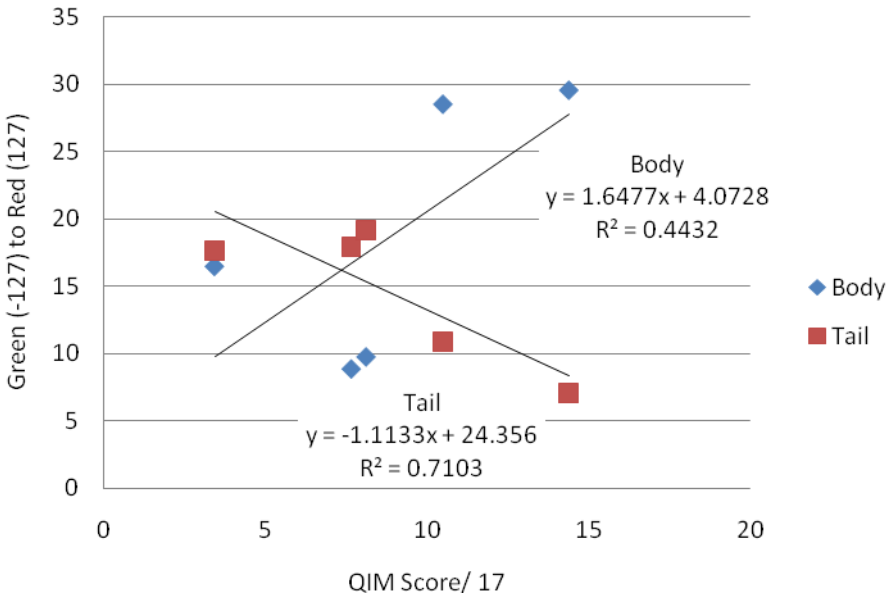


Figure 4.8: Colour change measured using the green to red colour.

The b* measurement (Figure 4.9) demonstrated a positive linear relationship for colour measurements obtained from the body saddletail snapper (y = 1.1077x +

1.5523, R^2 = 0.7085). As storage time increased, yellow values increased for saddletail snapper. The tail did not have such a strong positive correlation, R^2 = 0.0279, with little change measured throughout storage.

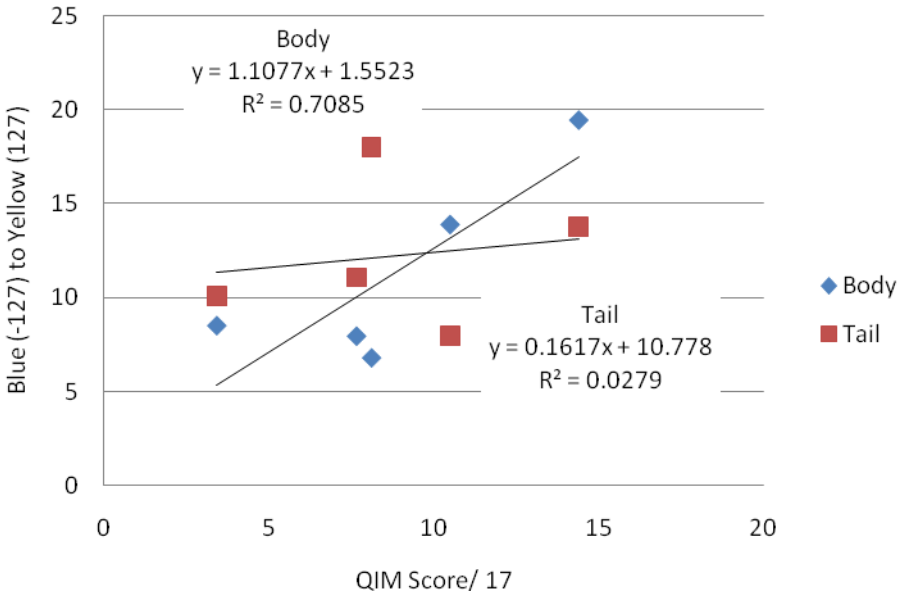


Figure 4.9: Colour change measured using the blue to yellow colour spectrum.

An increase yellow values was noticeable for the body of saddletail snapper, correlating positively with an increase in the QI score and an increase in microbial growth. The increase in yellowness was related to the increase in cloudiness of the mucus present on the skin as the fish deteriorates. This attribute is commonly associated with spoiling fish. It is most likely that the increase in cloudiness is in turn related to the increase in microbial growth. Overall, the strongest colour change observed for the tail of saddletail snapper was that it decreased in redness. By the end of storage the colour change had almost reached 0, indicating negligible amounts of red pigment was observed. This confirmed the use of the colour of the tail fin as another a sensory attribute to be included in the QIM.

Texture

Texture was measured by a Stable Micro Systems (SMS) texture analyser (TA ·XT2i) during storage. The readings for the head, body and tail were measured along the dorsal line of the fish, using an aluminum cylinder probe. The pressure was kept constant for 60 sec after which the relaxation profile was measured. All data points in

Figure 4.10 show a negative linear relationship between the force needed to compress the fish as storage time increased. The head (y = -13.167x + 369.78, R^2 = 0.7711), body (y = -10.525x + 263.78, R^2 = 0.7326) and tail (-12.083x + 242.78, R^2 = 0.8459) measurements all had strong negative correlations.

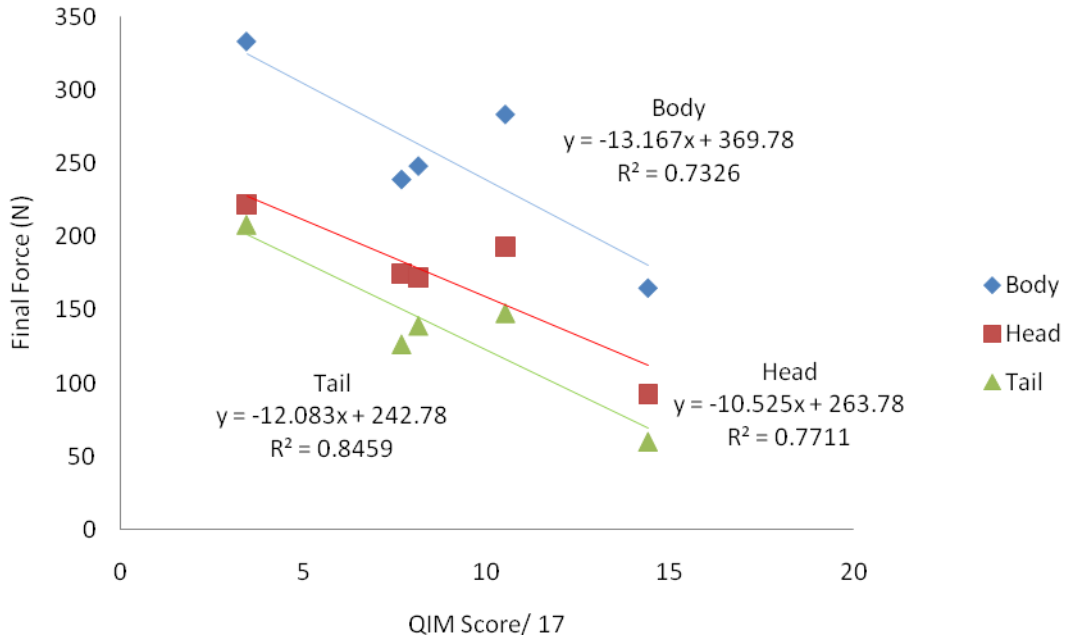


Figure 4.10: Texture analysis of saddletail snapper compared to the QI score

The texture of the fish goes through many changes post-harvest. The fish flesh is soft and elastic immediately after catch. The fish then experiences rigor mortis where stiffness occurs. During storage, proteolysis causes the flesh to soften and become watery (Schubring, 2002a). Similar to a previous texture study (Schubring, 2002a), these results show that with prolonged storage the texture of the fish becomes softer. Figure 4.10 shows that as the QI score increases the texture of the fish changes. Although the general trend was for the firmness of the fish flesh to become softer over time, the data points correlating to the QIM score of 11 appear to have increased. Many factors affect the texture of a fish including species, pH, age, size and post harvest handling (Hyldig & Nielsen, 2001). No other studies have previously identified this anomaly and so it is most likely a result of a variety of different sized fish being tested, or post harvest handling. Standardising the texture analysis by ensuring all samples are the same size and caught from the same source at the same time would eliminate sampling error.

Bacterial counts

A sample was taken from every fish examined by sensory analysis in the validation sessions using the flesh extraction method. The standard plate count for mesophilic bacteria, psychrotrophic plate count and the number of specific spoilage organisms were determined using the spiral plate method. Figure 4.11 shows the results of all bacterial counts. The enumeration of specific spoilage organisms (SSOs) was determined using iron agar. The initial count of SSOs present at storage day 0 was approximately 4 x 10^3 CGU / g. The number of organisms increased exponentially, peaking at 8 x 10^{10} CFU / g at day 24 of storage on ice (QI score =13.5). The number of SSOs decreased after this time point. The total plate count of mesophilic bacteria was determined using plate count agar incubated at 30˚C. It followed a trend similar to the SSOs, with the bacterial population increasing from 2.22 x 10^5 CFU / g to its highest level of 2 x 10^{11} CFU / g during day 24 of storage on ice (QI score= 13.5). Long and hammer agar was used to determine the total plate count for psychrotrophic bacteria. The counts began at approximately 6 x 10^5 CFU / g with the highest concentration of psychrotrophic bacteria counted during day 17 of storage (QI score of approximately 10), reaching 2.55 x 10^{12} CFU / g.

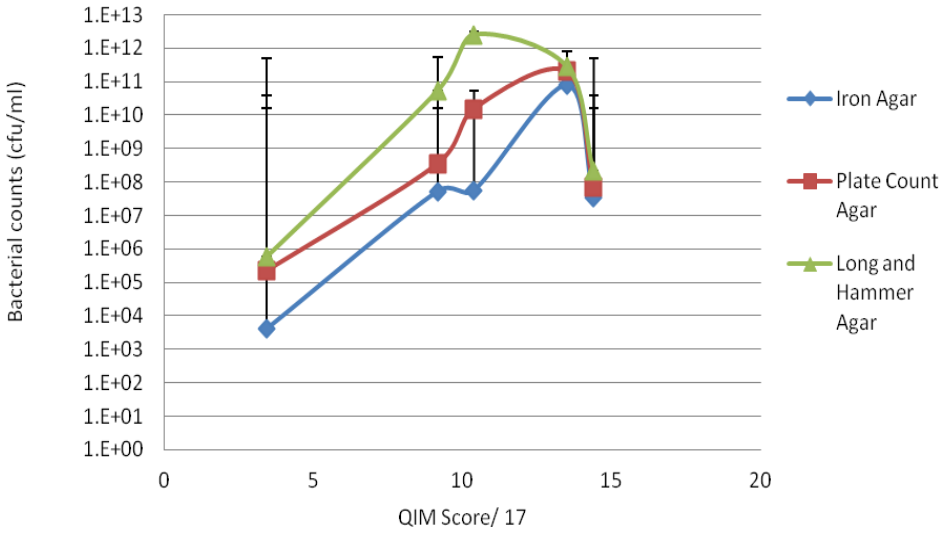


Figure 4.11: Total plate count, psychrotrophic count and number of spoilage organisms present on saddletail snapper in comparison to the QIM score .

The initial numbers of SSOs present on saddletail snapper were lower than the total plate counts of mesophilic and psychrotrophic bacteria. The sulphide producing SSOs were initially present at low numbers when fish were first harvested. It is under cold storage conditions that they proliferate and become responsible for fish spoilage (Vogel, Venkateswaran et al. 2005). The results illustrated in Figure 4.11 support this theory. The psychrotrophic plate counts, on the Long and Hammer media, exhibited the highest counts of all three media tested. The high psychrotrophic counts are a likely consequence of the cold storage environment the fish are exposed to post harvest. The total counts for mesophilic bacteria were fairly high, the consequence of a higher load of mesophilic bacteria initially present on the fish in their natural habitat (Gram & Huss, 1996). Contamination from human handling and poor temperature control may also have contributed to higher mesophilic counts (Shikongo-Nambabi, Chimwamurombe, & Venter, 2010). It is also likely that the bacterial populations are not completely independent of each other. The spoilage organisms are all likely to be present in the psychrotrophic counts and it is possible that some of the psychrotrophic bacteria have contributed to the mesophilic counts, since psychrotrophic bacteria have an optimum growth temperature of around 20 - 25°C (Morita, 1975). An increase in the bacterial counts mirrored an increase in the QI score. The psychrotrophic counts continued increasing until the fish reached a QI score of 10, after which time the counts decreased. The spoilage organisms and mesophilic bacteria increased until a QI score of approximately 14 was reached. It was estimated from the data that saddletail snapper had a shelf life of 24 days. There was a drop in bacterial growth occurring just after the fish was considered spoiled (QIM score = 12). It is most likely that the nutrients of the fish flesh had depleted to such a low level that bacterial growth was no longer supported. Other factors such as water activity and pH may have affected the growth rate of the organisms.

Conclusion

It was necessary to validate the newly developed QIM to ensure that the index components listed were suitable for West Australian caught saddletail snapper. Validation of the QIM involved using trained panellists to perform sensory analysis on whole fish and cooked fillets from different stages of storage. Physical tests and microbiological analyses were also performed to support the findings of the sensory analyses.

A positive relationship was observed between the QIM score and storage days on ice, indicating that the QIM score increased with storage time. The correlation for the linear relationship observed was R= 0.9299, which was similar to those obtained in the initial QIM studies (R^2 = 0.9398, R^2 = 0.9832, R^2 = 0.9851) (Boulter, et al., 2006), which used saddletail snapper caught off the east coast of Australia. A negative relationship was observed for the Torry scheme for odour and flavor indicating that the fish became less fresh as storage time increased. Previous studies (Bremner, 1985; Huss, 1995) have shown that a decrease in the Torry score for odour and flavor is expected as storage time is increased, due to the biochemical and microbiological changes occurring on the fish as it spoils. The results from these sensory analyses confirmed that the QIM for saddletail snapper is able to be used for saddletail snapper caught in Western Australia.

The results from the physical and microbiological analyses supported the findings of the sensory analyses. A negative relationship existed between the texture of the fish and storage time, indicating that the fish flesh became softer throughout storage. A previous texture study (Schubring, 2002a), concluded similarly that with prolonged storage the texture of the fish becomes softer. The results from the colour measurement indicated a decrease in redness which correlated with attributes already considered on the QIM and the microbiological analyses confirmed that as storage time increased so did the microbial flora and SSOs. Microbiological spoilage is a common cause of fish spoilage and an increase in the number of SSOs present on saddletail snapper indicates the fish have reached its useable shelf life. In conclusion, the QIM for saddletail snapper was successfully validated using West Australian caught saddletail snapper.

CHAPTER 5: COMPARISON OF SPOILAGE BETWEEN GOLDBAND AND SADDLETAIL SNAPPER

Introduction

Spoilage is a common problem within the finfish industry due to the structure and properties of fish muscle (Pedrosa-Menabrito & Reichelt, 1988). Although intrinsic properties can affect shelf life, environmental factors, such as on-board handling and processing, can be a major contributor to fish spoilage (Fraser & Sumar, 1998a).

Sensory, physical, biochemical and microbiological methods are employed to monitor and measure the spoilage of fish. Sensory evaluation is a non-invasive process that quantifies the degree and progress of spoilage, using sight, smell, touch and taste. The Quality Index Method (QIM) and the Torry scheme are commonly used for sensory analysis in the seafood industry. Sensory analysis is objectively verified by physical tests using scientific instruments. Texture and colour tests are physical tests commonly performed to assess fish deterioration (Macagnano, et al., 2005). Chemical tests are also able to determine the degree of spoilage by measuring volatile compounds, such as trimethylamine (TMA) and total volatile bases (TVB-N), often found in varying concentrations in fish throughout the spoilage process (Huss, 1988). The growth of microorganisms impacts greatly on the rate of fish spoilage. Microbiological analysis determines fish spoilage by assessing the number of total microorganisms present on the fish as well as the number of specific spoilage organisms present throughout storage (Dalgaard, 2003; Jeyasekaran, et al., 2005).

In this chapter a comparison of the spoilage process in goldband and saddletail snapper was undertaken.

Methods

Samples

Goldband snapper samples were sourced from two fishing companies. One company from Broome supplied samples during the months of March and June harvested using the trap method. The other company, based in Exmouth, supplied samples during March and harvested using the longline method. All samples were assumed to be 3 days old when received in Perth, as mentioned in Chapter 3. Nine fish smaples were used to collect data for sensory, microbiological and chemical analysis.

Saddletail snapper samples were sourced from two fishing companies from Exmouth. One company supplied samples during the months of September and October which had been harvested using the trawl method. The other company supplied samples during March which had been harvested using the longline method. Again, all samples were assumed to be 3 days old on receipt in Perth. Twelve fish samples were used to collect data for sensory, microbiologcal and chemical analysis.

QIM

The organoleptic changes occurring on goldband and saddletail snapper were recorded using the QIM developed for each species. Each QIM measured the overall appearance, texture and odour of the fish, the appearance of the eyes and the appearance and odour of the gills (Appendices 1 & 3). Each of the sensory attributes was monitored and changes were recorded every 7 days. The data collected throughout the spoilage process allowed comparisons between the spoilage rates of each species to be made.

Texture

Texture analysis is one way of measuring fish deterioration throughout storage life. Changes in texture were measured for both species over a month, using a texture analyser fitted with an aluminium cylindrical probe, as discussed in Chapter 3. The method of texture analysis used to perform this experiment was based on a paper by Macagnanoet al.(2005).

Colour

Depending on the species, colour measurement is another way of monitoring sensory changes that occur throughout storage life. Changes in colour were measured for both species over a month, using the Minolta spectrophotometer (CM-500i/CM-500C), as discussed in Chapter 3. The determination of any colour changes was based on the method developed by Macagnanoet al. (2005).

TMA

Measuring of trimethylamine (TMA) is commonly used to determine fish deterioration. All fish contain low amounts of TMA when they are caught and it accumulates as the fish spoils (Huss, 1988). TMA analysis was performed over 4

weeks, using the method developed by Baixas-Nogueraset al. (2001) which is based on the Dyer (1959) method, as discussed in Chapter 3.

Total Volatile Bases (TVB-N)

Measuring the levels of total volatile basic compounds, including trimethylamine, dimethylamine and ammonia, is an alternate technique for evaluating fish deterioration. TVBN analysis is useful in determining later stages of fish spoilage, since the compounds measured do not accumulate until late in the spoilage process (Howgate, 2010). TVBN analysis was performed on samples taken at different stage of storage over a period of 4 weeks. Testing was performed using a distillation and titration method (Baixas-Nogueras, et al., 2001), as discussed in Chapter 3.

Bacterial counts

Bacterial counts were performed to determine the changes experienced by the microbial populations on each species throughout storage. Saddletail and goldband snapper were sampled throughout 4 weeks of storage. Sampling was performed using the swabbing and flesh extraction method at days 3, 10, 17, 24, 31 of storage. Appropriate dilutions were plated onto long and hammer agar plates, plate count agar plates and iron agar plates using the Whitley Automated Spiral Plater. All methods and materials used in bacterial enumeration are detailed in Chapter 3.

Results and Discussion

QIM

The QIM scores for goldband snapper displayed a strong positive relationship (R = 0.9895) with storage days (Figure 5.1). At day 31, the highest score for goldband snapper was 18, well below the QIM limit of 25.

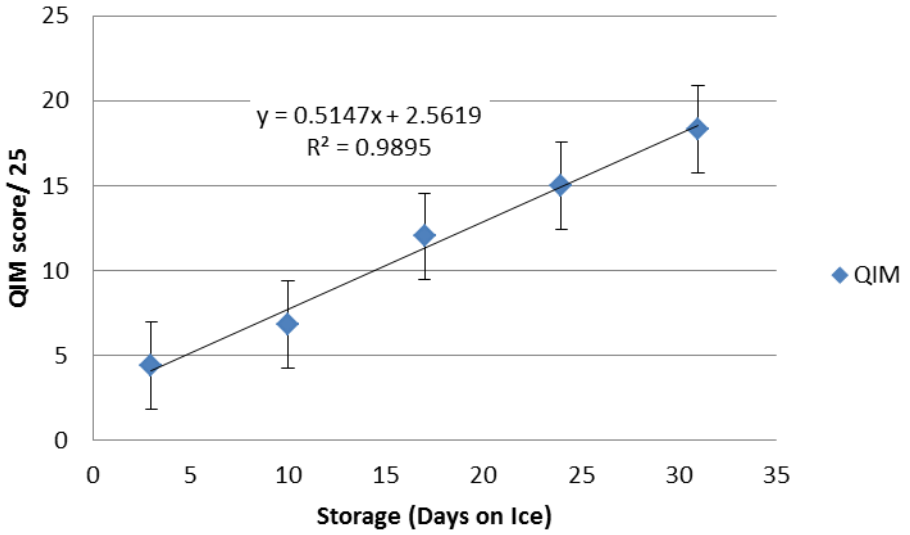


Figure 5.1: Average QIM score for goldband snapper .

The QIM scores for saddletail snapper also displayed a strong positive relationship (R = 0.9299), with storage days (Figure 5.2). At day 31 the highest score for saddletail snapper was 14, also below the QIM limit of 17.

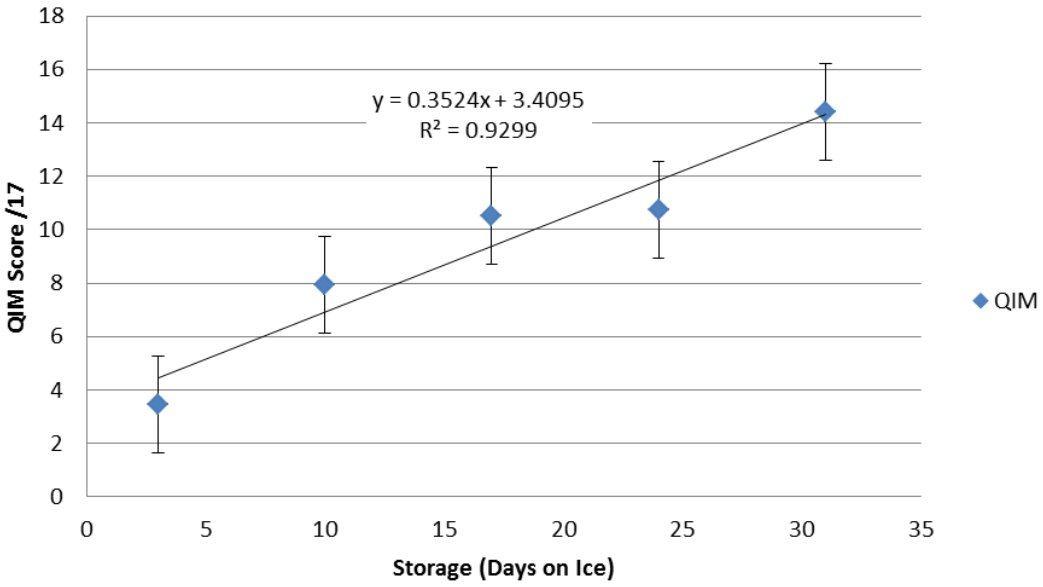


Figure 5.2: Average QIM scores for saddletail snapper.

Figures 5.1 and 5.2 demonstrate that the Quality Indices for goldband and saddletail snapper are well suited to determining the rate of spoilage for each species. There was no need for the Torry scheme for goldband snapper to be performed in this project since the QIM scheme had been validated however, data from a previous

study (Ames & Curran, 1985) showed that panellists considered the cooked goldband snapper fillets acceptable up until 35 days of storage (Ames & Curran, 1985).

The QIM scheme for saddletail snapper was new and had not yet been validated. To be able to use this scheme as a comparative measure against the goldband snapper scheme, validation sessions were performed, as previously described in Chapter 4. The Torry scheme was used to help validate the saddletail snapper QIM with West Australian caught saddletail snapper. These validation sessions showed that the panellists considered the cooked saddletail snapper fillets acceptable up until 24 days of storage.

The QIM score at the end of shelf life for saddletail snapper was approximately 12.5 out of a possible 17. The end of shelf life, determined by the acceptability of the taste panel, was day 35 for goldband snapper (Ames & Curran, 1985). The QIM score for goldband snapper at day 35 would have been approximately 22 out of a possible 25. Thus, goldband snapper appears to be acceptable for longer than saddletail snapper.

Texture

The textural analysis in Figure 5.3 shows a negative relationship between the number of storage days and the force used for compression analysis of saddletail snapper. The linear relationship observed, $y = -3.53x + 244.71$ (x=Days on ice, y= final force used to measure texture), and the correlation ($R^2 = 0.59$) supported a strong negative relationship. Goldband snapper did not exhibit a strong positive or negative relationship between storage days and force used for compression analysis.

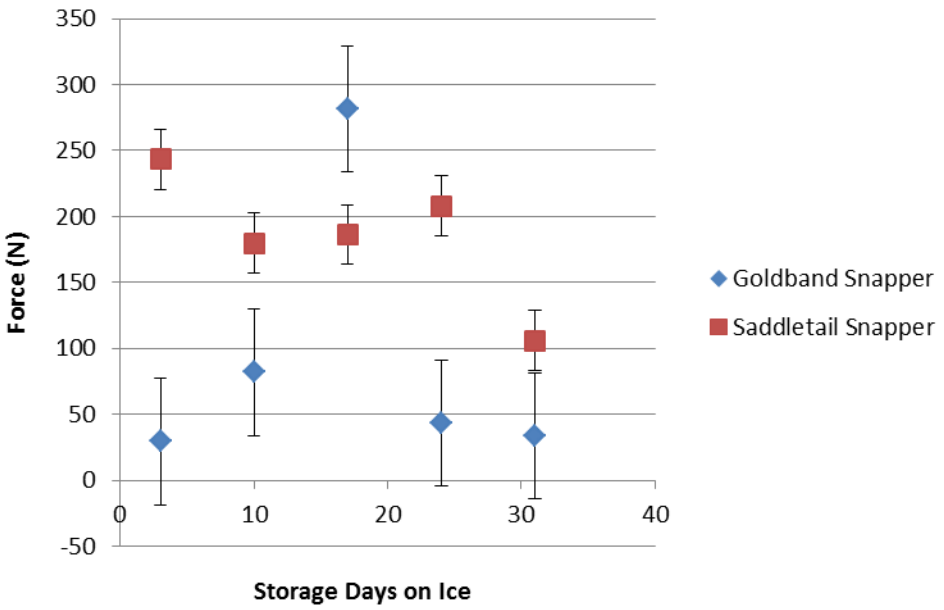


Figure 5.3: Texture analysis of goldband and saddletail snapper throughout spoilage.

The force used to compress goldband snapper peaked at approximately day 17, with a reading of 270 N. All other data points remained below 100 N. The force used to compress saddletail snapper ranged from approximately 250 N at day 3 to 100 N by day 31.

With the exception of day 17, the texture of goldband snapper exhibited different properties to that of saddletail snapper. Texture results are very dependent on the individual condition of the fish and also the handling conditions of the fish before they reach the laboratory (Mai, et al., 2009). The conditions of the fish may be affected by seasons. The experiments conducted for saddletail and goldband snapper occurred at different times of the year with goldband snapper samples harvested from May to June and saddletail snapper samples harvested from September to October. The results from the texture analysis confirmed that the longer saddletail snapper are stored, the less firm their flesh becomes. Goldband snapper begin with a fairly soft texture and appeared to maintain this throughout storage.

Tropical snappers have an extended spawning season, with saddletail snapper spawning recorded between September and February (Roelofs, 2010). Goldband snapper spawn from January to April with a peak in March (Newman, et al., 2008). Pedrosa-Menabrito and Regenstein (1990b) noted that after spawning fish have a

thinner, flabbier appearance. Saddletail snapper were harvested during their spawning season, whereas goldband snapper were harvested a few months after. If the goldband snapper harvested had already spawned, this may explain the low amount of force needed to measure their texture however, further analysis is necessary to determine the exact cause. The two species also came from different fishing companies and were also harvested using different methods of catch: goldband snapper were trap caught and saddletail snapper was trawl caught. Although storage and handling conditions were similar, how fish are treated post-harvest is very important in maintaining quality. These post-harvest differences may have affected the quality of the fish flesh, thereby affecting the texture results.

Colour

Colour intensity was monitored throughout the storage process for both goldband and saddletail snapper. The white to black (L*), green to red (a*) and blue to yellow (b*) colour spectrums were all measured using a Minolta spectrophotometer. The L* measurements indicated that overall the goldband snapper had a darker appearance than saddletail snapper with little variation occurring between the two species throughout spoilage (Table 5.1).

Table 5.1: Changes in lightness measured dorsally on goldband and saddletail snapper during ice storage.

Storage Days on Ice	3	10	17	24	31
CIE L* measurements for goldband snapper	63.62458	62.99778	61.80694	61.25583	59.7
CIE L* measurements for saddletail snapper	41.93611	43.57361	41.98389	45.64944	49.38

The a* measurements showed no significant changes in colour (Table 5.2). Goldband snapper experienced an increase in red values, followed by a declining slope until the end of the experiment. Saddletail snapper had a natural red colour and so its measurements were higher to begin with. Red values were lower at day 17 of storage than previously measured. After this time the measurements recorded increased.

Table 5.2: Changes in redness measured dorsally on goldband and saddletail snapper during ice storage.

Storage Days on Ice	3	10	17	24	31
CIE a* measurements for goldband snapper	1.71625	2.651111	1.864722	1.25	0.423056
CIE a* measurements for saddletail snapper	12.51722	11.47361	11.18486	16.62481	16.47261

The "b" measurements shown in Table 5.3 display data for goldband and saddletail snapper samples, throughout storage. Values for yellow were higher for saddletail snapper in comparison to goldband snapper. Goldband snapper did show an increase in yellow values but it was not as visually apparent as the changes on sadlletail snapper.

Table 5.3: Changes in yellowness measured dorsally on goldband and saddletail snapper during ice storage.

Storage Days on Ice	3	10	17	24	31
CIE b* measurements for goldband snapper	6.2175	7.3925	4.635556	5.535278	8.008333
CIE b* measurements for saddletail snapper	7.352222	8.824333	10.99819	10.78556	15.88148

Figure 5.4 shows the increased greenness seen on the tail of goldband snapper $y = -0.2429x + 6.835$, (y= storage days on ice, x= colour spectrum measured) and $R^2 = 0.71$). Saddletail snapper displayed a decrease in red values measured towards the end of storage ($y = 0.4043x + 21.4$, $R^2 = 0.71$). The increase inthe measurement of green was only apparent on goldband snapper and may be related to the populations of microbes colonising on the tail. Certain *Pseudomonas* species produce green pigments as a result of increased growth and also fluoresce under UV light (Rhodes, 1959).

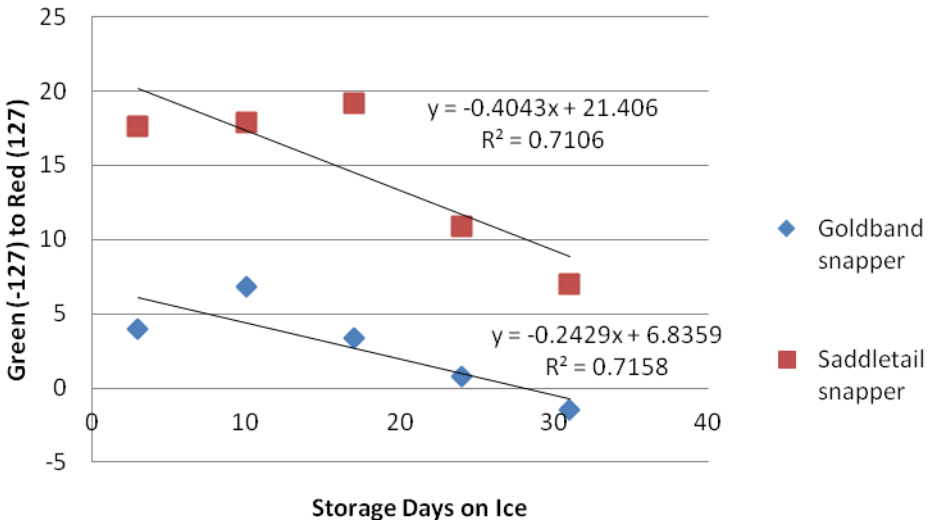


Figure5.4: Minolta "a" measurements for the tail of goldband and saddletail snapper.

Figures 5.5 and 5.6 illustrate the Minolta “b” measurements for the head and body of goldband and saddletail snapper. These measurements determine the colour of the sample on the blue/ yellow colour spectrum. Figure 5.5 shows that as storage days increase, the colour of the head of saddletail snapper increased in yellow values (y = 0.3533x + 2.818, R^2 = 94). There was hardly any change in colour throughout storage for goldband snapper (y = -0.0553x + 6.68, R^2 = 0.14). As storage time increased goldband snapper decreased in yellow values, however, from about day 17 the data points began to increase towards the yellow end of the spectrum. The increase in yellow values was possibly due to an increase in microbial growth.

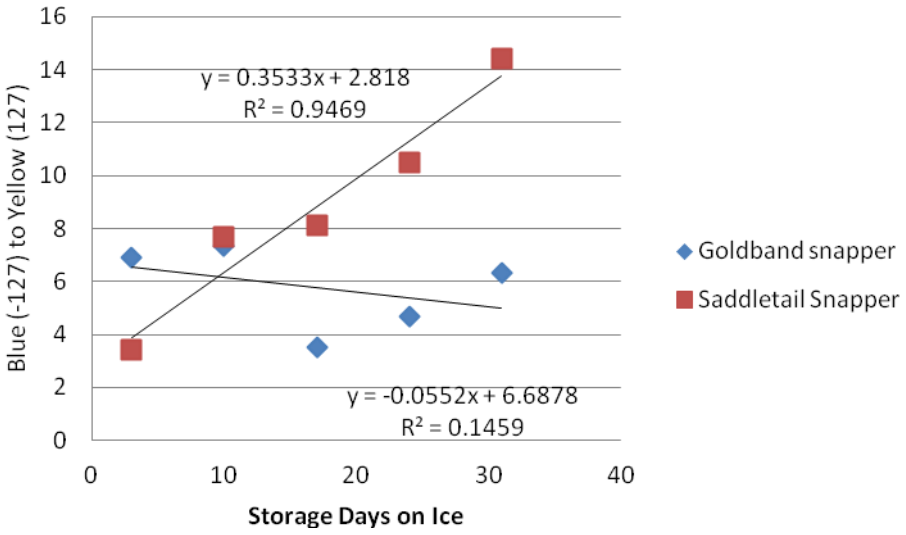


Figure 5.5: Minolta "b" measurements for the head section of goldband and saddletail snapper

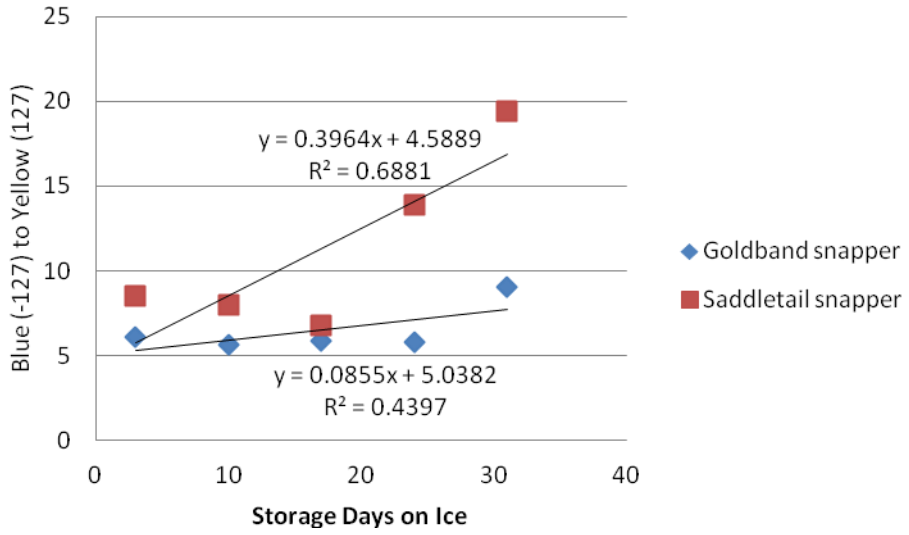


Figure 5.6: Minolta "b" measurements for the body section of goldband and saddletail snapper.

The “b” measurement for the body of goldband and saddletail snapper (Figure 5.6) indicated a positive relationship for both species. The relationship observed for goldband snapper was y = 0.085x + 5.03, R^2 = 0.43 and the relationship observed for saddletail snapper was y = 0.3964x + 4.588, R^2 = 0.68. An increase in yellow values was noticed for both species from day 25 until the end of the experiment. The increase in yellow colour would correlate with the increase in microbial growth. As the fish began spoiling a noticeable slime layer began forming on the fish due to an increase in microbial growth. Some of the colour analysis was indicative of microbial growth, however overall the colour analysis showed no consistent indication of spoilage throughout storage.

Microbiological analysis

The microbiological analysis was performed alongside other sensory and biochemical tests. The number of specific spoilage organisms (SSO) present on both goldband and saddletail snapper increased at a similar rate, until approximately day 24, after which growth decreased (Figure 5.7). The initial load for goldband snapper was 5 x10^4 CFU / g compared to the saddletail snapper initial load of 5 x 10^3 CFU / g. SSOs peaked on goldband snapper at day 17 (1 x10^8 CFU / g) before beginning to decrease at day 24. The number of SSOs present on saddletail snapper did not peak until day 24 (1 x 10^{11}CFU / g). There were a greater number of SSOs on saddletail

snapper than on goldband snapper. During iced storage, Gram negative, psychrotrophic, spoilage organisms, such as *Shewanella* species, proliferate and become dominant. If a higher proportion of psychrotrophic spoilage organisms was initially present on saddletail snapper in comparison to goldband snapper, a higher bacterial load would be expected (Vogel, et al., 2005).

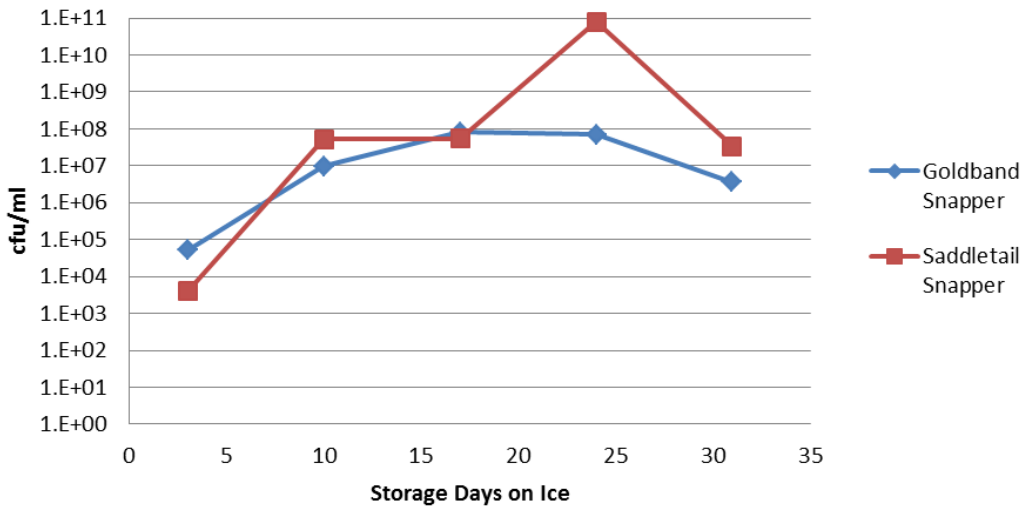


Figure 5.7: Number of specific spoilage organisms present on goldband and saddletail snapper.

The total numbers of mesophillic aerobic organisms present on goldband and saddletail snapper throughout iced storage are presented in Figure 5.8. Once again, the pattern of microbial growth was similar for both species however, goldband snapper had a reduced bacterial load. The bacterial load for goldband snapper peaked at 1 x 10^8 CFU / g on day 31. These results were similar to those obtained from Ames & Curran (1985). The bacterial load for saddletail snapper peaked at day 24, 1 x 10^{11} CFU / g. The difference in the bacterial load between goldband and saddletail snapper may be influenced by the total number of bacteria present. Ames & Curran (1985) determined that the maximum bacterial load for goldband snapper was 1.5 x 10^8 CFU / g after 30 days. This was similar to the results obtained in this study, leading to the conclusion that goldband snapper have a lower bacterial load than saddletail snapper.

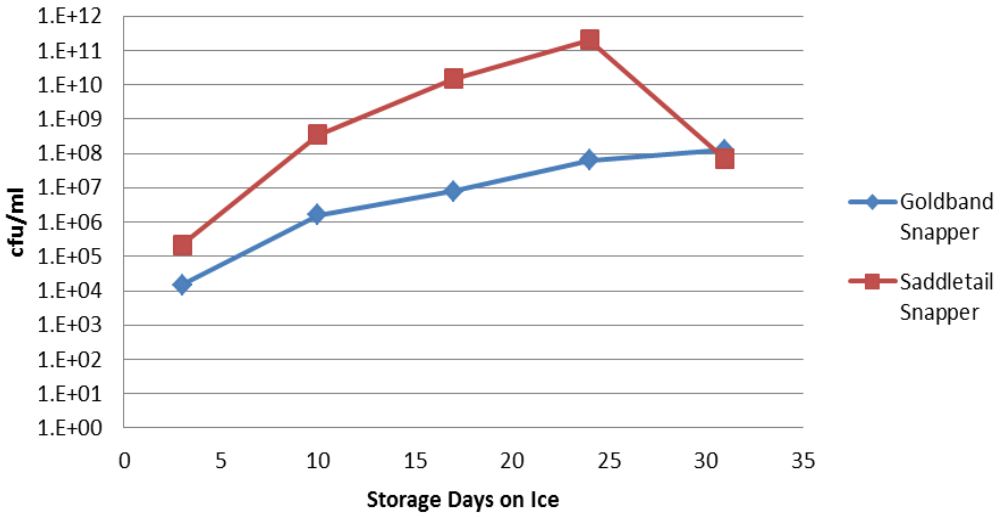


Figure 5.8: Total number of aerobic mesophillic bacteria present on goldband and saddletail snapper.

Figure 5.9 shows the total number of aerobic psychrotrophic bacteria present on goldband and saddletail snapper. Saddletail snapper had an increased psychrotrophic count compared to goldband snapper. Saddletail snapper reached its maximum pyschrotrophic count on day 17, with 1 x 10^{12} CFU / g, compared to goldband snapper which did not reach the maximum until day 31, with 1 x 10^{8} CFU / g. The difference in the bacterial load between goldband and saddletail snapper may be influenced by the total amount of bacteria present at the time of harvest and the different species of bacteria present on the fish. The cold storage conditions would be favourable for the "cold-loving" psychrotrophic organisms promoting a higher growth rate and so if there was a higher proportion of psychrotrophic organisms initially present on saddletail snapper compared to goldband snapper, a higher bacterial load would be expected.

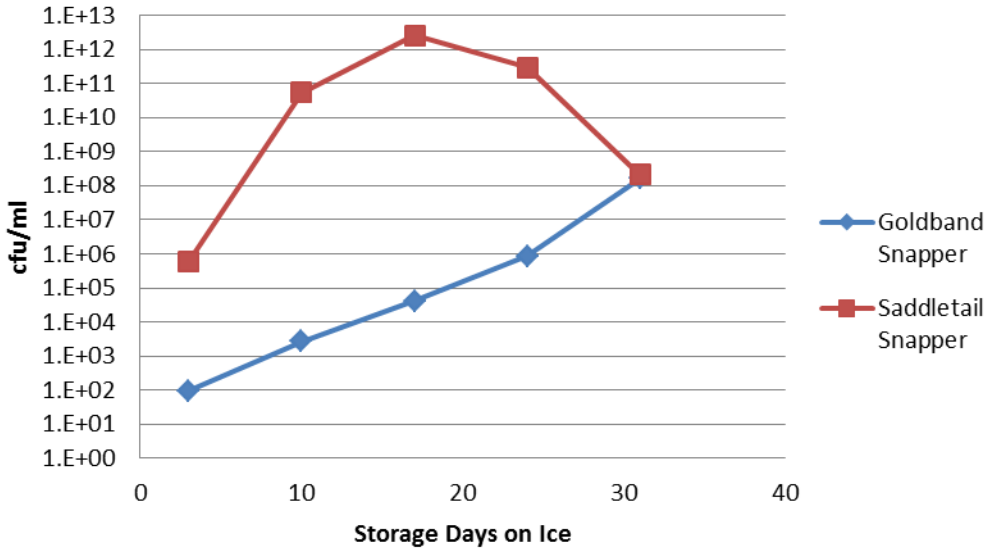


Figure 5.9: Total number of aerobic psychrotrophic bacteria present on goldband and saddletail snapper.

Collectively, the bacterial counts suggest that goldband snapper had a lower bacterial load than saddletail snapper. The aerobic mesophilic counts of saddletail snapper were significantly higher until day 30. The difference between the two species may be attributed to their habitat. Saddletail snapper inhabit shallower waters compared to goldband snapper (Newman, et al., 2008) and so this may offer an explanation for the higher counts of mesophilic bacteria. However, in some instances the fish were caught from the same depths. If the handling techniques were not replicated and the fish were not iced immediately this may have also impacted on the number of mesophilic bacteria isolated from each fish. Surti, et al. (2002) showed that delaying the icing time of tropical fish decreased their shelf life considerably. The storage temperatures on board the boat were not ideal due to a warm climate and temperatures not being regulated during transportation. These extrinsic factors may explain the mesophilic bacterial counts continuing to increase so rapidly after harvest.

The total plate count commonly used to represent the total bacterial load measures mesophillic bacteria and since the fish are being stored in a cold environment the psychrotrophic count is the preferred and more accurate indicator of the total bacterial load present on iced fish. Saddletail snapper had a significantly higher

psychrotrophic load than goldband snapper. The growth of both psychrotrophic and mesophilic organisms was delayed on goldband snapper. Isolates from all culture media were collected and stored, and further analysis was performed as described in Chapter 6 to determine the cause for a lower bacterial load seen with goldband snapper.

Throughout storage, the bacterial counts of SSOs were similar between the two fish species. There was a significant difference between the number of SSOs present on saddletail and goldband snapper at day 24 however, it is uncertain whether this would have impacted on storage life as it occurred towards the end of the experiment. SSOs play an important role in fish spoilage due to their ability to produce TMA (Gram & Huss, 1996). The similar bacterial loads between the two fish species makes it difficult to explain the difference experienced in shelf life, although overall the numbers of SSOs on saddletail snapper were consistently higher than on goldband snapper.

Further analysis of the types of spoilage organisms isolated from each species may help to identify a cause. It may be possible that one SSO is creating a probiotic effect, preventing other bacterial species from colonising the fish and causing further spoilage.

Biochemical analysis

Biochemical analysis was performed to monitor spoilage and observe any differences between goldband and saddletail snapper. Biochemical analysis is affected by different factors throughout spoilage, including autolysis, stress from capture and bacterial invasion. TMA and TVB-N were the primary biochemical tests performed to assess deterioration of the fish. Hypoxanthine levels and K values were also measured. The TMA values of freshly caught fish average 2 mg / 100 g, however it can range from 1- 4 mg / 100 g (Oehlenshlager, 1997). Figure 5.10 shows goldband and saddletail snapper with low levels of TMA at the beginning of storage. The levels of TMA on saddletail snapper rose consistently throughout storage, compared to goldband snapper which remained consistently low until day 17. By day 24 the TMA levels of goldband snapper rose sharply until the end of storage.

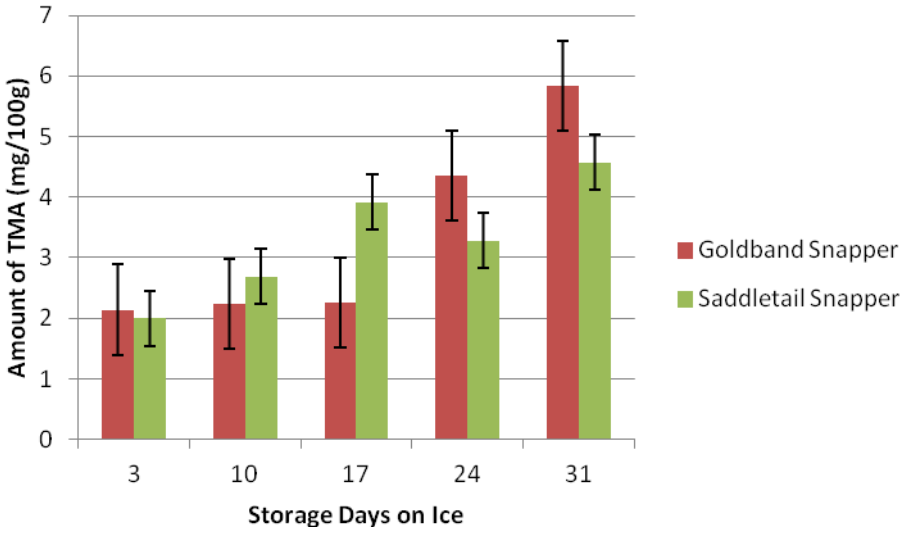


Figure 5.10: Amount of TMA mg /100 g of fish over 28 days of storage.

The TMA limit for human consumption is 10 – 15 mg / 100 g (Huss, 1988). The TMA concentrations for neither goldband nor saddletail snapper exceeded that limit after 31 days of storage. TMA is not generally used as a deterioration indicator as it does not indicate changes in fish until approximately 10-12 days of storage (Rehbein, et al., 1994). However, it is useful in indicating the stages of spoilage and when microbial spoilage has occurred. The levels of TMA in Figure 5.10 correlate with the microbial counts for goldband and saddletail snapper seen in Figures 5.8 and 5.9. Figure 5.11 shows the relationship between spoilage bacteria and the levels of TMA recorded. Overall the numbers of spoilage organisms for saddletail snapper were higher than goldband snapper, however, goldband snapper produced a higher concentration of TMA. The concentration of TMA produced in goldband snapper remained consistently low until day 17, which corresponded to the lower bacterial load of the spoilage organisms enumerated from goldband snapper. The concentration of TMA produced followed the general trend of the growth rate of the spoilage organisms found on saddletail snapper. The concentration of TMA accumulated in the flesh appeared to take approximately 7-14 days before they reflected the exponential increase in spoilage organisms.

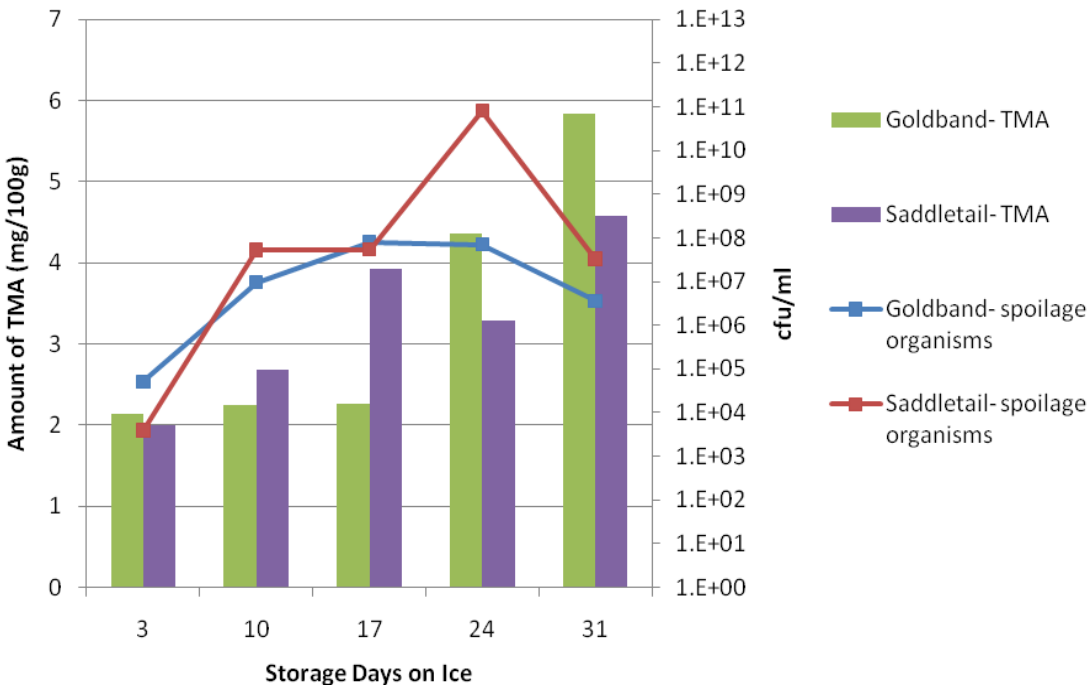


Figure 5.11: TMA values for goldband and saddletail snapper (mg N / 100 g) compared against the total number of spoilage organisms at different storage days.

TVB-N is also commonly used to measure later stages of spoilage since it takes time for each of the compounds to accumulate to measureable levels within the fish flesh (Howgate, 2010). TVB-N is a good indicator of spoilage, measuring the amounts of TMA, DMA and ammonia present in the fish. Figure 5.12 shows the amount of TVB-N present in saddletail snapper samples throughout iced storage. Amounts of TVB-N ranged from 20 mg N /100 g at the beginning of storage to 25mg N / 100 g by day 17, reaching 44 mg N / 100 g by day 31. The amount of TVB-N present in goldband snapper samples started slightly higher than the saddletail snapper. Concentrations began at approximately 23 mg N / 100 g and steadily increase to 35 mg N / 100 g. The levels of TVB-N then decreased by day 31 to approximately 27 mg N / 100g. These results were similar to those obtained in the study performed by Ames & Curran (1985).

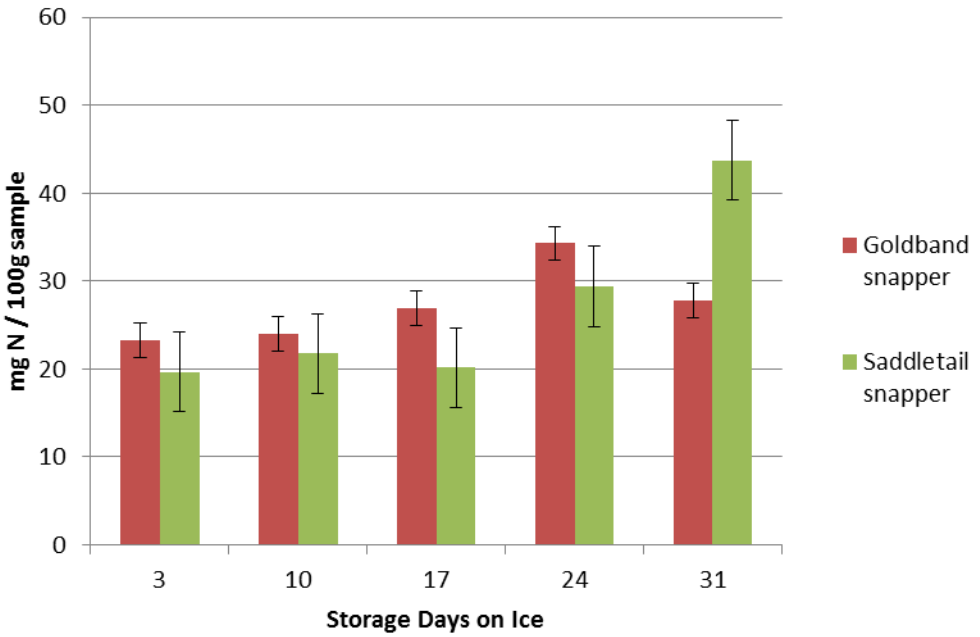


Figure 5.12: TVB-N values for goldband and saddletail snapper (mg N / 100 g) against storage days.

The suggested concentrations of TVB-N for rejection of fish are 30 mg/ 100 g flesh as this is regarded as the limit of acceptability for consumers (Castro, et al., 2006; Oehlenchläger, 1992). By day 24, goldband snapper would have been rejected and saddletail snapper approximately 1 or 2 days after. Figure 5.13 shows the relationship between the spoilage organisms enumerated and the amount of TVB-N measured in goldband and saddletail snapper. The amount of TVB-N accumulated mimics the growth rate of the spoilage organisms enumerated from both species throughout storage.

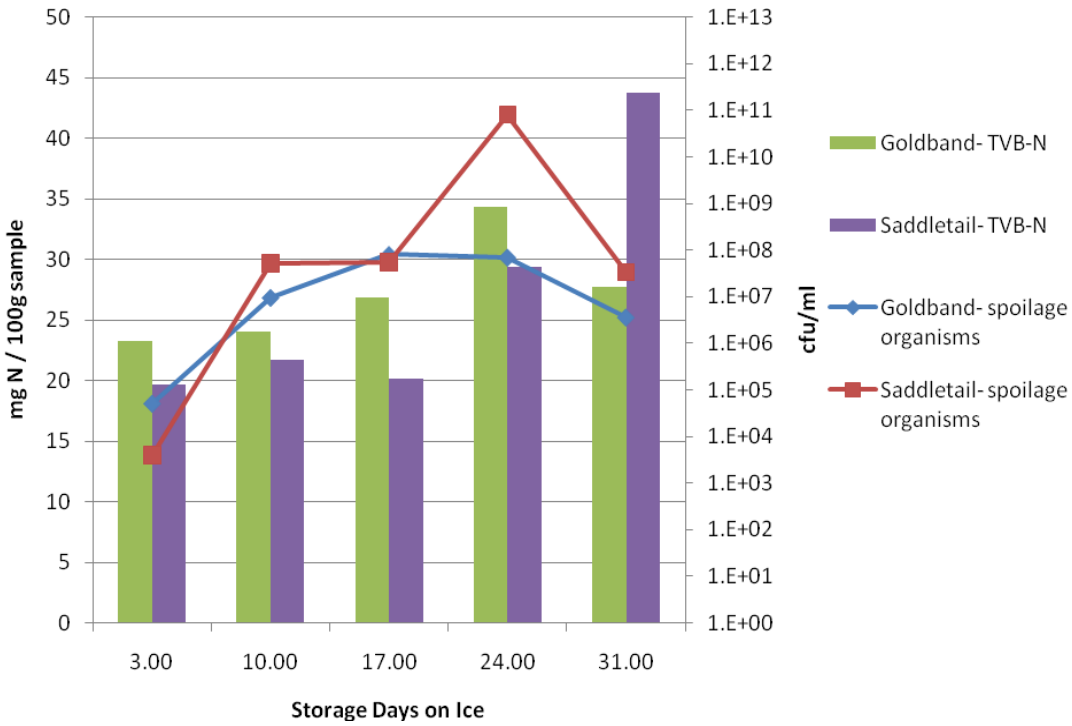


Figure 5.13: TVB-N values for goldband and saddletail snapper (mg N / 100 g) compared against the total number of spoilage organisms at different storage days.

Hypoxanthine is viewed a useful indicator for fish deterioration. Figure 5.14 shows the hypoxanthine concentrations for saddletail snapper remained consistently low, compared to goldband snapper which increased dramatically between day 10 and 17.

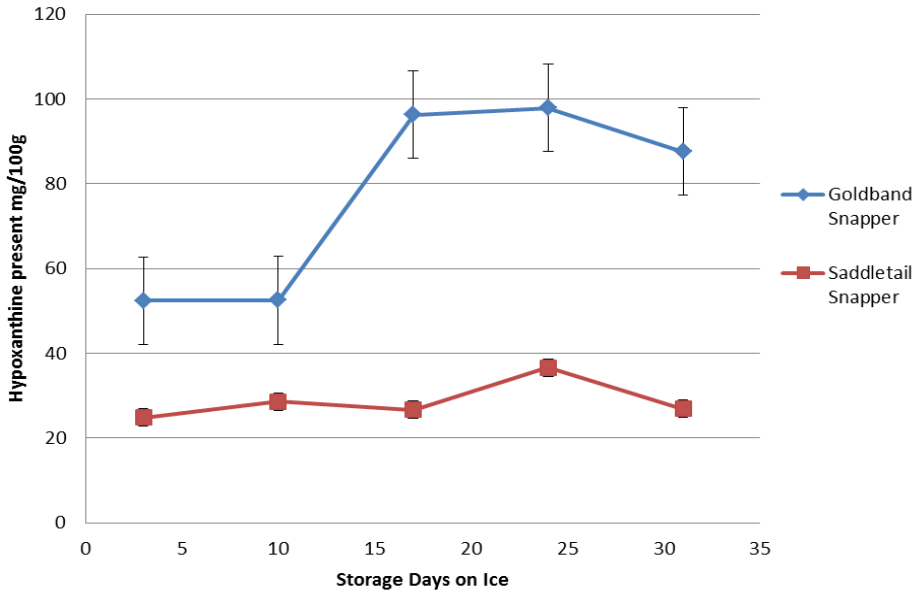


Figure 5.14: Levels of hypoxanthine measured in goldband and saddletail snapper throughout storage.

The initial concentrations of hypoxanthine for goldband snapper were also higher than those of saddletail snapper. Across the different experiments the handling, storage and harvesting process and even size of the fish differed, perhaps causing this difference between the two species. The hypoxanthine concentrations differed dramatically between the two species. Most fish reach the highest concentrations of hypoxanthine by day 10-18, a level reached with the goldband snapper. The onset of bacterial growth did not appear to affect saddletail snapper as it did goldband snapper. A fall in the concentration of hypoxanthine occurs in most species as the flesh becomes inedible (Leisner & Gram, 2000).

Investigation into the Hx/ inosine ratio was determined for saddletail and goldband snapper. However, because the data could not be repeated the graph is determined from only one sample for each storage day.

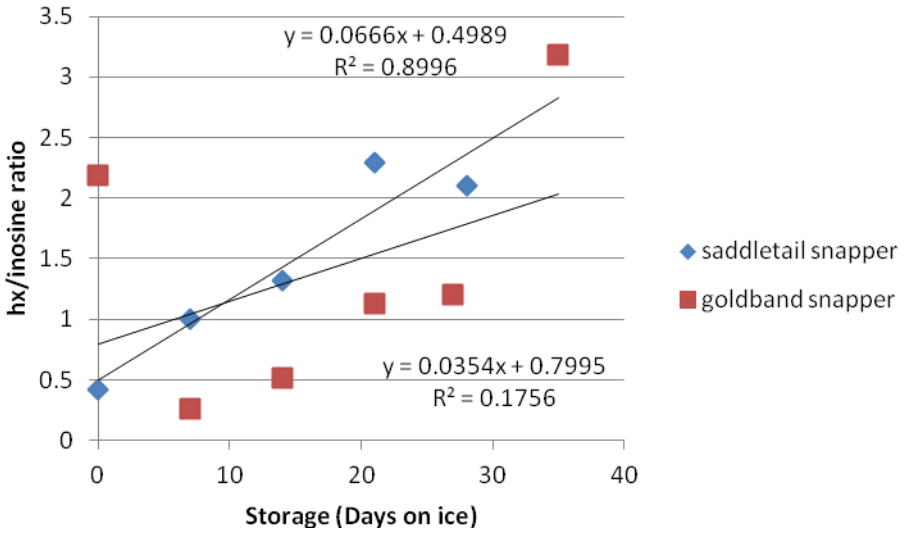


Figure 5.15: Hx/ inosine ratio measured in goldband and saddletail snapper throughout storage.

The results presented in Figure 5.15 show an increase in the Hx/ inosine ratio for saddletail snapper, confirming that saddletail snapper is a hypoxanthine producer. Goldband snapper has a high ratio at day 0 of storage but the Hx / inosine ratio then drops in value, supporting that goldband snapper is an inosine producer. Hypoxanthine produces a bitter flavour in comparison to inosine which produces a fairly neutral flavour (Fletcher, 1990). This may also explain why goldband snapper is acceptable to consumers for longer in comparison to saddletail snapper.

The results for the K-value for goldband snapper and saddletail snapper were inconclusive since insufficient data was collected throughout the project. The HPLC analysis did not provide results for all the components necessary for determination of K-value. Appendix 12 and 13 lists the results obtained from the HPLC analysis.

Conclusion

Investigation into the spoilage of goldband and saddletail snapper was important in determining the difference in shelf life experienced by the two fish species. Sensory, physical, microbiological and chemical analyses were used to determine the spoilage of goldband and saddletail snapper.

Results from the sensory analyses of goldband and saddletail snapper confirmed that a difference existed between the shelf life of the two fish species. Both exhibited a strong positive relationship between the QIM scores and storage days however, data from the Torry scheme concluded that saddletail snapper only had a shelf life of 24 days in comparison to the shelf of 35 days exhibited by goldband snapper (Ames & Curran, 1985).

Investigation into spoilage using physical analyses such as texture and colour gave further insight into the differences existing between goldband and saddletail snapper. Textural analysis of saddletail snapper displayed a strong negative relationship with storage time, indicating that the fish became softer throughout storage. A similar study (Hyldig, et al., 2009) concluded similar findings, so it was unusual to see that the texture of goldband snapper was soft and remained soft throughout storage, with the exception for a peak at day 17. This unusual phenomenon has not been recorded in the literature to date and it is unknown why it has occurred. Further biochemical analysis into the structure of the flesh of goldband snapper may provide answers. The colour intensity monitored throughout storage also determined differences between the two species. Overall goldband snapper was recorded as being slightly darker in appearance than saddletail snapper. Saddletail snapper increased in redness throughout storage and produced higher data points due to its natural red pigment. Goldband snapper appeared to decrease in redness and towards the end of storage was shown to increase in greenness. The increased green colour was only apparent

on the goldband snapper and may be related to the types of bacterial populations present. Certain *Pseudomonas* species produce green pigments as a result of increased growth and also fluoresce under UV light (Rhodes, 1959). Overall, both goldband and saddletail snapper increased in yellowness.

Microbiological analysis investigated the populations of spoilage bacteria, mesophillic bacteria and psychrotrophic bacteria present on each fish species. Overall saddletail snapper had a higher number of SSOs than goldband snapper, which may explain the difference in shelf life. Up until day 24 of storage, the populations of spoilage organisms were similar between the two species however at day 24 the population peaked for saddletail snapper (1×10^{11} CFU / g), in comparison to goldband snapper which peaked at day 17 (1×10^{8} CFU / g). The total bacterial load of mesophillic organisms was higher for saddletail snapper than goldband snapper. Saddletail snapper reached its maximum bacterial load at day 24 (1×10^{11} CFU/g). Goldband snapper reached 1×10^{8} CFU / g at day 31 and had not yet reached its maximum bacterial load. The levels of psychrotrophic bacteria were considerably different between the two fish species. The bacterial load of saddletail snapper peaked at day 17 ($1x\ 10^{12}$ CFU / g) in comparison to goldband snapper which appeared to still be increasing at day 31 reaching 1×10^{8} CFU / g. Overall across all the different microbial counts, the bacterial load present on saddletail snapper was much higher than on goldband snapper. Storage and handling techniques may be responsible for this however it is more likely a result of the type of bacteria present on each fish species.

Chemical analysis was successful in determining a difference in the spoilage of goldband and saddletail snapper. The concentrations of TMA appeared to follow the general growth trend of spoilage organisms. Specific spoilage organisms, such as *Shewanella* species, are responsible for reducing TMAO to TMA (Vogel, et al., 2005) and so it is assumed a higher proportion present on the fish would elicit a higher TMA concentration. Goldband snapper had a lower bacterial load of spoilage organisms which mirrors the consistently low TMA concentration experienced until day 17. Saddletail snapper experienced a higher bacterial load which mirrors with the increase in TMA levels at the beginning of storage. Neither species exceeded the 10-15 mg / 100g limit of TMA for human consumption (Huss, 1988). TVB-N is

commonly used to measure later stages of spoilage since it takes time for each of the compounds to accumulate to measureable levels within the fish flesh (Howgate, 2010). The total amount of TVB-N produced by goldband snapper was less than saddletail snapper. However, goldband snapper exceeded the maximum limit of TVB-N for human consumption (30 mg / 100 g flesh) at day 24 of storage saddletail snapper did not exceed the limit until approximately 1 or 2 days later. The levels of hypoxanthine remained consistently low for saddletail snapper in comparison to goldband snapper which increased at day 17 and remained high until the end of storage. Results for TVB-N and hypoxanthine analysis show that goldband snapper spoilt faster than saddletail snapper.

In conclusion, results from the sensory, physical, microbiological and chemical analyses performed, determined that there was a difference between the spoilage of goldband and saddletail snapper. A definitive cause for this difference was not established however it appears that the type of microflora present on the skin of goldband and saddletail snapper may provide further answers.

CHAPTER 6: BACTERIA IDENTIFIED FROM GOLDBAND AND SADDLETAIL SNAPPER

Introduction

Fish are a proteinaceous food, serving as a good substrate for microbial growth due to high moisture content, neutral to low pH and high nutritional value (Huis in't Veld, 1996). The highest loads of bacteria in and on fish originate from the gills, intestines and skin. The flesh of the fish is sterile for a newly harvested fish (Huss, 1995).

Fish harvested from warmer waters appear to have a higher bacterial load than those harvested from colder waters (Chattopadhyay, 1999; Shewan, 1977). However, the rate of spoilage is not always dependant on the total number of bacteria present on the fish. It is instead the number of specific spoilage organisms (SSOs) present on the fish that determine the rate of spoilage. The SSOs for iced marine fish are *Shewanella* species including *S. putrefaciens, S. algae and S. baltica* (Fonnesbech Vogel, Venkateswaran, Satomi, & Gram, 2005). 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 being susceptible to the same SSOs when stored in chilled aerobic conditions (Gram, 1996).

Most tropical fish species possess a relatively long shelf life (Bremner, Olley, Statham, & Vail, 1988) however, the shelf life of goldband snapper is unusually long in comparison, still being considered acceptable to panellists after 5 weeks of storage (Ames & Curran, 1985). In Chapter 5, the number of SSOs present on goldband snapper was not significantly different to those isolated from saddletail snapper, which had a shelf life of approximately 24 days. Therefore, the difference between the shelf life of saddletail snapper and goldband snapper is likely to be a result of the difference in the microbial communities populating each fish and the frequency in which they are isolated. In this chapter those differences are explored.

Methods

Samples

Goldband snapper samples were sourced from two fishing companies. One company from Broome supplied samples during the months of March and June harvested using the trap method. The other company, based in Exmouth, supplied samples during March and harvested using the longline method. All samples were assumed to

be 3 days old when received in Perth, as mentioned in Chapter 3. Twelve samples were used to determine the microbial flora present on goldband snapper.

Saddletail snapper samples were sourced from two fishing companies from Exmouth. One company supplied samples during the months of September and October which had been harvested using the trawl method. The other company supplied samples during March which had been harvested using the longline method. Again, all samples were assumed to be 3 days old on receipt in Perth. Thirteen samples were used to determine the microbial flora present on saddletail snapper.

Bacterial enumeration

Different sampling methods were used to enumerate the bacteria present on the skin of goldband snapper. Flesh extraction, described in Chapter 3, was performed on fish stored in the laboratory. The swabbing method, as described in Chapter 3, was introduced when sampling was completed on a commercial fishing boat where normal laboratory equipment was unavailable. Serial dilutions were performed on all laboratory samples and then inoculated onto media using the spiral plater. The spread plate method was used to inoculate dilutions onto media for bacterial enumeration when sampling took place on the commercial fishing vessel.

All samples were plated onto three different types of media. Plate count agar was used to measure the mesophilic counts, long and hammer agar was used to measure the psychrotrophic counts and iron agar was used to measure the number of spoilage organisms present.

Each organism was preliminarily identified using simple techniques including Gram stain, oxidase test, catalase test, motility, colony morphology, oxidase / fermentative test and optimum growth temperature, further explained in Chapter 3.

Results and Discussion

Spoilage flora isolated from Goldband Snapper

Figure 6.1 shows the bacteria isolated and identified from goldband snapper throughout 40 days of storage clearly indicating *Pseudomonas* species dominating the bacterial flora towards the end of storage. The microbial flora at the beginning of

storage was varied and included a higher proportion of *Staphylococcus* species, *Bacillus* species and *Psychrobacter* species, in comparison to saddletail snapper.

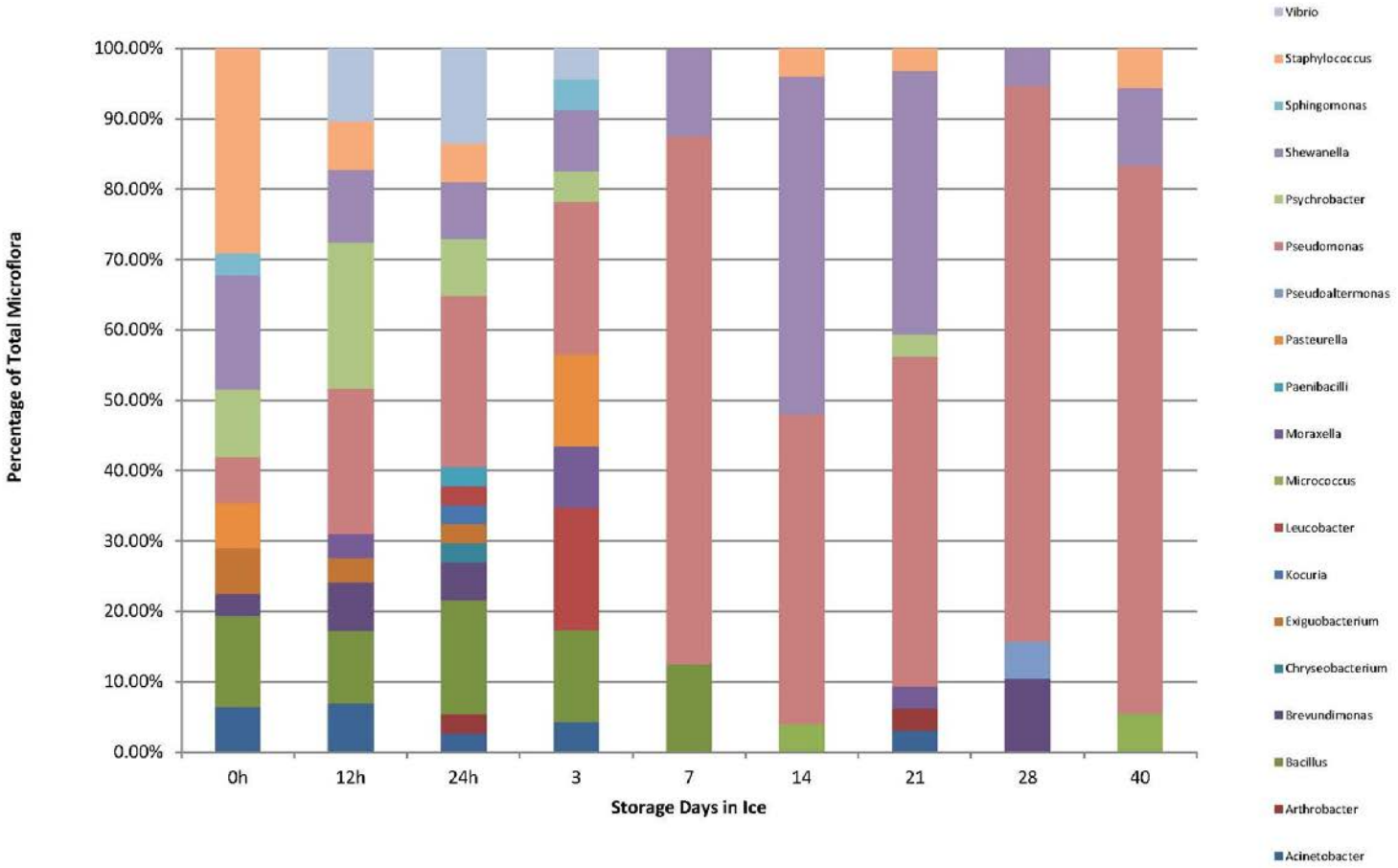


Figure 6.1: The proportion of bacteria present in the microflora isolated at different times during storage of goldband snapper.

Many different species were identified from goldband snapper immediately post harvest. Of the total microbial population, *Staphylococcus* species represented 29 %, *Shewanella* species (16 %), *Psychrobacter* species (9.6 %), *Pseudomonas* species (6.5 %), *Pasteurella* species (6.5 %), *Exiguobacterium* species (6.5 %), *Bacillus* species (13 %), and *Acinetobacter* species (6.5 %). *Sphingomonas* and *Brevundimonas* species were present in small numbers.

After 12 h of storage in an ice slurry, the microbial population was isolated and identified. The microbial flora had changed with *Staphylococcus* species representing only 7 % of the total population in comparison to the 29 % of the initial microflora identified immediately post-harvest. There was also an increase in *Pseudomonas* and *Psychrobacter* species, with both now representing 20 % of the total microbial population. *Vibrio* species and *Moraxella* species were not detected in the initial microflora, however they had increased to represent 10 % and 3.5 % , respectively. *Shewanella* species decreased to 10 % from the initial 16 % present in the microflora with the other species contributing (*Bacillus* species 10 %, *Acinetobacter* species 7 %, *Exiguobacterium* species 3.5 % and *Brevundimonas* species 7 %).

There was little difference in the microflora between 12 and 24 h of storage, with all species identified after 12 h of storage, present after 24 h of storage. *Vibrio* species, *Pseudomonas* species and *Bacillus* species increased representing 13.5 %, 24 % and 16 % of the total microflora, respectively. *Staphylococcus* species *Shewanella* species and *Exiguobacterium* species showed little variation from the 12 h post harvest sample, representing 5.4 %, 8 % and 2.7 % of the total microbial population, respectively. There was a decrease in the number of *Psychrobacter* species (8 %), *Brevundimonas* species (5.4 %) and *Arthrobacter* species (2.7 %) identified from the total microflora. Although the majority of the microflora remained unchanged, there were a few new organisms identified, these included *Paenibacilli* species, *Leucobacter* species, *Kocuria* species, *Chryseobacterium* species and *Arthrobacter* species, collectively representing 13.5 % of the total microbial flora identified on goldband snapper after 24 h of storage.

After 3 days of storage there was a significant increase in *Sphingomonas*, *Pasteurella* and *Leucobacter* species, totalling 35 % of the microbial population. The remaining

species identified, representative of the total microbial population, included *Vibrio* species (4 %), *Shewanella* species (8.7 %), *Psychrobacter* species (4.3 %), *Pseudomonas* species (21.7 %), *Moraxella* species (8.7 %), *Bacillus* species (13 %) and *Acinetobacter* species (4.3 %).

At day 7 of storage there were only three genera identified; *Shewanella* species and *Bacillus* species each representing 12.5 % of the total microbial population, and *Pseudomonas* species representing 75 %. Day 14 of storage showed an increase in *Shewanella* species and a decrease in *Pseudomonas* species. *Shewanella* species now represented 48 % of the total microbial flora and *Pseudomonas* species represented 44 %. *Staphylococcus* species and *Micrococcus* species were also identified at day 14, each representing 4 % of the total microbial population.

There was a further change in the microflora identified on goldband snapper after 21 days of storage. *Shewanella* species and *Pseudomonas* species dominated the microflora representing 37.5 % and 46 %, respectively. *Staphylococcus*, *Psychrobacter*, *Moraxella*, *Arthrobacter* and *Acinetobacter* species were also present in low numbers. At day 28 of storage *Pseudomonas* species dominated the microflora, representing 79 % of isolates. Other organisms identified included *Shewanella* species (5 %), *Brevundimonas* species (10 %) and *Pseudoaltermonas* species (5 %). With the exception that *Pseudomonas* species were more dominant, the microflora at day 40 looked similar to the microflora at day 14.

Gram positive bacteria versus Gram negative bacteria isolated from goldband snapper

The ratio of Gram positive to Gram negative organisms changed throughout the storage of goldband snapper as seen in Figure 6.2.

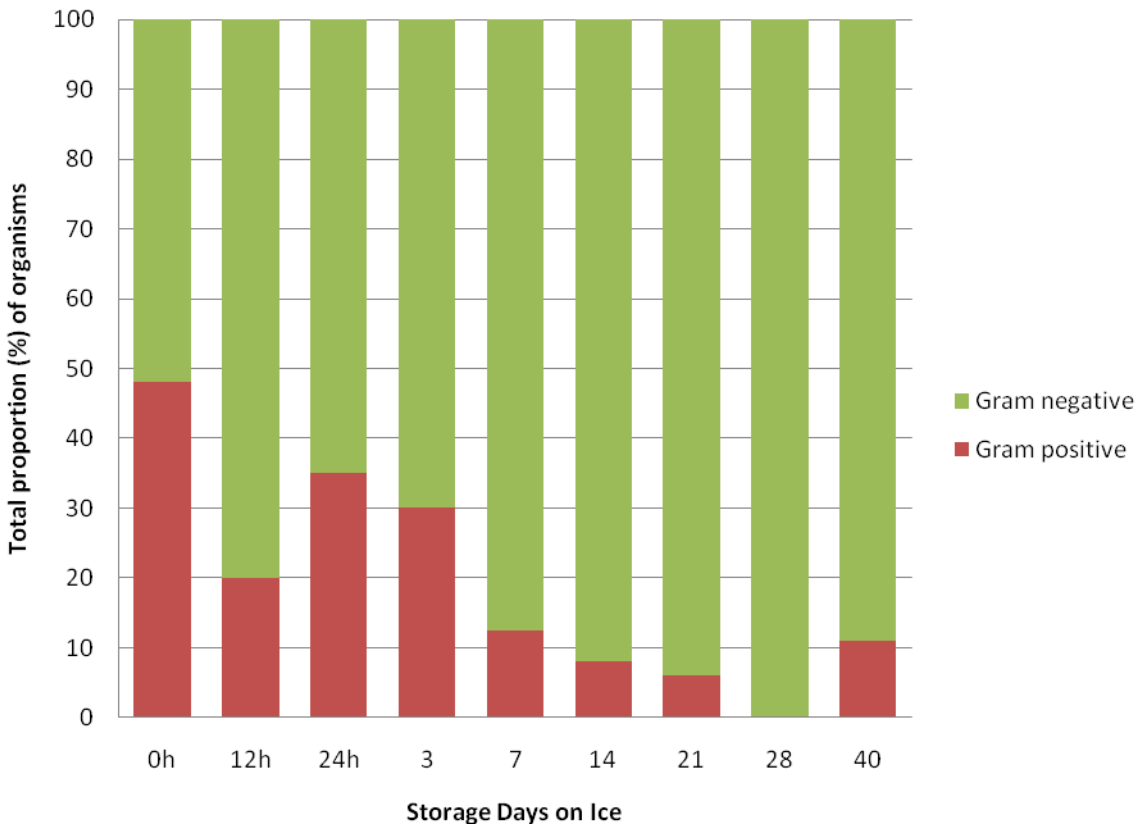


Figure 6.2: Proportions (%) of Gram positive and Gram negative organisms isolated from goldband snapper throughout storage.

Immediately post harvest the ratio of Gram negative to Gram positive organisms was approximately even. After 12 h of storage, approximately 80 % of the organisms isolated from goldband snapper were Gram negative. After 24 h of storage the number of Gram positive organisms increased to approximately 35 % however, numbers continued to decrease until day 14, at which time less than 10 % of the organisms isolated from goldband snapper were Gram positive. Tropical fish species appear to carry a higher load of Gram positive organisms and enteric bacteria in comparison to temperate water fish (Gram, 1996), which may explain the high Gram positive bacterial counts post harvest. The psychrotrophic Gram negative bacteria responsible for spoilage in aerobically stored iced fish begin to dominate towards the end of storage since they are better suited to proliferate in the colder storage conditions (Gram, 1996).

Specific Spoilage Organisms isolated from Goldband Snapper

Shewanella putrefaciens and *Pseudomonas* species are the specific spoilage organisms isolated from fish stored aerobically on ice (Gram & Melchiorsen, 1996). Figure 6.3 shows the relative proportions of *Pseudomonas* and *Shewanella* species throughout the storage of goldband snapper.

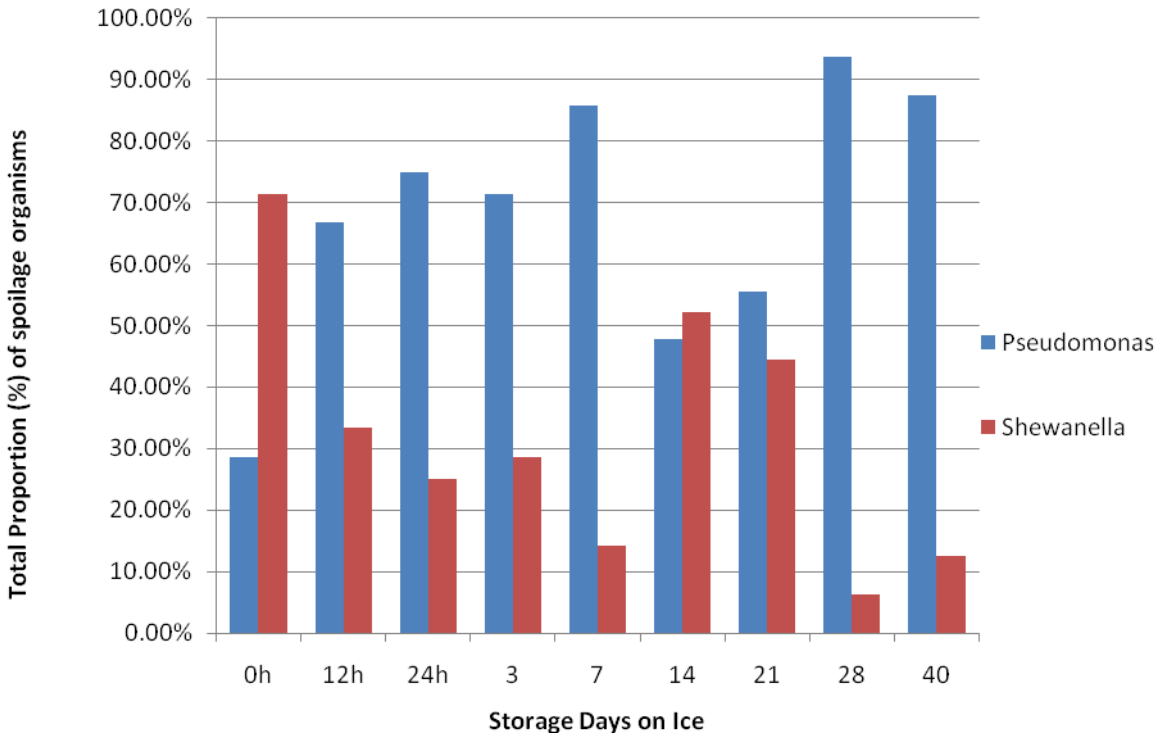


Figure 6.3: Relative proportions (%) of *Pseudomonas* and *Shewanella* species throughout the storage of goldband snapper.

Shewanella was the dominant species immediately post harvest. There was a sudden decline in numbers 12 h post harvest, with *Pseudomonas* species remaining the dominant species of the two spoilage organisms until day 7. The proportion of *Shewanella* species isolated during days 14 and 21 increased to constitute approximately 50 % of the spoilage organisms identified on goldband snapper. However, this decreased considerably by day 28. The difference between the specific spoilage organisms may be related to the siderophores and other compounds produced by some *Pseudomonas* species (Gram & Melchiorsen, 1996). Gram and Melchiorsen (1996) reported that aquatic *Pseudomonas* species, under iron limiting

conditions, are inhibitory to *Shewanella putrefaciens*. Fish muscle contains only 1-2 µmol 1^{-1} iron which is not available in free form. Therefore, fish muscle supports the production of siderophores from *Pseudomonas* and *Shewanella* species isolated from fish (Gram, 1996). One study identified the siderophore-producing isolates all belonged to *P. fluorescens / putida* group (Gram & Melchiorsen, 1996). At least 58 % of the *Pseudomonas* species were identified as either *P. fluorescens* or *P. putida*.

Spoilage flora isolated from saddletail snapper

Figure 6.4 represents the 795 isolates colonies isolated and identified from saddletail snapper throughout 40 days of storage. Many different organisms were isolated and identified at the beginning of storage with less variation occuring towards the end of storage. *Shewanella* and *Pseudomonas* species were the dominant organisms isolated from saddletail snapper throughout storage.

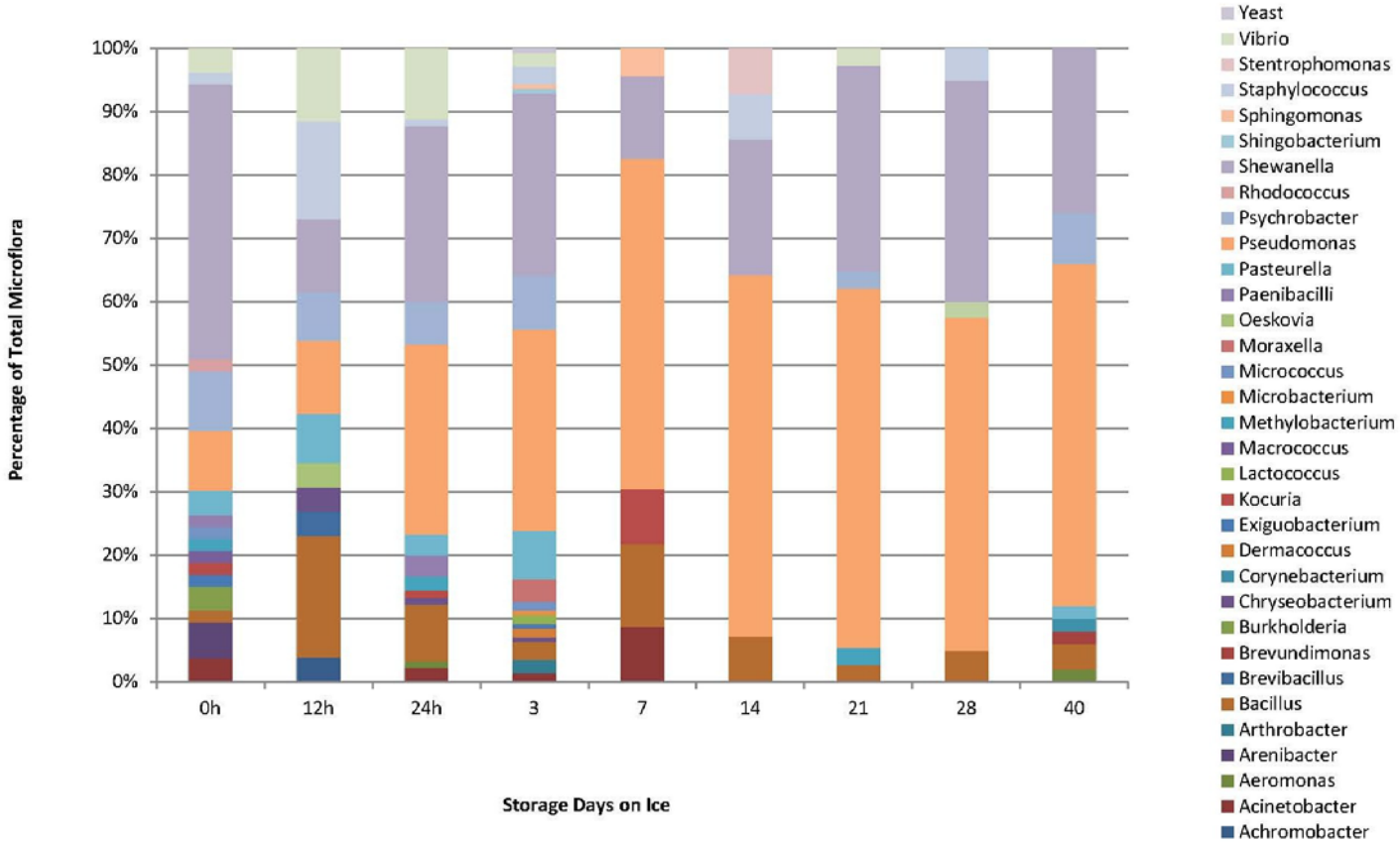


Figure 6.4: The proportion (%) of bacteria present in the microflora isolated at different times during storage for saddletail snapper.

Many different species were identified from saddletail snapper immediately post harvest. Of the total microbial flora, *Shewanella* species represented 43 % of identified isolates, *Pseudomonas* and *Psychrobacter* species represented 9 % each. The other species identified represented a total of approximately 36 % of the total cultured microflora. The genera identified included *Vibrio, Staphylococcus, Rhodococcus, Pasteurella, Paenibacilli, Micrococcus, Methylobacterium, Macrococcus, Kocuria, Exiguobacterium, Burkholderia, Bacillus, Arenibacter* and *Acinetobacter* species.

After 12 h of storage there was a change in the microflora present on saddletail snapper. *Vibrio*, *Bacillus* and *Staphylococcus* species increased to represent 11.5 %, 15 % and 19 %, respectively. *Shewanella* species decreased approximately 32 % representing only 11.5 % of the total bacterial population. Other species present in low numbers included *Psychrobacter, Pasteurella, Oeskovia, Chryseobacterium, Brevibacillus* and *Achromobacter* species.

Within 24 h of storage, the levels of *Pseudomonas* and *Shewanella* species had increased so that together they represented over 50 % of the total microbial population identified on saddletail snapper. The number of *Staphylococcus* species had diminished considerably, representing only 1 % of the total population. The total number of *Vibrio* and *Bacillus* species isolated had also decreased in numbers. Other bacterial species identified were isolated in low numbers; these included *Psychrobacter, Pasteurella, Paenibacilli, Methylobacterium, Kocuria, Chryseobacterium, Aeromonas* and *Acinetobacter* species. Day 3 of storage provided the most diversity out of all the storage days sampled however, *Pseudomonas* and *Shewanella* species still dominated the microflora. The remaining organisms isolated from saddletail snapper were present in low numbers, these included *Vibrio, Staphylococcus, Sphingomonas, Shingobacterium, Exiguobacterium, Dermacoccus, Bacillus, Arthrobacter*, *Psychrobacter*, *Moraxella, Micrococcus, Microbacterium, Lactococcus, Pasteurella, Paenibacilli,* and *Acinetobacter* species.

By day 7, the diversity within the microflora isolated had decreased with only 6 genera isolated. The species identified included *Sphingomonas* species (4 %),

Shewanella species (13 %), *Pseudomonas* species (52 %), *Kocuria* species (8.7 %), *Bacillus* species (13 %) and *Acinetobacter* species (8.7 %). A slight change in the microflora was seen by day 14 of storage. The genera identified included *Stentrophomonas, Staphylococcus, Shewanella, Pseudomonas* and *Bacillis*. The microflora was dominated by *Pseudomonas* species which represented over 50 % of the total bacterial population. *Pseudomonas* species continued to dominate the microflora until the end of the experiment. *Shewanella* species were the next dominant bacterial species contributing between 26 % and 35 % between days 21 and 40 of storage.

Vibrio, Psychrobacter, Methylobacterium and *Bacillus* species were isolated from saddletail snapper after 21 days of storage and *Staphylococcus,* and *Bacillus* species were isolated along with *Pseudomonas* and *Shewanella* species after 28 days of storage. There was more variation at day 40 with *Psychrobacter, Pasteurella, Corynebacterium, Brevundimonas, Bacillus* and *Aeromonas* species being isolated in low numbers.

Gram positive bacteria versus Gram negative bacteria isolated from Saddletail Snapper

The ratio of Gram positive to Gram negative organisms changed throughout the storage of saddletail snapper, as shown in Figure 6.5.

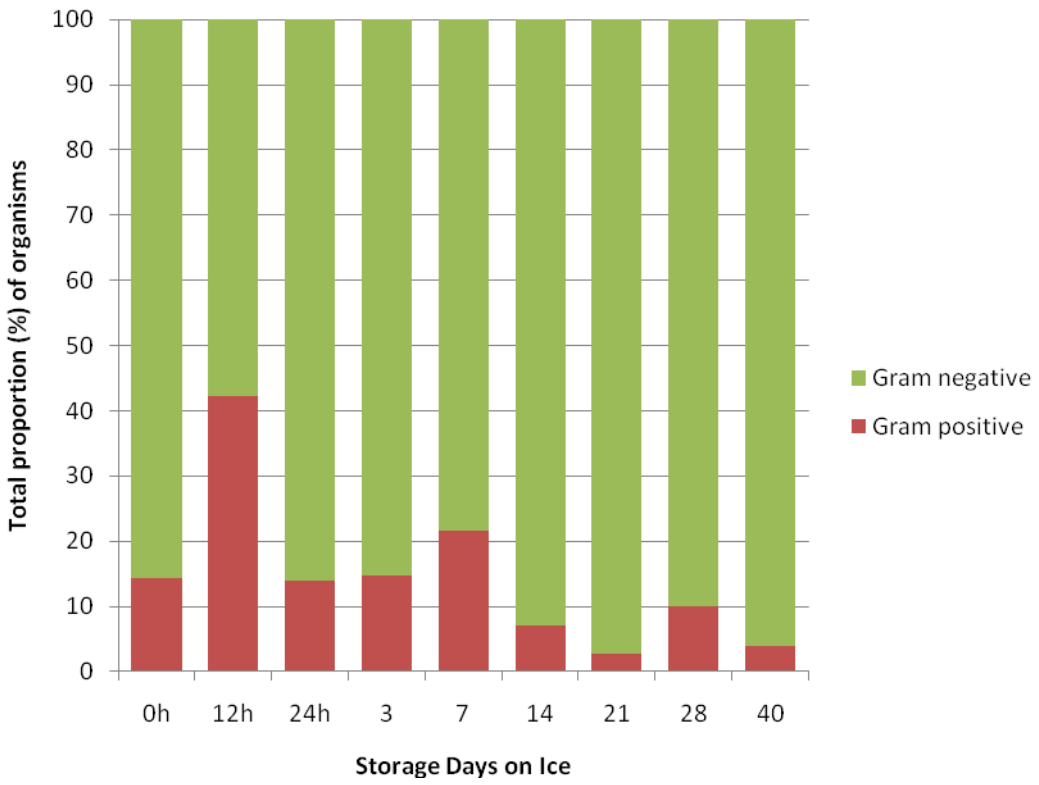


Figure 6.5: Proportion (%) of the Gram positive and Gram negative organisms isolated from saddletail snapper throughout storage.

Gram negative organisms dominated the microflora of saddletail snapper. Tropical fish harvested from warmer waters have been documented as having a higher load of Gram positive organisms, the initial flora of saddletail snapper did not follow this trend (Fraser & Sumar, 1998b; Gram, 1996). Saddletail snapper were caught at depths of approximately 120 m at a temperature of approximately 24°C; therefore the fact that the initial microflora was so heavily dominated with Gram negative organisms was not expected. There appeared to be an increase in Gram positive organisms after 12 h of storage, with them totalling just over 40 %. For the remainder of the experiment the number of Gram positive organisms remained below 25 %. The storage boxes the fish were kept in were not regularly checked and iced and so it is possible the sudden increase in the number of Gram positive organisms is explained by a spike in the storage temperature. The Gram positive organisms isolated from saddletail snapper are mesophilic in nature, which means they would have adapted to the increase in temperature very quickly in comparison to the Gram negative organisms which are psychrotrophic in nature preferring the colder environments. An increase in Gram negative bacteria throughout storage was

expected since the SSOs for aerobically stored fish are psychrotolerant Gram negative bacteria (Gram & Dalgaard, 2002).

Specific spoilage organisms isolated Saddletail Snapper

Shewanella putrefaciens and *Pseudomonas* species are the SSOs responsible for spoilage of fish stored aerobically on ice (Gram & Melchiorsen, 1996). Figure 6.6 shows relative proportions of *Pseudomonas* and *Shewanella* species throughout the storage of saddletail snapper.

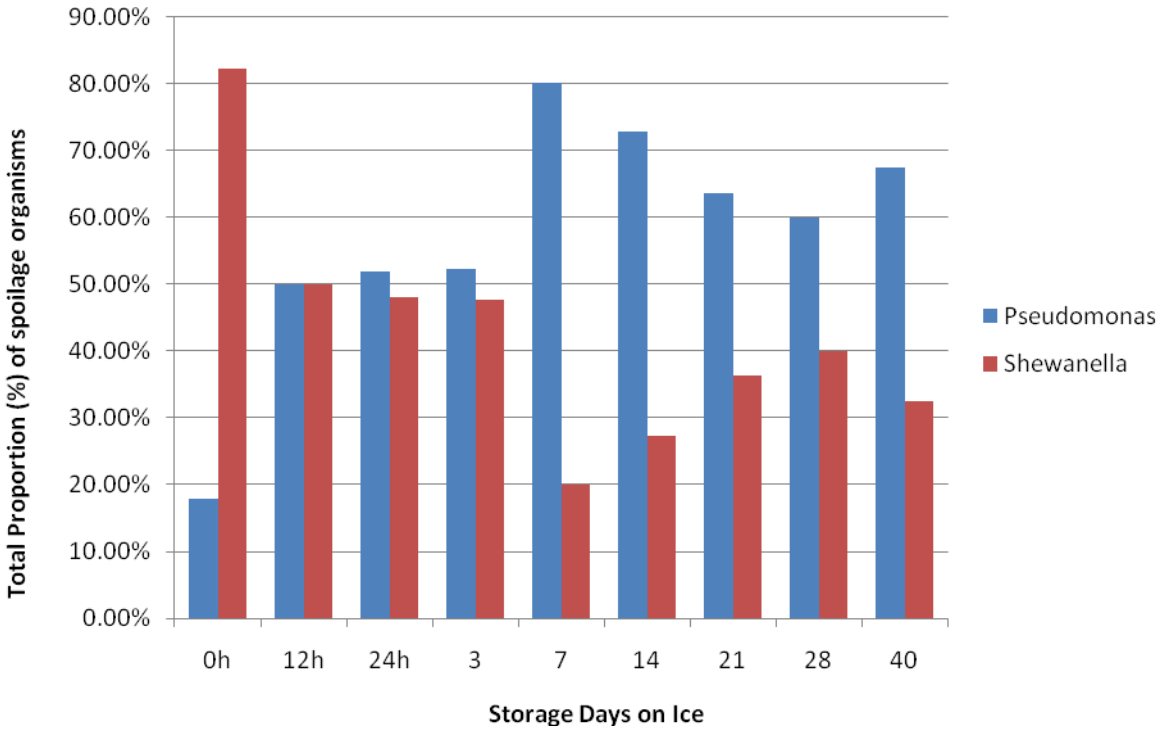


Figure 6.6: Relative proportions (%) of *Pseudomonas* and *Shewanella* species throughout the storage of saddletail snapper.

Shewanella species were the dominant spoilage organisms, immediately post harvest. However, after 12 h of iced storage, and until day 3, the number of *Pseudomonas* species increased until each species contributed approximately 50 % to the total spoilage organisms present on saddletail snapper. By day 7 the *Pseudomonas* population had significantly increased and was responsible for 80 % of the spoilage organisms on saddletail snapper. Although the *Pseudomonas* population dominated for the remainder of the experiment there was a decrease in numbers after day 7 as

the *Shewanella* species began to increase. This trend continued until day 28, with 40 % of the total spoilage organism being *Shewanella* species and the remainder belonging to *Pseudomonas* species. By day 40, *Pseudomonas* species had begun to increase in numbers again.

Conclusion

Overall, from the investigation into the spoilage of goldband snapper, using saddletail snapper as the control, it was concluded that the microbiological populations present on the skin significantly affected the shelf life of each fish species. Earlier work showed that the SSOs of iced fresh fish, responsible for producing the "off" odours and flavours commonly associated with spoilt fish, were *Shewanella* and *Psuedomonas* species (Vogel, et al., 2005; Gram & Melchiorsen, 1996). Both *Shewanella* and *Pseudomonas* species were isolated from goldband and saddletail snapper in the present study. The proportions in which the SSOs were isolated throughout storage appear to be the predominant cause for the difference between the shelf life of the two fish species.

Spoilage flora

There appeared to be more variation within the microflora for both fish species within the first 3 days of storage. The spoilage flora isolated from goldband snapper appeared to be more evenly distributed in comparison to saddletail snapper. The SSOs (*Shewanella* and *Pseudomonas* species) for goldband snapper totalled 22.58 % of the total flora isolated in comparison to the 52.83 % isolated from saddletail snapper. Due to the lower numbers of SSOs present on goldband snapper immediately post harvest, a variety of other organisms are able to colonise the surface of the skin in greater numbers, as similarly concluded in another study (Gram & Dalgaard, 2002). Although a greater number of bacterial species was identified from saddletail snapper they did not comparatively contribute as much to the total bacterial population as those present on goldband snapper. The number of SSOs decreased 12 h post harvest, however by 24 h post harvest the total number of SSOs present on saddletail snapper had increased to over 58 % of the total bacterial population.

Gram positive versus Gram negative

The proportion of Gram positive organisms as a total of the microbial flora was comparatively higher on goldband snapper than saddletail snapper. The number of Gram positive organisms peaked immediately post harvest for goldband snapper and then followed a downward trend. The number of Gram positive organisms were fairly low immediately post harvest for saddletail snapper. The numbers then peaked at 12 h of storage, followed by a sharp decrease in numbers which remained low for the remainder of the experiment.

The total microbial flora on tropical fish species is expected to be dominated by Gram negative organisms with a slightly higher load of Gram positive organisms than is expected on cold water fish (Fraser & Sumar, 1998b). Although saddletail snapper comes from a reef associated environment with warmer temperatures it is exhibiting a microflora dominated by Gram negative organisms which would be more similar to a fish caught from colder waters.

Specific Spoilage Organisms

The SSOs for goldband and Saddletial snapper are *Shewanella* and *Pseudomonas* species (Gram & Vogel, 1999; Morita, 1975; Shewan, 1977). The relationship between *Shewanella* and *Pseudomonas* species changed throughout the storage of both fish species. The *Shewanella* population identified on saddletail snapper was identified in high numbers, constituting approximately 43 % of the total microbial population, immediately post harvest (Table 6.1). This population was reduced to 20 % by day 7 of storage and then gradually increased to 35 % by day 28, with *Pseudomonas* species becoming the more dominant spoilage organism. An even ratio of *Shewanella* species to *Pseudomonas* species was displayed for the first 3 days of storage for goldband snapper, excluding immediately after harvest. Until day 7 of storage both *Shewanella* and *Pseudomonas* species constituted only a small proportion of the total microbial population for goldband snapper (Table 6.2). The proportion of *Shewanella* species increased to represent approximately 50 % of the spoilage organisms at days 14 and 21 of storage however, by day 28 *Shewanella* species had declined rapidly and *Pseudomonas* species were dominant on goldband snapper. The increased *Pseudomonas* population at the beginning of the storage for goldband snapper may explain why this fish species has an extended shelf life.

Table 6.1: Percentage of SSOs for saddletail snapper and goldband snapper

Day	Saddletail Snapper (% SSOs of total flora)				Goldband Snapper (% SSOs of total flora)			
	Shewanella	*Pseudomonas*	*SSO*	*Other*	*Shewanella*	*Pseudomonas*	*SSO*	*Other*
0hr	43	10	53	47	16	6	22	78
12hr	12	11	23	77	10	21	31	79
24hr	28	30	58	42	9	6	15	85
3	29	32	61	39	9	24	33	67
7	13	53	66	34	12	75	87	13
14	21	57	78	22	48	45	93	7
21	32	57	89	11	38	48	86	14
28	35	53	88	12	5	78	83	17
40	26	55	81	19	12	77	89	11

Table 6.2: Percentage of Shewanella and Pseudomonas species for saddletail snapper and goldband snapper

Day	Saddletail Snapper (% *Shewanella* and *Pseudomonas* of Total SSOs)		Goldband Snapper (% *Shewanella* and *Pseudomonas* of Total SSOs)	
	Shewanella	*Pseudomonas*	*Shewanella*	*Pseudomonas*
0hr	82	18	71	29
12hr	50	50	33	67
24hr	48	52	26	74
3	47	53	29	71
7	20	80	14	86
14	27	73	52	48
21	36	63	45	55
28	40	60	6	94
40	32	68	12	88

CHAPTER 7: GENERAL DISCUSSION

The purpose of this project was to investigate and determine why goldband snapper experiences such an extended shelf life. Different aspects of spoilage were investigated and different techniques employed to help determine a cause for the extension of shelf life. Microbiological, chemical, physical and sensory analyses were used to assess the different components of spoilage and determine which, if any, were responsible for the extended shelf life seen with goldband snapper.

Saddletail snapper, the control for the project, was initially chosen because it met the necessary requirements relevant to determining why goldband snapper has an extended shelf life. Saddletail snapper is genetically similar to goldband snapper with both species being members of the *Lutjanidae* family and belonging to the same order (*Perciformes*) and class (*Actinopterygii*). Selecting a control sample genetically similar to the experimental sample allows genetic factors to be eliminated as a cause for the difference in shelf life between the two fish species. Although genetically similar, the two fish species inhabit different environments. Goldband snapper is a demersal fish distributed between 40-245m below sea level (Newman, et al., 2008). Saddletail snapper is a reef associated fish distributed between 12-100m below sea level (Newman, et al., 2008). It was initially hypothesised that the microflora present on the two fish species was responsible for the difference in shelf life. Goldband snapper is harvested from a temperate environment and so it was thought that a reef associated fish harvested from warmer waters would have a different microflora and that this difference may influence shelf life.

To measure the differences in quality observed throughout the spoilage process between goldband and saddletail snapper a Quality Index Method (QIM) was employed. Goldband snapper has a published QIM (Boulter, et al., 2006), currently available in the Australian Quality Index Manual which is widely distributed within the Australian fishing industry. At the commencement of this project the QIM for saddletail snapper had only recently been developed and it was being introduced to the industry by the Sydney Fish Market (Boulter, et al., 2006). Genetic variation can occur within fish species caught from different locations (Ovenden, et al., 2004). The QIM for saddletail snapper had been developed using saddletail snapper caught from

the east coast of Australia. Validation of the QIM was necessary to ensure it was applicable for saddletail snapper caught from the west coast of Australia. Sensory, physical and microbiological methods were employed to determine if the QIM developed could be used for saddletail snapper caught in Western Australia.

Sensory analysis was performed by a trained panel that assessed whole saddletail snapper at different stages of spoilage using the QIM and the Torry Scheme. The results from these sensory analyses confirmed that the QIM for saddletail snapper will accurately measure the spoilage of saddletail snapper caught in Western Australia. These results were further validated using physical analyses, including colour measurements and texture analysis of the whole fish.

Microbiological analyses of saddletail snapper throughout storage were used to determine how micro-organisms affected spoilage. Further investigation into using SSO and psychrotrophic counts instead of total plate counts should be investigated so that industry, retailers and consumers have a truer picture of the quality of the produce they are producing, selling and buying, respectively. This knowledge may also promote better storage and handling habits for producers and consumers.

Spoilage of goldband and saddletail snapper

Investigation into the spoilage of goldband and saddletail snapper, using sensory, physical, microbiological and biochemical methods, was necessary to determine why these two fish have different shelf lives.

Sensory analyses performed detected a difference between the two fish species however, no reason for the difference was identified. The QIM produced strong correlation coefficients for both goldband snapper (R^2 = 0.9895) and saddletail snapper (R^2 = 0.9299). The Torry scheme was performed in conjunction with the QIM to determine the shelf life of saddletail snapper. The cooked fillets of saddletail snapper were considered acceptable to eat until day 24 of storage. A previous study had already performed a shelf life evaluation on goldband snapper and identified a shelf life of 35 days (Ames & Curran, 1985). For this reason the Torry scheme was not performed on goldband snapper in this project. The difference in shelf life was

confirmed by the sensory analyses however, further physical, biochemical and microbiological analyses are needed to determine why this difference is occurring.

Differences were also identified between goldband and saddletail snapper using textural analysis. Saddletail snapper showed a negative relationship between the number of storage days and the force used for compression analysis with a correlation coefficient of $R^2 = 0.59$. This relationship demonstrates that as the storage days increased the flesh of saddletail snapper become softer and less force was needed to compress it. This trend was also observed from the validation sessions used for the QIM of saddletail snapper. Goldband snapper exhibited completely different textural properties. There appeared to be no defined relationship between the storage days and the force used for compression analysis. With the exception of day 17 of storage, all data points remained below 100 N. In comparison saddletail snapper began at approximately 250N and was measured just above 100N at day 31 of storage. The measurements taken for goldband snapper were much lower. At day 17 of storage the texture measurements for goldband snapper peaked at over 250 N. These results were obtained from two independant trials so the findings are unlikely to be the result of neither experimental error nor differences in storage at the laboratory. It is unclear why there was a peak at day 17 for goldband snapper. Overall the differences between the two fish were evident however, the cause could be innate or environmental. Different sized fish were sampled throughout this experiment so it seems unlikely that this could be the cause for the variation in texture experienced by goldband snapper. Spawning season is also known to affect the fish flesh making it softer and flabbier (Pedrosa-Menabrito & Regenstein, 1990a). Saddletail snapper collected for this project were harvested during their spawning season, whereas goldband snapper were harvested a few months after. If the goldband snapper harvested had already spawned, this may explain the low amount of force needed to measure their texture. Environmental factors such as where the fish were caught, the harvest method used and storage and handling immediately after harvest could also have an impact on the textural results. Further analysis of the structural properties of the fish flesh for both saddletail and goldband snapper is necessary before being able to determine the exact cause of the difference in texture between the two fish species.

Colour, another physical method used to assess the spoilage of goldband and saddletail snapper, produced some clear differences between the two species. Saddletail snapper appeared to increase in redness throughout storage and produced higher data points probably due to its natural red pigment in comparison to goldband snapper. Goldband snapper increased in green pigment throughout storage. The increase in green pigment is possibly related to the types of *Pseudomonas* species isolated from goldband snapper throughout storage. Certain *Pseudomonas* species produce green pigments (pyocyanins) as a result of increased growth and also fluoresce under UV light (Rhodes, 1959). It is unclear why this phenomenon did not occur on saddletail snapper. It may be related to the specific strains of *Pseudomonas* species colonising goldband snapper or the type of spoilage flora present on goldband snapper which allows these species to grow (see below). Further analysis on the microflora colonising each fish species may help determine why this colour change occurs. Overall colour measurement confirmed the difference in shelf life however, it did not provide conclusive evidence as to why this difference exists.

The microbiological analysis investigated the populations of spoilage bacteria, mesophillic bacteria and psychrotrophic bacteria present on goldband and saddletail snapper. Saddletail snapper had a greater proportion of spoilage organisms than goldband snapper. Until day 24 of storage, the populations of spoilage organisms were similar between the two species, however, at day 24 the population peaked for saddletail snapper (1×10^{11} CFU / g), in comparison to goldband snapper which peaked earlier at day 17 (1×10^{8} CFU / g). The SSOs population for goldband snapper appeared to plateau at day 17 of storage and did not decrease until day 31 of storage. The SSOs population for saddletail snapper appeared to plateau at day 10 of spoilage until day 17 after which the population increased to 1×10^{11} CFU / g at day 24 of storage. This increase in spoilage organisms correlated with the shelf life of saddletail snapper ending at 27 days of storage. Overall saddletail snapper had a higher load of spoilage organisms during the later stages of storage. During iced storage, Gram negative, psychrotrophic, spoilage organisms, such as *Shewanella* species, proliferate and become dominant. If a higher proportion of psychrotrophic spoilage organisms were initially present on saddletail snapper in comparison to goldband snapper, a higher bacterial load would be expected (Vogel, et al., 2005) however, this was not the case. Goldband snapper had the higher load of spoilage

organisms at the beginning of storage. Further analysis of the types of spoilage organisms present on goldband and saddletail snapper may provide further clarification as to why saddletail snapper had a higher load of spoilage organisms at the later stage of spoilage. *Pseudomonas* and *Shewanella* species are common spoilage organsisms isolated from iced fish and some strains of *Pseudomonas* have been shown to inhibit the growth of *Shewanella* species. It may be possible that goldband snapper have a higher load of these *Pseudomonas* strains which are capable of inhibiting the growth of *Shewanella* species. This would then impact on the amount of spoilage organisms detected on iron agar, since it is primarily used to identify *Shewanella* species (Gram, et al., 1987).

The total numbers of mesophillic aerobic organisms present on goldband and saddletail snapper throughout iced storage were different between the two species. A reduced bacterial load was seen for goldband snapper compared to saddletail snapper. The bacterial load for goldband snapper peaked at 1×10^8 CFU / g on day 31 compared to saddletail snapper which peaked at day 24 at 1×10^{11} CFU / g. The difference in the shelf life between goldband and saddletail snapper may be influenced by the total amount of bacteria present. A previous study performed by Ames & Curran (1985) determined that the maximum bacterial load for goldband snapper was 1.5×10^8 CFU / g after 30 days. This was similar to the results obtained in this study, leading to the conclusion that goldband snapper have a lower bacterial load than saddletail snapper.

The psychrotrophic bacteria measured on saddletail snapper showed the greatest variation between the two species. Overall saddletail snapper had a greater population of psychrotrophic bacteria present throughout storage compared to goldband snapper. Saddletail snapper began storage with an initial load of approximately 1×10^6 CFU / g, compared to goldband snapper which started with an approximate initial load of 1×10^2 CFU / g. Saddletail snapper then peaked at day 17 of storage with 1×10^{12} CFU / g, whilst goldband snapper had only reached $1 \times 10^{4.5}$ CFU / g at that timepoint. By day 31 of storage the number of psychrotrophic bacteria present on saddletail snapper had decreased and the psychrotophic population on goldband snapper had increased so they both measured approximately 1×10^8 CFU / g. The cold storage conditions would be favourable for the “cold-

loving" psychrotrophic organisms promoting a higher growth rate and so if there was a higher proportion of psychrotrophic organisms initially present on saddletail snapper compared to goldband snapper, a higher bacterial load would be expected. Across all the different microbial counts, the bacterial load present on saddletail snapper was much higher than on goldband snapper. Initially it was thought that the reason for this could be the location the fish were harvested from, the storage and handling conditions or the initial state of the fish. Further investigation has confirmed that the difference between the bacterial populations may be related to the type of bacteria colonising each fish.

Chemical analysis was also successful in determining a difference in the spoilage of goldband and saddletail snapper. The concentrations of TMA appeared to follow the general growth trend of spoilage organisms. The SSOs for goldband and saddletail snapper are *Shewanella* and *Pseudomonas* species (Gram & Vogel, 1999; Morita, 1975; Shewan, 1977). Spoilage of aerobically stored fish only occurs when SSOs reach levels of 10^8 to 10^9 CFU / g (Gram & Huss, 1996). Once spoilage organisms reach these levels measurable amounts of TMA are produced along with the "off" odours and flavours associated with fish spoilage. The number of spoilage organisms present on goldband snapper did not reach 10^8 CFU / g until day 17 of storage. Goldband snapper had a low level of TMA (approximately 2mg / 100g fish) until day 17, mirroring the lower spoilage organism counts. The spoilage organism count for saddletail snapper reached 10^8 CFU / g at day 10 of storage, explaining the increased levels of TMA, almost reaching 4 mg / 100g fish at day 17. By the end of storage goldband snapper had reached almost 6 mg TMA / 100g fish compared to the lower 4.5g TMA / 100g fish for saddletail snapper. Variation between fish due to spawning, environmental factors and innate qualities, may be responsible for the lower TMA values seen in saddletail snapper at the end of the experiment. Importantly, both fish species remained well below the human limit of 10-15 g TMA / 100g fish.

Previous studies have also shown a linear correlation between the levels of TMA and hypoxanthine, formed from autolytic decomposition and bacterial formation (Gram & Huss, 1996). The rate of bacterial growth is higher than autolysis and so bacterial spoilage is the most common cause of hypoxanthine production. Several spoilage

organisms, including *Shewanella* species, *Pseudomonas* species and *Photobacterium phosphoreum*, are responsible for producing hypoxanthine (Dalgaard, Mejlholm, & Huss, 1996; Jorgenson, Gibson, & Huss, 1988; van Spreekens, 1977). The hypoxanthine levels measured in goldband snapper were considerably higher than in saddletail snapper from day 17 of storage onwards. The difference in hypoxanthine levels appeared to correlate with the number of spoilage organisms isolated from each fish species. The proportion of *Pseudomonas* and *Shewanella* species combined for goldband snapper totalled 87.5% of the total bacterial population identified at day 7 of storage, compared to the 65.21% of spoilage organisms present on saddletail snapper. The total SSOs present on goldband snapper totalled 92% of the total microbial population identified compared to 78.57% present on saddletail snapper. The increased total number of spoilage organisms present on goldband snapper appears to have caused the increased levels of hypoxanthine.

Investigation into the spoilage of goldband and saddletail snapper confirmed that a difference between the two fish species existed. The microbiological results suggested that saddletail snapper produced higher bacterial counts through storage. Further investigation into the microbial isolates present on each fish will illustrate a broader picture of what happens during spoilage. After investigation into the spoilage of each fish species it was thought that the difference in bacterial loads and the types of bacteria present on each fish may offer an explanation and help define a cause for the difference in shelf life.

Bacteria isolated and identified from goldband and saddletail snapper

From previous chapters it was concluded that there was a difference in the rate of spoilage between goldband and saddletail snapper however sensory, physical, and biochemical analyses, and preliminary microbiological analysis, were not sufficient to provide an answer as to why this difference was occurring. It was hypothesised that the types of bacteria colonising the fish species may be different and this may offer an explanation for the difference in shelf life. Further investigation into identifying the bacterial isolates present on each fish species from immediately after harvest to the end of storage life was undertaken. The general spoilage flora was investigated as well as the proportions of Gram negative isolates versus Gram

positive isolates on each fish and, finally, the differing proportions of spoilage organisms present.

The spoilage flora isolated from the two fish species contained many of the same bacterial isolates in differing proportions. A greater diversity of isolates was identified from saddletail snapper compared to goldband snapper. The majority of isolates identified were collected from fish caught from a commercial long line fishing boat off the West Australian coast, near Exmouth. Isolates collected during the validation of the Quality Index for saddletail snapper were also identified and grouped together with the Exmouth isolates. By grouping the isolates together a larger sample pool was created for saddletail snapper allowing a more accurate analysis of the microflora present throughout storage. However, grouping the isolates also introduced factors such as variation of origin. Isolates identified from saddletail snapper were grouped according to the location of harvest, and bacterial populations were compared. Isolates were sourced initially from Broome (trap method) and then Exmouth (longline harvest method and trawl method). Samples for Broome were from a preliminary study and so were only taken at the beginning of storage and at the end of storage. *Shewanella* species were present in very low numbers compared to *Pseudomonas* species at the beginning of storage. By the end of storage *Pseudomonas* dominated the total microflora followed closely by *Shewanella* species. The isolates identified at the beginning of storage for Exmouth (trawl method) were dominated equally by *Shewanella* and *Pseudomonas* species until the end of storage and overall approximately 70% of the bacterial population identified were SSOs. The pattern of SSOs present on samples from the Exmouth (longline harvest method) showed *Shewanella* species as the dominant organism at the beginning of storage. However, *Pseudomonas* species appeared to dominate towards the end of storage. Although the types of bacteria were similar, slight differences existed between the proportions of isolates identified from each location. Approximately 500 isolates from Exmouth (trawl method), 250 isolates from Exmouth (longline harvest method) and 150 isolates from Broome (trap method) were combined to illustrate the proportions of bacterial populations present on saddletail snapper. Although the contributions were not evenly spread, all locations followed a similar trend and so location of origin did not appear to be a factor influencing variation. Colwell (1962) suggested that the methods of handling fish

and their pre-capture environment played a role in affecting the composition of the microflora present on the skin. The number of organisms identified for saddletail snapper (795 isolates) was considerably more than goldband snapper (280 isolates). The difference in the number of isolates identified was also reflected in the number of genera identified: saddletail snapper had 24 different bacterial genera identified throughout storage and goldband snapper had 19 different genera. Although saddletail snapper had a greater diversity of bacterial isolates present, the variation was not as evenly distributed. For example, immediately post harvest the total microbial flora for saddletail snapper consisted of *Shewanella* species representing 43% of identified isolates, and *Pseudomonas* and *Psychrobacter* species representing 9% each. The other species identified represented a total of approximately 36% of the total cultured microflora. The total microbial population isolated from goldband snapper consisted of *Staphylococcus* species representing 29%, *Shewanella* species (16%), *Psychrobacter* species (9.6%), *Pseudomonas* species (6.5%), *Pasteurella* species (6.5%), *Exiguobacterium* species (6.5%), *Bacillus* species (13%), and *Acinetobacter* species (6.5%). *Sphingomonas* and *Brevundimonas* species were present in small numbers. Specifically, the difference between the two fish species related to the number of SSOs present immediately post harvest. The number of SSOs isolated from goldband snapper totalled 22.58% of the total flora isolated compared to the 52.83% isolated from saddletail snapper. The lower bacterial load of SSOs present on goldband snapper may be responsible for the more equal spread of spoilage flora isolated immediately post harvest. Gram & Dalgaard (2002) concluded that lower numbers of SSOs allow other organisms to colonise the surface of the skin in greater numbers.

The Gram negative to Gram positive ratio also differed between goldband and saddletail snapper. Goldband snapper had a fairly even ratio of Gram negative and Gram positive organisms immediately post harvest. This changed over time with Gram positive organisms decreasing as Gram negative organisms began to dominate the spoilage flora. Saddletail snapper had low levels of Gram positive organisms throughout storage, contributing less than 20% of the total microbial population. There was a spike 12 h post harvest, however this event was most likely caused by poor storage and handling. The microflora of saddletail snapper was heavily dominated by Gram negative organisms throughout storage, and this may contribute

to its shorter shelf life. Saddletail snapper were caught from approximately 150m below the sea surface. The average sea temperature of the catchment location at 150m was approximately 24°C. It is likely that the lower temperatures from which saddletail snapper were caught influenced the type of microflora which colonised on the skin of the fish. The Gram negative organisms commonly isolated from fish during spoilage are frequently psychrotrophic. Since psychrotrophic bacteria optimally grow between 15°C and 30°C, the environment from which the fish were caught would have been good for the psychrotrophic bacteria to grow and dominate the microflora.

It is still unclear why goldband snapper does not experience the same microflora as saddletail snapper, since they were harvested from similar environments. It appears that the spoilage organisms present on the skin of each fish species may affect the overall microflora present on each fish species. Further investigation into the spoilage organisms was performed to determine if this is in fact the case. Numerous studies have concluded that *Pseudomonas* and *Shewanella* species are the SSOs responsible for the spoilage of fresh fish (Gram & Vogel, 1999; Morita, 1975; Shewan, 1977). These species were isolated from goldband and saddletail snapper in large numbers compared to other bacterial species, especially towards the end of storage. Investigation into the proportions of these species present on goldband and saddletail snapper resulted in a pattern which may explain the difference in shelf life seen with the two fish species. The proportion of SSOs present on saddletail snapper immediately post harvest was over 50% of the total microbial population identified. Goldband snapper did not reach these proportions until day 7 of storage. The delay in the total amount of SSOs present on goldband snapper appears to have had a positive effect on its shelf life. Further analysis of the SSOs showed that as well as having lower levels of SSOs, goldband snapper has also displayed low levels of *Shewanella* species until day 14 of storage. Comparing the SSOs populations, *Shewanella* species was the dominant organism post harvest however, for every time point measured after that *Pseudomonas* species were the dominant SSOs for goldband snapper. The SSOs present on saddletail snapper also had *Shewanella* species as the dominant spoilage organisms immediately post harvest. For the next 3 storage days, *Shewanella* and *Pseudomonas* species maintained an even ratio. After day 3 *Pseudomonas* species were the dominant spoilage organism present. Antagonistic

interactions between *Shewanella* and *Pseudomonas* species may explain the larger *Pseudomonas* population isolated from both fish species. A study performed by Gram and Melchiorsen (1996) showed that *Pseudomonas* species are capable of inhibiting the growth of *Shewanella* species on fish extracts and fish tissue depending on their ability to produce siderophores. It appears that the siderophores produced by *Pseudomonas* species chelate iron from the surrounding environment more efficiently than the siderophores some *Shewanella* species produce. Fluorescent *Pseudomonas* species also possess antimicrobial properties (Uzair, Ahmed, Kousar, & Edwards, 2006; Vachee, Mossel, & Leclerc, 1997). The increased *Pseudomonas* population from day 0 to day 7 of storage reported in the goldband snapper species provides a reason as to why goldband snapper experienced a longer shelf than saddletail snapper.

Future Work

The results from this project provide some insight into why goldband snapper has a longer shelf life than other tropical fish. However, there are many variables which still need to be investigated before these results can be confirmed.

To eliminate some of the variables seen throughout this project, future experiments should use fish from the same fishing company and these fish should be approximately the same size, harvested using the same method of catch and stored and processed together. A laboratory setup closer to where the fish are unloaded would also reduce extrinsic factors experienced throughout processing, such as temperature spikes during transportation and unnecessary handling. Temperature data loggers should also be implemented so that changes in temperature can be monitored automatically and continuously.

Investigation into the microflora of one or two other tropical fish species also needs to be completed. Monitoring the microflora of other species throughout storage will determine if the results from this project were correct in assuming that it was the *Pseudomonas* population that was responsible for goldband snapper having a longer shelf life than saddletail snapper. Monitoring the variety of species from the one catch at the same location would also be valuable to determine if location is the influencing factor rather than a species related factor.

The *Pseudomonas* isolates were identified to a species level. Due to time constraints no further molecular testing was carried out to determine if there were any genetic differences between each of the isolates from within each species subset. This project should be a precursor for further work investigating the antimicrobial properties for each *Pseudomonas* strain, allowing us to determine exactly which *Pseudomonas* strain(s) were responsible for inhibiting *Shewanella* species. This information would enable exploration of antimicrobial treatments which may be applied to various other fish species. Further studies may include examining the application of an antimicrobial treatment to fillets as well as whole fish. If these studies are successful then testing may be performed to determine if these antimicrobial treatments could be manufactured for commercial use. It would also be beneficial to determine the spoilage potential for each *Pseudomonas* strain. The spoilage potential would be measured by the organisms' ability to cause sensory and chemical changes similar to those experienced by spoiling fish. Spoilage potential of the *Pseudomonas* species could be performed using raw fish juice (Lerke, Adams, & Farber, 1963) or sterile muscle blocks (Herbert, Hendrie, Gibson, & Shewan, 1971). Calculating the amount of TMA produced per cell surface or volume will determine the spoilage potential of the organism, allowing us to determine if it plays an important role in spoilage (Gram & Melchiorsen, 1996).

Examination of the innate properties of goldband snapper should also be further investigated. Although the microbiological results concluded that the presence of *Pseudomonas* species in larger numbers delayed spoilage of goldband snapper, results from other tests did not fully support this theory. The texture of Goldband Snapper for example remained soft the entire experiment with little change, the TVB-N also accumulated faster in goldband than saddletail snapper. Investigations into the muscle structure of goldband snapper as well as other intrinsic factors such as the pH, which were not performed due to time constraints, are required. These may provide answers about why the results from previous studies could not be replicated.

Conclusion

This project achieved many outcomes whilst determining the cause for goldband snapper having a longer shelf life which include:

- Successfully validated the QIM for saddletail snapper originally developed and used with saddletail snapper caught off the east coast of Australia.
- Confirmed the shelf life of saddletail snapper to be 24 days when stored at 0°C, which was longer than originally estimated.
- Concluded that textural properties and colour did not clearly indicate spoilage nor did they support any evidence as to why goldband snapper may experience a longer shelf life.
- Concluded that the types of bacterial populations were indicative of the shelf life of each fish species.
- Chemical analysis supported a difference between the spoilage of goldband and saddletail snapper.
- Lower numbers of SSOs present on goldband snapper immediately post harvest allowed a variety of other organisms to colonise the surface of the skin in greater numbers changing the spoilage flora isolated from goldband snapper to be more evenly distributed in comparison to saddletail snapper
- The proportion of Gram positive organisms as a total of the microbial flora was comparatively higher on goldband snapper than saddletail snapper
- There was an increased number of *Pseudomona*s species initially present on goldband snapper in comparison to saddletail snapper, which appeared to have impacted on positively on the shelf life for goldband snapper.

The outcomes delivered were promising and encourage future work to be continued within this area. The microbiological analysis provided the most conclusive data supporting the hypothesis that the types of bacteria present on the skin of the fish does in fact affect the shelf life.

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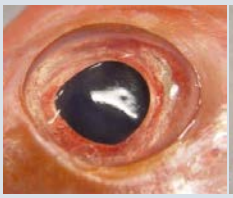
Appendix 1

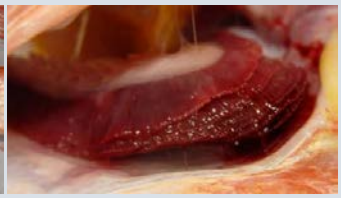
Quality Index scheme for saddletail snapper

			Score	1	2	3	4	5
Skin	Colour / appearance	Iridescent colour (red, pink, silver). External gill flap bright red	0					
		Fading colour around belly. External gill flap pale red	1					
		Colours fading all over. External gill flap patchy white	2					
		Colour mostly gone with possible yellow patches / tinge. External gill flap mostly white with possible yellow tinge	3					
	Tail fin?	Red, pink	0					
		Faded with possible white tips	1					
		Pale with yellow discolouration	2					
	Mucus	None / Natural clear	0					
		Cloudy	1					
	Odour	Fresh sea	0					
		Neutral, slightly stale	1					
		Sour, natural gas	2					
		Sulphur, ammonia, cabbage, off odour	3					
Eyes	Form	Convex / Flat / Bulging	0					
		Completely Sunken	1					
Gills	Colour	Uniform red or pink when cut	0					
		Discoloured / Blotchy	1					
		Brown / Grey colour present	2					
	Mucus	Transparent / Slightly cloudy	0					
		Milky / Opaque	1					
		Brown, bloody	2					
	Odour	Fresh sea	0					
		Not so fresh, stale	1					
		Sour, vegetable, metallic	2					
		Rotten, strong off odour	3					

Attribute photos for saddletail snapper (*Lutjanus malabaricus*)

Day 0



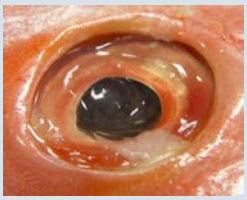


SKIN: Iridescent colour with bright red gill flap. Red to pink tail with clear mucus (if present) and a fresh sea odour

EYES: Flat, convex to bulging

GILLS: Uniform red or pink, transparent to slightly cloudy mucus and fresh sea odour

Day 9

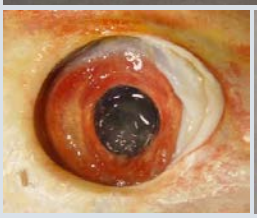


SKIN: Fading colour with gill flap pale red to patchy white. Faded tail with possible white tips, cloudy mucus and a slightly stale to sour odour

EYES: Sunken

GILLS: Discoloured or blotchy with opaque to milky mucus and stale/sour odour

Day 19



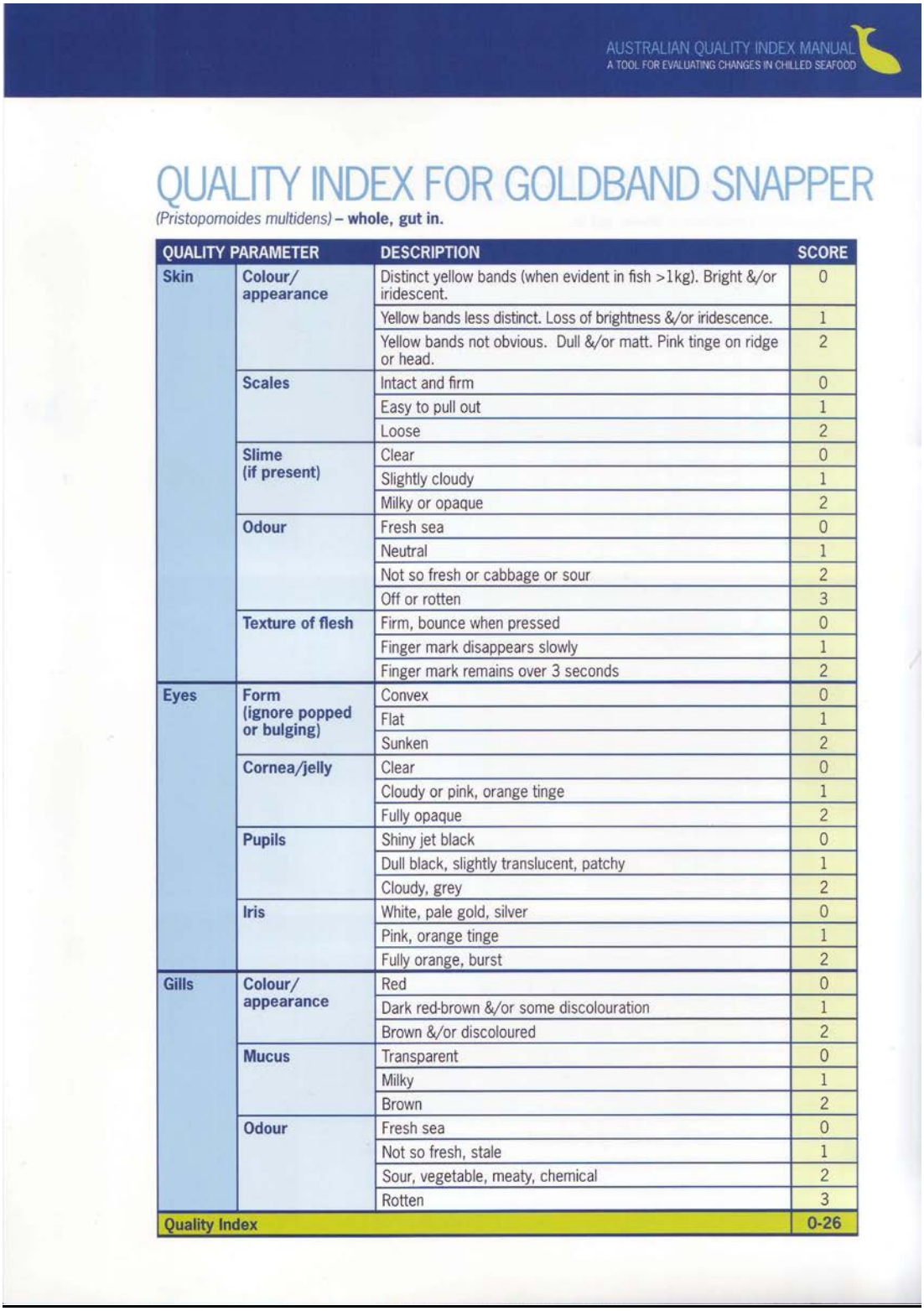
SKIN: Colour mostly gone with possible yellow tinge. Pale tail with yellow discolouration, cloudy mucus and sulphur/ammonia odour

EYES: Sunken

GILLS: Brown or grey present, brown or bloody mucus and rotten odour

Appendix 2

Quality Index for goldband snapper



QUALITY INDEX FOR GOLDBAND SNAPPER

(Pristopomoides multidens) – **whole, gut in.**

QUALITY PARAMETER		DESCRIPTION	SCORE
Skin	**Colour/ appearance**	Distinct yellow bands (when evident in fish >1kg). Bright &/or iridescent.	0
		Yellow bands less distinct. Loss of brightness &/or iridescence.	1
		Yellow bands not obvious. Dull &/or matt. Pink tinge on ridge or head.	2
	Scales	Intact and firm	0
		Easy to pull out	1
		Loose	2
	Slime (if present)	Clear	0
		Slightly cloudy	1
		Milky or opaque	2
	Odour	Fresh sea	0
		Neutral	1
		Not so fresh or cabbage or sour	2
		Off or rotten	3
	Texture of flesh	Firm, bounce when pressed	0
		Finger mark disappears slowly	1
		Finger mark remains over 3 seconds	2
Eyes	**Form (ignore popped or bulging)**	Convex	0
		Flat	1
		Sunken	2
	Cornea/jelly	Clear	0
		Cloudy or pink, orange tinge	1
		Fully opaque	2
	Pupils	Shiny jet black	0
		Dull black, slightly translucent, patchy	1
		Cloudy, grey	2
	Iris	White, pale gold, silver	0
		Pink, orange tinge	1
		Fully orange, burst	2
Gills	**Colour/ appearance**	Red	0
		Dark red-brown &/or some discolouration	1
		Brown &/or discoloured	2
	Mucus	Transparent	0
		Milky	1
		Brown	2
	Odour	Fresh sea	0
		Not so fresh, stale	1
		Sour, vegetable, meaty, chemical	2
		Rotten	3
Quality Index			**0-26**

Appendix 3

Torry Scheme for Freshness Evaluation of Cooked Lean Fish

Torry Scoresheet for Freshness Evaluation of Cooked Lean Fish such as Cod, Haddock and Pollock

Odour	Flavour	Score
Initially weak odour of sweet, boiled milk, starchy, followed by strenghtening of these odours	Watery, metallic, starchy. Initially no sweetness but meaty flavours with slight sweetness may develop	10
Shellfish, seaweed, boiled meat	Sweet, meaty, characteristic	9
Loss of odour, neutral odour	Sweet and characteristic flavours but reduced in intensity	8
Woodshavings, woodsap, vanillin	Neutral	7
Condensed milk, boiled potato	Insipid	6
Milk jug odours, reminiscent of boiled clothes	Slight sourness, trace of "off"-flavours	5
Lactic acid, sour milk, TMA	Slight bitterness, sour, "off"-flavours, TMA	4
Lower fatty acids (eg acetic or butyric acids) decomposed grass, soapy, turnipy, tallowy	Strong bitterness, rubber, slight sulphide	3

Appendix 4

Consent form used for QIM validation sessions

Informed consent

The aim of the study is to develop a Quality Index (QI) scheme for fresh whole, Saddletail Snapper (*Lutjanus malabaricus*) and to validate the scheme using a shelf life study.

Participants will be asked to attend three sessions to validate the established Quality Index Scheme for Saddletail Snapper.

Training will involve becoming familiar with the QI scheme developed by Sue Poole in conjunction with Sydney Fish Market. Participants will be asked to evaluate the appearance of the skin, eyes, gills, odour, colour, texture and any surface slime if present of Saddletail Snapper. Using the Saddletail Snapper QI scheme participants will score each attribute using a scoring system where 0 is equal to fresh to 3 equal to spoiled. The total sum of all scores will give a Quality Index (QI) score.

To validate the QI scheme participants will be asked to assess cooked fish fillets using the Torry scheme. Panellists will be asked to taste fish stored for days to weeks but fit for human consumption. The Torry scheme is a recognized industry standard for sensory evaluation of spoilage in fish and will be used to validate the final QI scheme. Participants will be asked to assess the appearance, odour, texture and flavour of the fillets using a list of sensory attributes to describe the samples

Subjects are required to fill in sensory evaluation forms at each session. The results are of importance to the Australian fishing industry. No personal information will be used for the study and all contact details will remain confidential, and will be stored in a secure environment at Curtin University of Technology.

Individuals will not be directly or indirectly pressured or coerced into participation through unequal power relationships or payments or inducements and are free to pull out of the study if they find it offensive for whatever reason.

I have read and understood this Informed Consent document and conditions of this project. I am of normal health, am not pregnant and **have no food allergies**. As I have had all my questions answered I agree to participate the sensory test held by the school of Public Health, Curtin University of Technology as outlined to me.

Name

Signature Date

Appendix 5

Primers used for 16srRNA sequencing

First-round primers	bp1 (5′-CGATGATCGTTGGCGCTT)
	bp4 (5′-CGTTGTGCCGTATTCCAAT)
First round PCR mix	0.5 units of *Taq* polymerase (Applied Biosystems, Foster City, CA)
	2 µl of buffer
	0.2 mM of pooled deoxynucleoside triphosphates
	1.5 mM of $MgCl_2$
	0.2 µM each of primers bp1 and bp4 (product = 302 bp)
First round cycling program	pre-PCR of 5 min at 94°C
	45 cycles of 30 s at 94°C (denaturation)
	30 s at 55°C (annealing)
	45 s at 72°C (extension)
	72°C for a further 7 min (following final cycle)

Second-round primers	bp1 (5′-ATTAGAGTCGAACAAT)
	bp3 (5′-ATTAGAGTCGAACAAT)
Second round PCR mix	0.5 units of *Taq* polymerase (Applied Biosystems, Foster City, CA)
	2 µl of buffer
	0.2 mM of pooled deoxynucleoside triphosphates
	1.5 mM of $MgCl_2$
	primers bp1 and bp3 (product = 285 bp)
Second round cycling program	pre-PCR of 5 min at 94°C
	45 cycles of 30 s at 94°C (denaturation)
	30 s at 50°C (annealing)
	45 s at 72°C (extension)
	72°C for a further 7 min (following final cycle)

Appendix 6

Reagents used for fatty acid analysis

Saponification Reagent

Ingredient	Amount	Manufacturer	Catalogue No.
Sodium Hydroxide	45 g	Sigma Aldrich	221465
Methanol	150 ml	Sigma Aldrich	34884
Deoinised distilled water	150 ml		

Add NaOH pellets to water and methanol while stirring. Stir until pellets are dissolved.

Methylation Reagent

Ingredient	Amount	Manufacturer	Catalogue No.
6.00N Hydochloric acid	325 ml	Mallinckrod	H 168-05
Methanol	275 ml	Sigma Aldrich	34884

Add acid to methanol while stirring.

Extraction Solvent

Ingredient	Amount	Manufacturer	Catalogue No.
Hexane	200 ml	Merick	1.04371.2500
Methyl-tert Butyl Ether	200 ml	Fisher Scientific	M/4496/17

Add MTBE to hexane and stir.

Base Wash

Ingredient	Amount	Manufacturer	Catalogue No.
Sodium Hydroxide	10.8 g	Sigma Aldrich	221465

Deionised distilled water	900 ml	Fisher Scientific	M/4496/17

Add pellets to water whilst stirring. Stir until pellets are dissolved.

Appendix 7

Potential Spoilage Flora not implicated in the spoilage of fish

Photobacterium species

Photobacterium species are Gram negative rods, which are halophilic and luminescent. *Photobacterium phosphoreum* is an organism commonly found in the gut of many marine fish species. It produces off odours in aerobically stored marine fish (van Spreekens, 1974). It appears to have a major impact on the shelf life of modified atmosphere fish products (Dalgaard, Mejlholm, Christensen, & Huss, 1997). A study performed by Dalgaard et al. (1997) showed that *P. phosphoreum* was found in all marine fish tested and produced strong off odours in all modified atmosphere packed (MAP) fish. In all MAP stored fish *P. phosphoreum* appeared to be an important spoilage organism (Dalgaard, et al., 1997).

Flavobacterium species

Flavobacterium species are a Gram negative rod, commonly recognised by the yellow, orange and red carotenoid pigments which they produce (Levin, 2009). *Flavobacterium* are commonly isolated from fresh and marine water, ocean sediments, food environments and foods, including seafood especially those caught in temperate waters. An early study was performed to investigate the spoilage potential of *Flavobacterium*, examining 245 *Flavobacterium* isolates taken from fish (Castell & Mappleback, 1952). A large percentage of orange pigmented isolates produced off odours in sterile fish tissue after 5-8 days (Castell & Mappleback, 1952). This study demonstrated that *Flavobacterium* have the potential to cause spoilage in fish however, upon refrigeration other psychrotrophic species, such as *Pseudomonas* and *Shewanella* species dominate (Castell & Mappleback, 1952; Garcia- Lopez, Santos, & Otero, 1999; Lobben & Lee, 1968).

Lactobacillus

Lactobacillus species are Gram positive non-spore-forming rods. *Lactobacillus* are readily isolated from seafood products (Castell & Mappleback, 1952) and increase in numbers when oxygen levels are lower,

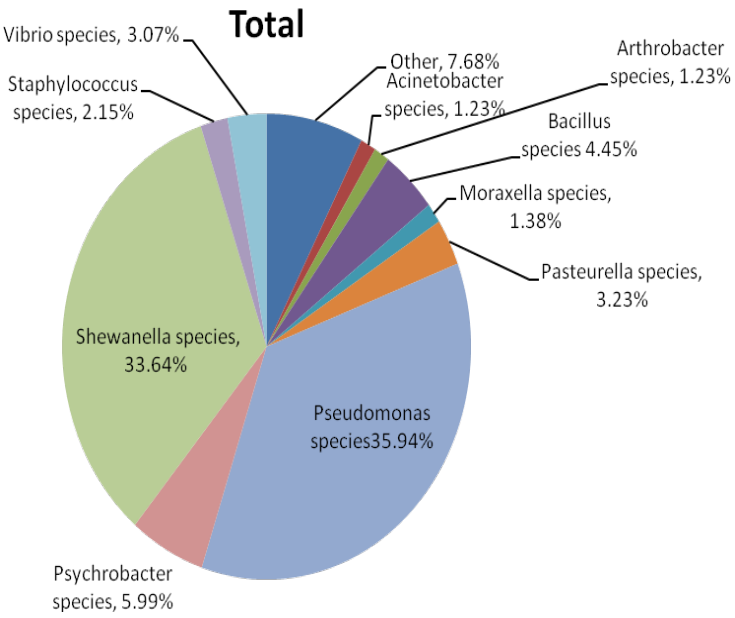
for example, in vacuum packed fish fillets and when food, such as fish, is stored in aerobic iced or refrigerated conditions. *Lactobacillus* constitute only a small percentage of the initial spoilage bacteria, and in a competitive environment cannot compete in conditions that support *Pseudomonas* species (Varnam, 2002). However, in a non-competitive environment *Lactobacillus* is capable of causing spoilage. A study performed by Joffraud et al. (2001) inoculated sterile cold smoked salmon fillets with *Lactobacillus,* which were then vacuum packed and stored in refrigerated conditions. After 40 days of storage, the fillets inoculated with *Lactobacillus* were considered to be grossly spoiled. The odours they gave off were described as "sour", "acid", "pungent" and "hydrogen sulphide" (Joffraud, et al., 2001). These organisms are able to grow faster than other organisms in these conditions because they are unaffected by pH levels and by the production of lactic acid (Nychas & Drosinos, 1999).

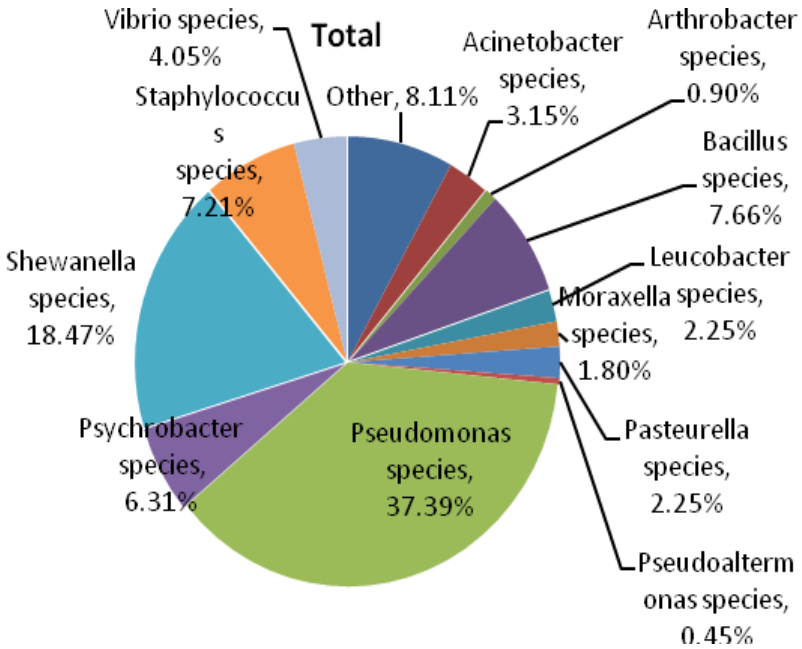
Corynebacterium

Corynebacterium has been found to be dominant in freshly caught fish from warmer waters. Gillespie and Macrae (1975) demonstrated that this dominance persisted throughout most of the handling process. However, *Corynebacterium* does not appear to be a major contributor to spoilage with only some strains from the small proportion found on spoiled fish able to produce off odours. Lerke, Adams & Farber (1965) concluded that some strains were able to produce volatile reducing substances without TMA, and thus were not considered spoilers.

Appendix 8

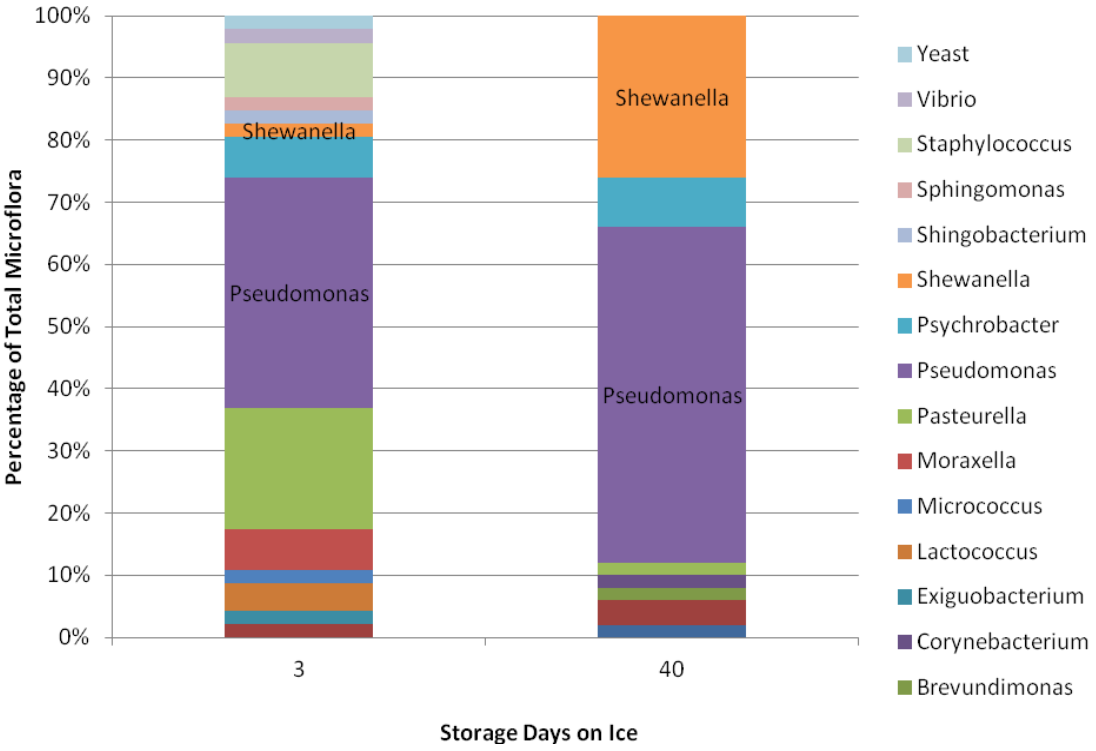
Total microflora found on saddletail snapper (top) and goldband snapper (bottom) harvested from Exmouth

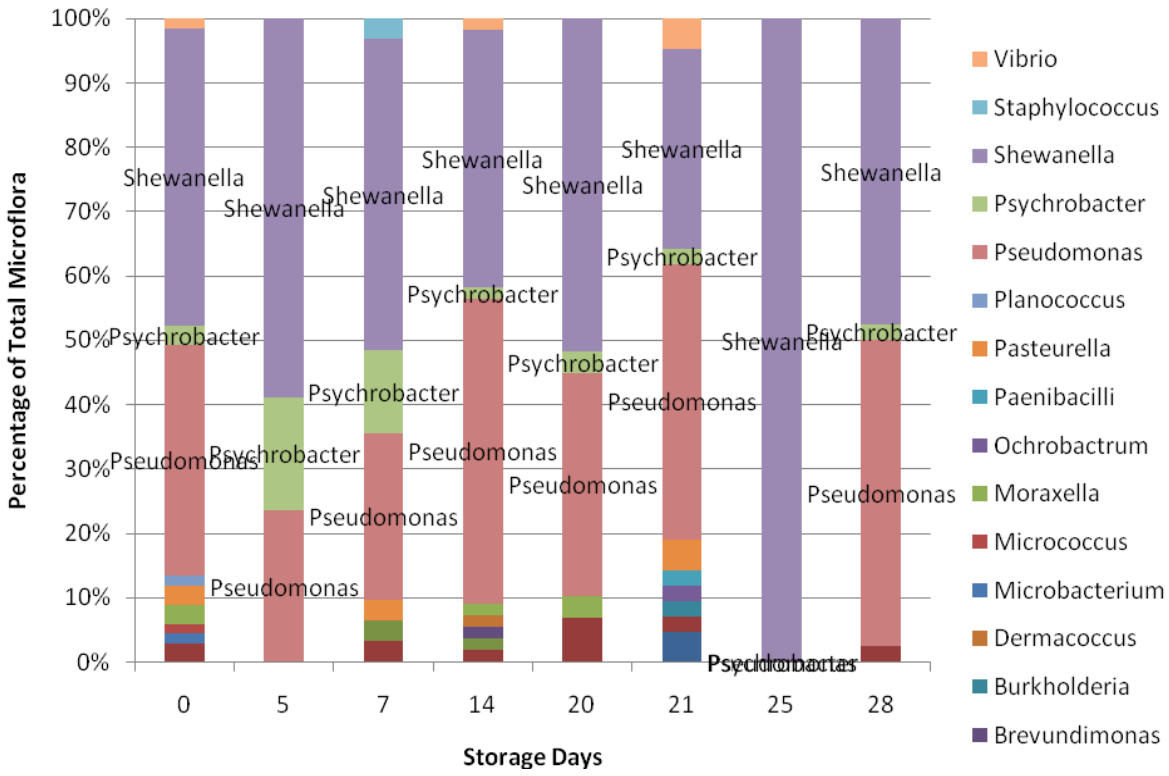




Appendix 9

Percentage of total microflora for saddletail snapper harvested in Broome, WA (top-trap method) and Exmouth, WA (bottom-trawl method)





Appendix 10

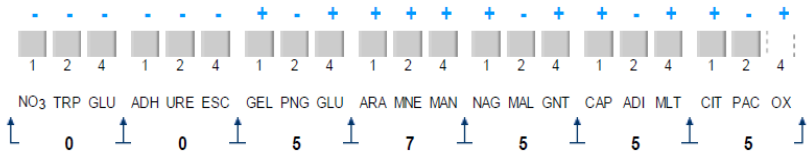
Results sheet for a bacterial colony using an API test

apiweb™ - Identification result https://apiweb.biomerieux.com/servlet/Identify

PathWest - Perth

apiweb

API 20 NE V7.0



REFERENCE	DATE
Q211	12/5/11

COMMENT

VERY GOOD IDENTIFICATION

Strip	API 20 NE V7.0
Profile	0 0 5 7 5 5 5
Note	

Significant taxa	% ID	T	Tests against
Pseudomonas fluorescens	99.7	0.87	ADH 80%

Next taxon	% ID	T	Tests against
Burkholderia cepacia	0.2	0.47	ADIa 93% PACa 99%

Close Print

Appendix 11

Preliminary Identification results for isolates identified from goldband and saddletail snapper

Sample	Gram	Gram notes	Mot	Cat	Ox	O/F	Temp	Media		Mot	Id	Fishy
E179	-	rod	+	+	+	O	30	PCA		52	Achromobacter denitrificans	Saddletail
						No						
E004	-	cocci	-	+	-	charge	30	PCA		2	Acinetobacter calcoaceticus	Goldband
E028	-	rod	-	+	-	O	30				Acinetobacter calcoaceticus	Goldband
						No						
E043	-	cocci	-	+	-	change	30	PCA		1	Acinetobacter calcoaceticus	Goldband
E160	-	rod	-	+	-	O	25		0	1	Acinetobacter calcoaceticus	Saddletail
E228	-	cocci	-	+	+	O	30				Acinetobacter johnsonii	Goldband
E197	-	cocci	-	+	+	O	30	PCA		4	Acinetobacter jonhsonii	Saddletail
E309	-	cocci	-	+	+	O	30	PCA		1	Acinetobacter jonhsonii	Saddletail
E199	-	rod	-	+	-	O	30				Acinetobacter junii	Saddletail
E326	-	rod	-	+	-	O	30				Acinetobacter junii	Goldband
E337	-	rod	-	+	-	O	30				Acinetobacter junii	Saddletail
						No						
E257	-	rod	-	+	+	change	25	LH		4	Acinetobacter species	Saddletail
E334	-	rod	+	+	-	O	30				Acinetobacter species	Saddletail
E469	-	rod	N	+	+	O	25		0	0	Acinetobacter species	Goldband
E515	-	rod	-	+	+	O	25	PCA		13	Acinetobacter species	Goldband
						No						
E402	-	rod	-	+	+	change	25	LH		2	Acinetocbacter species	Saddletail
2420	-	rod	+	+	+	F	30	IA			Aeromonas salicida	Saddletail
Q401	-	rod	+	+	+	F	25	LH		12	Aeromonas species	Saddletail
Q431	-	rod	+	+	+	F	30	LH		12	Aeromonas species	Saddletail
						No						
E641	-	rod	-	+	+	change	30	LH		4	Arenibacter species	Saddletail

E642	-	rod	-	+	+		25			Arenibacter species	Saddletail
E648	-	rod	-	+	+	No change	30			Arenibacter species	Saddletail
E243	-	rod	-	+	+	F	30	LH	2	Arthrobacter agilis	Goldband
E518	-	rod	-	+	+	F	30	PCA	2	Arthrobacter agilis	Goldband
Q134	+	rod	+	+	+	F	25	PCA	5	Arthrobacter agilis	Saddletail
Q135	+	rod	+	+	-	F				Arthrobacter agilis	Saddletail
Q147	+	rod	+	+	-	F				Arthrobacter agilis	Saddletail
Q183	+	rod	-	-	-	F	25	PCA		Arthrobacter agilis	Saddletail
Q310	+	rod	-	+	-	F	30			Arthrobacter agilis	Saddletail
Q325	+	rod	-	+	+	O	25			Arthrobacter bergerei	Saddletail
Q489	+	rod	-	+	+	O	30			Arthrobacter bergerei	Saddletail
Q129	+	rod	+	+	-	F	30	PCA		Arthrobacter globiformis	Saddletail
2193	+	rod	+	+	+	F	30	PCA		Bacillus cereus	Goldband
2187	+	rod	+	+	+	F	30	PCA		Bacillus cereus group	Saddletail
E034	+	rod	+	+	+	F	30			Bacillus cereus group	Goldband
E134	-	rod	+	+	+	O	25	IA	7	Bacillus cereus group	Saddletail
E156	+	rod	+	+	+	F	30			Bacillus cereus group	Saddletail
E169	+	rod	+	+	+	F	30			Bacillus cereus group	Saddletail
E184	+	rod	+	+	+	F	30			Bacillus cereus group	Saddletail
E310	+	rod	+	+	+	F	30			Bacillus cereus group	Saddletail
E312	-	rod	+	+	+	F	30	PCA	5	Bacillus cereus group	Saddletail
E321	+	rod	+	+	-	F	30			Bacillus cereus group	Saddletail
E323	+	rod	+	+	+	F	30			Bacillus cereus group	Saddletail
E502	+	rod	+	+	+	F	0	PCA	18	Bacillus cereus group	Saddletail
E045	+	cocci	-	+	-	F	30	PCA	6	Bacillus luciferensis	Goldband
E046	+	cocci	+	+	-	F	30			Bacillus luciferensis	Goldband

2194	+	rod	+	+	+	F	30	PCA			Bacillus mycoides	Goldband
E166	+	rod	+	+	+	F	30				Bacillus mycoides	Saddletail
E339	+	rod	+	+	+	F	30				Bacillus mycoides	Goldband
E346	+	rod	+	+	+	F	30				Bacillus mycoides	Saddletail
2216	+	rod	+	+	+	F	30	PCA			Bacillus pumilus	Saddletail
E370	+	rod	+	+	+	F	25		0	18	Bacillus pumilus/ Bacillus megatarium	Saddletail
E529	+	rod	+	+	+	F	30				Bacillus pumulis	Saddletail
Q175	+	rod	+	+	+	F	30				Bacillus pumulis	Saddletail
Q215	+	rod	-	+	-	F	25				Bacillus pumulis	Saddletail
E022	+	rod	-	+	-	O	30	PCA		12	Bacillus spaericus	Goldband
E174	+	rod	-	+	+	O	30	PCA		2	Bacillus sphaericus	Saddletail
E314	+	rod	-	+	+	O	30				Bacillus sphaericus	Goldband
2221	+	rod	+	+	+	F	25	PCA			Bacillus species	Saddletail
E006	+	rod	N	+	-	O	30				Bacillus species	Goldband
E015	+	rod	+	+	+	F	30				Bacillus species	Goldband
E017	+	rod	+	+	+	F	30				Bacillus species	Goldband
E027	-	rod	-	+	+	F	30				Bacillus species	Goldband
E041	+	rod	+	+	+	O	30	PCA		61	Bacillus species	Goldband
E064	+	rod	+	+	+	F					Bacillus species	Goldband
E065	+	rod	+	+	-	F					Bacillus species	Goldband
E066	+	rod	+	+	-	F					Bacillus species	Goldband
E093	+	rod	+	+	+	F	25				Bacillus species	Saddletail
E158	-	rod	+	+	+	O	25	PCA		15	Bacillus species	Saddletail
E167	+	rod	+	+	-	F	30				Bacillus species	Saddletail
E255	+	rod	+	+	-	F	25				Bacillus species	Goldband
E280	+	rod	+	+	+	O	30				Bacillus species	Saddletail
E293	+	rod	+	+	-	F	25				Bacillus species	Saddletail

E294	+	rod	+	+	-	F	30				Bacillus species	Saddletail
E376	+	rod	+	+	-	F	25				Bacillus species	Saddletail
E411	+	rod	+	+	+	O	30				Bacillus species	Saddletail
E596	+	rod	-	+	-	F	30				Bacillus species	Saddletail
E600	-	rod	+	+	+	O	30	PCA		40	Bacillus species	Saddletail
E183	+	rod	-	+	-	No change	30	PCA		16	Brevibacillus laterosporous	Saddletail
E573	-	rod	N	+	+	F	25				Brevundimonas diminuta	Goldband
E012	-	rod	+	+	+	O	30	PCA		63	Brevundimonas nasdae	Goldband
E032	-	rod	N	+	+	F	25				Brevundimonas nasdae	Goldband
E044	+	rod	-	+	-	No change	30	PCA		2	Brevundimonas nasdae	Goldband
Q259	-	rod	+	+	+	F					Brevundimonas psychrophila	Saddletail
2241	+	cocci	+	+	-	F	25	LH			Brevundimonas species	Saddletail
E585	+	cocci	+	+	-	F	25				Brevundimonas vasicularis	Goldband
E035	-	rod	+	+	+	O	30	PCA		65	Brevundimonas vesicularis	Goldband
E215	-	rod	-	+	+	No change	25	LH		10	Brevundimonas vesicularis	Goldband
E152	+	rod	-	+	-	F	30		0	0	Burkholderia cepacia / Pseudomonas cepacia	Saddletail
E192	+	rod	-	+	-	F	30				Burkholdia cepacia	Saddletail
Q293	-	rod	+	+	-	O	25				Burkholdia cepacia	Saddletail
E159	-	rod	-	+	+	O	30	PCA		14	Chryseobacterium jejuense	Saddletail
E010	-	rod	-	+	+	O	30	PCA		14	Chryseobacterium species	Goldband
E407	-	rod	-	+	+	O	30	LH		8	Chryseobacterium species	Saddletail
E164	-	rod	-	+	+	No change	30	PCA		14	Chryseobacterium taichungense	Saddletail
2464	+	rod	-	+	-	O	30	PCA			Corynebacterium propinquum	Saddletail

Q117	+	rod	DNG	+	-	F	25			Dermacoccus nishinomiydensis	Saddletail
E344	-	rod	+	+	+	O	30			Dermacoccus nishinomyaensis	Saddletail
E062	+	rod	+	+	+	F				Exiguobacterium aestuarii	Goldband
2281	+	rod	+	+	+	F	30	PCA		Exiguobacterium indicum	Saddletail
E201	+	rod	+	+	+	F	25			Exiguobacterium oxidotolerans	Goldband
E653	+	cocci	-	+	-	F	30	LH	14	Exiguobacterium profundum	Saddletail
E005	+	rod	+	+	-	No charge	30			Exiguobacterium species	Goldband
E055	+	rod	+	+	+	F				Exiguobacterium species	Goldband
E191	+	cocci	-	+	-	F	30	PCA	10	Kocuria palustris	Saddletail
E308	+	cocci	-	+	-	F	30	PCA	11	Kocuria rosea	Saddletail
E347	+	cocci	-	+	-	F	30	PCA	12	Kocuria varians	Saddletail
E218	+	rod	+	+	+	F	30			Kocuria- varians	Goldband
E635	-	rod	-	+	+	O	30	LH	12	Kocuria- varians	Saddletail
2195	+	cocci	-	-	-	+	30	PCA		Lactococcus garvieae	Saddletail
2196	+	cocci	-	-	-	+	30	PCA		Lactococcus garvieae	Saddletail
E233	+	cocci	-	+	+	F	30			Leucobacter alluvii	Goldband
E303	+	rod	+	+	-	O	25			Leucobacter alluvii	Goldband
E315	+	rod	+	+	-	O	30			Leucobacter alluvii	Goldband
E317	+	rod	+	+	-	O	25			Leucobacter alluvii	Goldband
E324	+	rod	+	+	-	O	30			Leucobacter alluvii	Goldband
E650	-	cocci	-	+	+	O	30	LH	7	Macrococcus caseolyticus	Saddletail
E532	+	rod	-	+	+	No change	25			Methylobacterium mesophilicum	Saddletail
E652	-	cocci	-	+	+	O	30			Methylobacterium mesophilicum	Saddletail
E659	-	cocci	-	+	+	O	30			Methylobacterium mesophilicum	Saddletail
E661	-	cocci	-	+	+	O	30	LH	1	Methylobacterium mesophilicum	Saddletail

Q487	+	rod	+	+	-	O	30			Microbacterium maritypicun	Saddletail
2186	+	cocci	-	+	+	O	30	PCA		Micrococcus luteus	Saddletail
E177	+	cocci	-	+	+	O	30	PCA	3	Micrococcus luteus	Saddletail
E455	+	cocci	-	+	-	O	30			Micrococcus luteus	Goldband
Q009	+	cocci/rod	-	+	+	No change	25			Micrococcus luteus	Saddletail
2212	+	cocci	-	+	-	O	30	PCA		Micrococcus lylae	Goldband
E425	+	rod	-	+	+	O	25			Moraxella catarrhalis	Goldband
Q180	+	cocci	-	+	+	O	25	PCA	13	Moraxella catarrhalis	Saddletail
2208	+	rod	-	+	+	F	25	LH		Moraxella lacunata	Goldband
2166	-	rod	-	+	+	O	30	PCA		Moraxella species	Saddletail
2174	-	rod	-	+	+	O	30	PCA		Moraxella species	Saddletail
2175	-	rod	-	+	+	O	30	PCA		Moraxella species	Saddletail
E061	+	rod	+	+	+	F	0	PCA	6	Moraxella species	Goldband
E413	+	rod	-	+	+	No change	25			Moraxella species	Saddletail
E417	+	rod	-	+	+	F	30			Moraxella species	Saddletail
E460	+	rod	-	+	+	O	25			Moraxella species	Goldband
Q108	+	rod	-	+	+	O	25			Moraxella species	Saddletail
Q116	-	rod	-	+	+	O	25	PCA	25	Moraxella species	Saddletail
Q136	-	rod	+	+	+	O	15			Moraxella species	Saddletail
Q433	+	cocci	-	+	+	O	25	LH	7	Ochrobactrum anthropi	Saddletail
E281	+	rod	+	+	+	F	30	LH	15	Oeskovia turbata	Saddletail
E172	+	rod	+	+	+	F	30	PCA	15	Paenibacilli lentimorbus	Saddletail
E282	+	rod	+	+	+	F	30			Paenibacilli lentimorbus	Saddletail
E237	+	rod	+	+	+	F	30			Paenibacilli species	Goldband
E658	+	cocci	-	+	+	O	25	LH	7	Paenibacillius lentimorbus	Saddletail

Q515	+	rod	-	+	-	F	15	LH		Paenibacillus polymyxa	Saddletail
2178	+	cocci	-	+	+	No change	25	IA		Pasteurella species	Saddletail
2197	+	cocci	-	+	+	No change	25	LH		Pasteurella species	Saddletail
2198	-	cocci	-	+	+	O	25	LH		Pasteurella species	Goldband
2200	+	cocci	-	+	+	No change	15	LH		Pasteurella species	Saddletail
2201	+	cocci	-	+	+	No change	25	LH		Pasteurella species	Saddletail
2202	+	cocci	-	+	+	No change	25	LH		Pasteurella species	Saddletail
2204	+	cocci	-	+	+	No change	25	LH		Pasteurella species	Saddletail
2207	-	cocci	-	+	+	O	30	LH		Pasteurella species	Goldband
2240	+	cocci	-	+	+	No change	25	LH		Pasteurella species	Saddletail
2344	+	cocci	-	+	+	No change	30	LH		Pasteurella species	Saddletail
2349	+	cocci	-	+	+	No change	25	LH		Pasteurella species	Saddletail
E023	-	rod	-	+	+	O	30	PCA	7	Pasteurella species	Goldband
E120	-	rod	+	+	+	O	25	IA	2	Pasteurella species	Saddletail
E240	-	cocci	-	+	+	O	25	LH	1	Pasteurella species	Goldband
E258	-	rod	-	+	+	O	25	LH	2	Pasteurella species	Saddletail
E262	+	cocci	-	+	+	O	30	LH	7	Pasteurella species	Saddletail
E265	-	cocci	-	+	+	O	25	LH	1	Pasteurella species	Saddletail
E631	+	rod/ cocci		+	+	O	25			Pasteurella species	Saddletail
E643	-	cocci/rod	-	+	+	O	30			Pasteurella species	Saddletail

Q014	+	cocci	-	+	+	O	30		3	Pasteurella species	Saddletail
Q302	-	rod	-	+	+	O	25	PCA	17	Pasteurella species	Saddletail
Q304	-	rod	-	+	+	O	25	PCA	17	Pasteurella species	Saddletail
Q409	+	cocci	-	+	+	No change	25			Pasteurella species	Saddletail
Q444	+	cocci	-	+	+	No change	25			Pasteurella species	Saddletail
E304	-	rod	+	+	-	F	30	PCA	2	Pasteurella species	Goldband
2389	+	cocci	-	+	+	No change	25	LH		Pasteurella species/ Moraxella lacunata	Saddletail
Q318	+	cocci	-	+	-	O	30			Planococcus donghaensis	Saddletail
E594	-	rod	+	+	+	No change	25	PCA	14	Pseudoalteromonas nigfifaciens	Goldband
2249	-	rod	+	+	+	O	25	LH		Pseudomonas alcaligenes	Goldband
2271	-	rod	+	+	+	O	25	LH		Pseudomonas alcaligenes	Goldband
E060	-	rod	+	+	+	O	30	PCA	50	Pseudomonas chloraphis	Goldband
E602	-	rod	+	+	+	No change	30	PCA	14	Pseudomonas chloraphis	Goldband
E533	+	rod	-	+	+	No change	25			Pseudomonas chloritidismutans	Saddletail
2173	-	rod	+	+	+	O	30	PCA		Pseudomonas fluorescens	Saddletail
2181	-	rod	+	+	+	O	30	IA		Pseudomonas fluorescens	Saddletail
2182	-	rod	+	+	+	O	30	IA		Pseudomonas fluorescens	Saddletail
2282	-	rod	+	+	+	O	30	PCA		Pseudomonas fluorescens	Saddletail
2287	-	rod	+	+	+	O	30	PCA		Pseudomonas fluorescens	Saddletail
2298	-	rod	+	+	+	O	30	PCA		Pseudomonas fluorescens	Saddletail
2431	-	rod	+	+	+	O	30	PCA		Pseudomonas fluorescens	Saddletail
E163	-	rod	+	+	+	O	30	PCA	48	Pseudomonas fluorescens	Saddletail

E170	-	rod		+	+	+	O		30			Pseudomonas fluorescens			Saddletail
E301	-	rod		+	+	+	O		30	PCA	48	Pseudomonas fluorescens			Saddletail
E319	-	rod		-	+	+	O		30	PCA	6	Pseudomonas fluorescens			Saddletail
E320	-	rod		+	+	+	O		30	PCA	48	Pseudomonas fluorescens			Saddletail
E333	-	rod		+	+	+	O		30			Pseudomonas fluorescens			Saddletail
E405	-	rod		+	+	+	O		25	LH	59	Pseudomonas fluorescens			Goldband
E465	-	rod		+	+	+	O		25	LH	38	Pseudomonas fluorescens			Goldband
E503	-	rod		+	+	+	O		30	PCA	33	Pseudomonas fluorescens			Goldband
E506	-	rod		+	+	+	O		30			Pseudomonas fluorescens			Saddletail
E507	-	rod		+	+	+	O		30	PCA	33	Pseudomonas fluorescens			Saddletail
E509	-	rod		+	+	+	O		30	PCA	44	Pseudomonas fluorescens			Saddletail
E510	-	rod		+	+	+	O		30	PCA	33	Pseudomonas fluorescens			Saddletail
E519	-	rod		+	+	+	O		30	PCA	47	Pseudomonas fluorescens			Goldband
E520	-	rod		+	+	+	O		30	PCA	48	Pseudomonas fluorescens			Saddletail
E587	-	rod		+	+	+	O		30			Pseudomonas fluorescens			Saddletail
E588	-	rod		+	+	+	O		30	PCA	44	Pseudomonas fluorescens			Saddletail
E589	-	rod		+	+	+	O		30	PCA	33	Pseudomonas fluorescens			Goldband
E592	-	rod		+	+	+	O		30	PCA	31	Pseudomonas fluorescens			Goldband
E599	-	rod		+	+	+	O		30	PCA	33	Pseudomonas fluorescens			Saddletail
E607	-	rod		+	+	+	O		30	PCA	48	Pseudomonas fluorescens			Saddletail
Q008	-	cocci/rod		+	+	+	O		25		20	Pseudomonas fluorescens			Saddletail
Q025	-	rod		-	+	+	O		25	PCA	27	Pseudomonas fluorescens			Saddletail
Q027	-	rod		+	+	+	O		30			Pseudomonas fluorescens			Saddletail
Q101	-	rod		+	+	+	O		25	PCA	9	Pseudomonas fluorescens			Saddletail
Q121	-	rod		+	+	+	N		30	PCA	31	Pseudomonas fluorescens			Saddletail
Q181	-	rod		+	+	+	O		30			Pseudomonas fluorescens			Saddletail
Q186	-	rod		-	+	+	O		25	PCA	18	Pseudomonas fluorescens			Saddletail

Q187	-	rod	+	+	+	No change	25	PCA	20	Pseudomonas fluorescens	Saddletail
Q190	-	rod	+	+	+	O	25	PCA	41	Pseudomonas fluorescens	Saddletail
Q191	-	rod	+	+	+	O	30	PCA	48	Pseudomonas fluorescens	Saddletail
Q200	-	rod	+	+	+	O	25	PCA	41	Pseudomonas fluorescens	Saddletail
Q211	-	rod	+	+	+	O	25	PCA	33	Pseudomonas fluorescens	Saddletail
Q212	-	rod	+	+	+	O	25	PCA	33	Pseudomonas fluorescens	Saddletail
Q214	-	rod	+	+	+	O	25	PCA	34	Pseudomonas fluorescens	Saddletail
Q313	-	rod	+	+	+	No change	25	PCA	36	Pseudomonas fluorescens	Saddletail
Q316	-	rod	+	+	+	No change	25	PCA	36	Pseudomonas fluorescens	Saddletail
Q402	-	rod	+	+	+	No change	15	LH	14	Pseudomonas fluorescens	Saddletail
Q491	-	rod	+	+	+	No change	30			Pseudomonas fluorescens	Saddletail
E252	-	rod	+	+	+	O	25	LH	1	pseudomonas fluroescens	Goldband
2250	-	rod	+	+	+	O	30	LH		Pseudomonas fragi	Goldband
2469	-	rod	+	+	+	O	25	LH		Pseudomonas grimonti	Saddletail
E266	-	rod	+	+	+	No change	25	LH	7	Pseudomonas grimontii	Saddletail
E342	-	rod	-	+	+	O	30	PCA	1	Pseudomonas grimontii	Saddletail
E345	-	rod	-	+	+	O	25	PCA	1	Pseudomonas grimontii	Saddletail
E371	-	rod	-	+	+	O	15	LH	1	Pseudomonas grimontii	Saddletail
E419	-	rod	-	+	+	O	25	LH	1	Pseudomonas grimontii	Goldband
E445	-	rod	-	+	+	O	25	PCA	1	Pseudomonas grimontii	Goldband
E511	-	rod	-	+	+	O	30	PCA	1	Pseudomonas grimontii	Saddletail
E512	-	rod	+	+	+	O	30	PCA	1	Pseudomonas grimontii	Saddletail

E535	-	rod	-	+	+	O	25	PCA		1	Pseudomonas grimontii	Saddletail
E590	-	rod	-	+	+	O	30	PCA		1	Pseudomonas grimontii	Goldband
E601	-	rod	-	+	+	O	30	PCA		1	Pseudomonas grimontii	Goldband
E604	-	rod	-	+	+	O	30	PCA		1	Pseudomonas grimontii	Saddletail
E608	-	rod	-	+	+	O	30	PCA		1	Pseudomonas grimontii	Saddletail
E664	-	rod / cocci	-	+	+	O	25	LH		1	Pseudomonas grimontii	Saddletail
Q056	-	rod	-	+	+	O	25	LH		1	Pseudomonas grimontii	Saddletail
Q062	-	rod	-	+	+	O	25	LH		1	Pseudomonas grimontii	Saddletail
Q111	-	rod	-	+	+	N	25	PCA		1	Pseudomonas grimontii	Saddletail
Q124	-	rod	+	+	+	N	30	PCA		1	Pseudomonas grimontii	Saddletail
Q172	-	rod	-	+	+	O	15	LH		1	Pseudomonas grimontii	Saddletail
Q242	-	rod	-	+	+	O	25	LH		1	Pseudomonas grimontii	Saddletail
Q255	-	rod	-	+	+	No change	25	LH		1	Pseudomonas grimontii	Saddletail
Q281	-	rod	-	+	+	No change	15	PCA		1	Pseudomonas grimontii	Saddletail
Q333	-	rod	-	+	+	O	25	PCA		1	Pseudomonas grimontii	Saddletail
Q371	-	rod	-	+	+	No change	25	IA		1	Pseudomonas grimontii	Saddletail
Q457	-	rod	+	+	+	No change	30	LH		1	Pseudomonas grimontii	Saddletail
E188	-	rod	+	+	+	O	25		0	36	Pseudomonas nigrifaciens	Saddletail
E632	-	rod	-	+	+	F	25	LH		5	Pseudomonas nigrifaciens	Saddletail
Q463	-	rod	+	+	+	F	25	LH		10	Pseudomonas psychrophila	Saddletail
2168	-	rod	+	+	+	O	30	PCA			Pseudomonas putida	Saddletail
2179	-	rod	+	+	+	O	30	IA			Pseudomonas putida	Goldband
2211	-	rod	+	+	+	O	30	PCA			Pseudomonas putida	Goldband
2218	-	rod	+	+	+	O	30	PCA			Pseudomonas putida	Saddletail

2219	-	rod	+	+	+	O	30	PCA		Pseudomonas putida	Saddletail
2223	-	rod	+	+	+	O	30	PCA		Pseudomonas putida	Saddletail
2224	-	rod	+	+	+	O	25	PCA		Pseudomonas putida	Saddletail
2229	-	rod	+	+	+	O	30	IA		Pseudomonas putida	Goldband
2230	-	rod	+	+	+	O	30	IA		Pseudomonas putida	Goldband
2233	-	rod	+	+	+	O	30	IA		Pseudomonas putida	Saddletail
2237	-	rod	+	+	+	O	30	IA		Pseudomonas putida	Saddletail
2238	-	rod	+	+	+	O	25	LH		Pseudomonas putida	Saddletail
2244	-	rod	+	+	+	O	25	LH		Pseudomonas putida	Saddletail
2245	-	rod	+	+	+	O	25	LH		Pseudomonas putida	Goldband
2246	-	rod	+	+	+	O	30	LH		Pseudomonas putida	Goldband
2432	-	rod	+	+	+	O	30	PCA		Pseudomonas putida	Saddletail
E013	-	rod	+	+	+	O	30	PCA	64	Pseudomonas putida	Goldband
E038	-	rod	+	+	+	No change	30	PCA	1	Pseudomonas putida	Goldband
E057	-	rod	+	+	+	O				Pseudomonas putida	Goldband
E068	-	rod	+	+	+	O	30	PCA	35	Pseudomonas putida	Goldband
E111	-	rod	+	+	+	No change	25	IA	1	Pseudomonas putida	Goldband
E187	-	rod	+	+	+	O	30	PCA	56	Pseudomonas putida	Saddletail
E212	-	rod	+	+	+	O	25			Pseudomonas putida	Goldband
E250	-	cocci	+	+	+	O	25			Pseudomonas putida	Goldband
E251	-	cocci	+	+	+	O	25			Pseudomonas putida	Goldband
E357	-	rod	+	+	+	O	25	LH	18	Pseudomonas putida	Goldband
E363	-	rod	+	+	+	O	25	LH	26	Pseudomonas putida	Saddletail
E367	-	rod	+	+	+	O	25	LH	18	Pseudomonas putida	Saddletail
E380	-	rod		+	+	O	25			Pseudomonas putida	Saddletail

E393	-	rod	+	+	+	O	25			Pseudomonas putida	Saddletail
E463	-	rod	+	+	+	O	30			Pseudomonas putida	Saddletail
E476	-	rod	+	+	+	O	30	LH	20	Pseudomonas putida	Saddletail
E477	-	rod	+	+	+	O	25	LH	18	Pseudomonas putida	Saddletail
E482	-	rod	+	+	+	O	25	LH	18	Pseudomonas putida	Goldband
E488	-	rod	-	+	+	No change	30	LH	1	Pseudomonas putida	Goldband
E490	-	rod	+	+	+	O	25	LH	36	Pseudomonas putida	Goldband
E496	-	rod	+	+	+	O	25	LH	20	Pseudomonas putida	Goldband
E500	-	rod		+	+	O	30			Pseudomonas putida	Saddletail
E516	-	cocci	-	+	+	O	25	PCA	6	Pseudomonas putida	Goldband
E517	-	cocci	+	+	+	O	30	PCA	6	Pseudomonas putida	Goldband
E562	-	rod	+	+	+	O	25	LH	38	Pseudomonas putida	Saddletail
E597	-	rod	+	+	+	O	25	PCA	45	Pseudomonas putida	Saddletail
E618	-	rod	+	+	+	No change	25	IA	1	Pseudomonas putida	Saddletail
E649	-	rod	+	+	+	O	30	LH	18	Pseudomonas putida	Saddletail
E669	-	rod	+	+	+	O	25	LH	2	Pseudomonas putida	Saddletail
Q015	-	rod	+	+	+	No change	25	PCA	10	Pseudomonas putida	Saddletail
Q047	-	rod	+	+	+	No change	25	LH	1	Pseudomonas putida	Saddletail
Q103	-	rod	+	+	+	N	15	PCA	11	Pseudomonas putida	Saddletail
Q104	-	rod	+	+	+	O	15	PCA	11	Pseudomonas putida	Saddletail
Q115	-	rod	+	+	+	N	30	PCA	12	Pseudomonas putida	Saddletail
Q145	-	rod	+	+	+	O				Pseudomonas putida	Saddletail
Q159	-	rod	+	+	+	O	30			Pseudomonas putida	Saddletail
Q216	-	rod	+	+	+	O	25	PCA	35	Pseudomonas putida	Saddletail

Q247	-	rod	+	+	+	O	25	LH	38	Pseudomonas putida	Saddletail
Q254	-	rod	+	+	+	O	25	LH	36	Pseudomonas putida	Saddletail
Q291	-	rod	+	+	+	O	25			Pseudomonas putida	Saddletail
Q311	-	rod	+	+	+	O	25	PCA	64	Pseudomonas putida	Saddletail
Q312	-	rod	+	+	+	O	25	PCA	64	Pseudomonas putida	Saddletail
Q317	-	rod	+	+	+	O	25	PCA	12	Pseudomonas putida	Saddletail
Q337	-	rod	+	+	+	O	25			Pseudomonas putida	Saddletail
Q390	-	rod	+	+	+	O	15			Pseudomonas putida	Saddletail
Q419	-	rod	+	+	+	No change	15	LH	34	Pseudomonas putida	Saddletail
Q459	-	rod	+	+	+	No change	25	LH	1	Pseudomonas putida	Saddletail
Q469	-	rod	+	+	+	O	25	LH	35	Pseudomonas putida	Saddletail
Q514	-	rod	+	+	+	O	15	IA	1	Pseudomonas putida	Saddletail
E444	-	rod	+	+	+	No change	30	PCA	13	Pseudomonas putida/ fluorescens	Goldband
E073	-	rod	+	+	+	O	25	IA	42	Pseudomonas sp.	Saddletail
Q001	-	rod	+	+	+	O	30		6	Pseudomonas sp.	Saddletail
2170	+	coccobacilli	+	+	+	F	30	PCA		Pseudomonas species	Saddletail
2176	-	rods	+	+	+	O	5	IA		Pseudomonas species	Saddletail
2177	-	rods	+	+	+	O	25	IA		Pseudomonas species	Saddletail
2180	-	rod	+	+	+	O	30	IA		Pseudomonas species	Goldband
2183	-	rod	+	+	+	O	30	IA		Pseudomonas species	Saddletail
2185	-	rod	+	+	+	O	30	IA		Pseudomonas species	Saddletail
2189	-	rod	+	+	+	O	25	IA		Pseudomonas species	Goldband
2190	-	rod	+	+	+	O	25	IA		Pseudomonas species	Saddletail
2199	-	rod	+	+	+	O	25	LH		Pseudomonas species	Saddletail
2210	-	rod	+	+	+	O	25	PCA		Pseudomonas species	Goldband

2215	-	rod	+	+	+	O		30	PCA		Pseudomonas species			Goldband
2220	-	rod	-	-	+			30	PCA		Pseudomonas species			Saddletail
2222	-	rod	+	+	+	O		30	PCA		Pseudomonas species			Saddletail
2225	-	rod	+	+	+	O		30	PCA		Pseudomonas species			Saddletail
2226	-	rod	+	+	+	O		30	IA		Pseudomonas species			Goldband
2227	-	rod	+	+	+	O		30	IA		Pseudomonas species			Goldband
2228	-	rod	+	+	+	O		25	IA		Pseudomonas species			Goldband
2231	-	rod	+	+	+	O		30	IA		Pseudomonas species			Saddletail
2232	-	rod	+	+	+	O		30	IA		Pseudomonas species			Saddletail
2234	-	rod	+	+	+	O		30	IA		Pseudomonas species			Saddletail
2235	-	rod	+	+	+	O		30	IA		Pseudomonas species			Saddletail
2236	-	rod	+	+	+	O		30	IA		Pseudomonas species			Saddletail
2239	-	rod	+	+	+	O		25	LH		Pseudomonas species			Saddletail
2251	-	rod	+	+	+	O		25	LH		Pseudomonas species			Goldband
2286	-	rod	+	+	+	O					Pseudomonas species			Saddletail
2345	-	rod	+	+	+	O		25	LH		Pseudomonas species			Saddletail
2346	-	rod	+	+	+	O		25	LH		Pseudomonas species			Saddletail
2429	-	rod	+	+	+	O		30	PCA		Pseudomonas species			Saddletail
2433	-	rod	+	+	+	O			PCA		Pseudomonas species			Saddletail
2434	-	rod	+	+	+	O		30	PCA		Pseudomonas species			Saddletail
2436	-	rod	+	+	+	O		30	PCA		Pseudomonas species			Saddletail
2471	-	rod	+	+	+	O		25	LH		Pseudomonas species			Saddletail
2529	-	rod	+	+	+	O		25	LH		Pseudomonas species			Saddletail
						No								
2530	-	rod	+	+	+	change		25	LH		Pseudomonas species			Saddletail
E014	-	rod	+	+	+	O		30	PCA		Pseudomonas species	41		Goldband
E037	-	rod	+	+	+	O		30	PCA		Pseudomonas species	9		Goldband

E040	-	rod	+	+	+	O	30	PCA	51	Pseudomonas species			Goldband
E051	-	rod	-	+	+	O				Pseudomonas species			Goldband
E110	-	rod	+	+	+	O	25			Pseudomonas species			Goldband
E196	-	rod	+	+	+	O	30	PCA	5	Pseudomonas species			Saddletail
E318	-	rod	+	+	+	O	30	PCA	31	Pseudomonas species			Saddletail
E322	-	rod	+	+	+	O	30	PCA	44	Pseudomonas species			Saddletail
E330	-	rod	+	+	+	O	30	PCA	9	Pseudomonas species			Goldband
E340	-	rod	+	+	+	O	30			Pseudomonas species			Goldband
E341	-	rod	+	+	+	O	30	PCA	9	Pseudomonas species			Goldband
E355	+	rod	+	+	+	O	25			Pseudomonas species			Goldband
E446	-	rod	+	+	+	O	30	PCA	61	Pseudomonas species			Goldband
E447	-	rod	+	+	+	O	30	PCA	9	Pseudomonas species			Saddletail
E448	-	rod	+	+	+	O	30	PCA	9	Pseudomonas species			Saddletail
E449	-	rod	+	+	+	O	30	PCA	9	Pseudomonas species			Saddletail
E504	-	rod	+	+	+	O	30	PCA	31	Pseudomonas species			Goldband
E508	-	rod	+	+	+	O	30	PCA	46	Pseudomonas species			Saddletail
E530	-	rod	+	+	+	O	30	PCA	42	Pseudomonas species			Saddletail
E534	-	rod	+	+	+	O	30	PCA	9	Pseudomonas species			Saddletail
E537	-	rod	+	+	+	O	30	PCA	9	Pseudomonas species			Saddletail
E539	-	rod	+	+	+	O	30	PCA	42	Pseudomonas species			Saddletail
E549	-	rod	+	+	+	O	25			Pseudomonas species			Saddletail
E554	-	rod	+	+	+	O	30			Pseudomonas species			Goldband
E559	-	rod	+	+	+	O	25	LH	6	Pseudomonas species			Saddletail
E567	-	rod	+	+	+	O	25	LH	6	Pseudomonas species			Saddletail
E572	-	rod	+	+	+	O	25	LH	6	Pseudomonas species			Goldband
E581	-	rod	+	+	+	O	25	LH	6	Pseudomonas species			Saddletail
E583	-	rod	+	+	+	O	30	LH	6	Pseudomonas species			Goldband

E584	-	rod	+	+	+	O		30	LH	6	Pseudomonas species		Goldband
E591	-	rod	+	+	+	O		30	PCA	40	Pseudomonas species		Goldband
E595	-	rod	+	+	+	O		30	PCA	44	Pseudomonas species		Saddletail
E598	-	rod	+	+	+	O		30	PCA	31	Pseudomonas species		Saddletail
E603	-	rod	+	+	+	O		30			Pseudomonas species		Saddletail
E609	-	rod	+	+	+	O		30	PCA	9	Pseudomonas species		Saddletail
Q010	-	rod	+	+	+	O		30	PCA	14	Pseudomonas species		Saddletail
Q013	-	rod	+	+	+	O		30	PCA	14	Pseudomonas species		Saddletail
Q024	-	rod	+	+	+	O		30	PCA	51	Pseudomonas species		Saddletail
Q112	-	rod	+	+	+	O		30	PCA	8	Pseudomonas species		Saddletail
Q122	-	rod	+	+	+	O		25	PCA	6	Pseudomonas species		Saddletail
Q123	-	rod	+	+	+	O		30	PCA	14	Pseudomonas species		Saddletail
Q128	-	rod	+	+	+	O		30	PCA	14	Pseudomonas species		Saddletail
Q136	-	rod	+	+	+	O		30	PCA	16	Pseudomonas species		Saddletail
Q137	-	rod	+	+	+	O		30	PCA	11	Pseudomonas species		Saddletail
Q139	-	rod	+	+	+	O		30	PCA	17	Pseudomonas species		Saddletail
Q184	-	rod	+	+	+	O		25	PCA	12	Pseudomonas species		Saddletail
Q194	-	rod	+	+	+	O		30	PCA	41	Pseudomonas species		Saddletail
							No						
Q203	-	rod	+	+	+	change		25	PCA	19	Pseudomonas species		Saddletail
Q204	-	rod	+	+	+	O		25	PCA	6	Pseudomonas species		Saddletail
Q283	-	rod	+	+	+	O		25			Pseudomonas species		Saddletail
Q288	-	rod	+	+	+	O		30	PCA	42	Pseudomonas species		Saddletail
Q296	-	rod	+	+	+	O		30	PCA	42	Pseudomonas species		Saddletail
Q306	-	rod	+	+	+	O		30	PCA	14	Pseudomonas species		Saddletail
Q307	-	rod	+	+	+	O		30	PCA	14	Pseudomonas species		Saddletail
Q315	-	rod	+	+	+	O		30	PCA	14	Pseudomonas species		Saddletail

Q326	-	rod	+	+	+	O	30			Pseudomonas species	Saddletail
Q327	-	rod	+	+	+	O	25			Pseudomonas species	Saddletail
Q334	-	rod	+	+	+	O	25	PCA	42	Pseudomonas species	Saddletail
Q336	-	rod	+	+	+	O	25	PCA	45	Pseudomonas species	Saddletail
Q341	-	rod	+	+	+	O	30	PCA	45	Pseudomonas species	Saddletail
Q342	-	rod	+	+	+	O	30	PCA	42	Pseudomonas species	Saddletail
Q347	-	rod	+	+	+	O	30	PCA	41	Pseudomonas species	Saddletail
Q448	-	rod	+	+	+	O	25	LH	6	Pseudomonas species	Saddletail
Q490	-	rod	+	+	+	O	25	PCA	45	Pseudomonas species	Saddletail
Q340	-	rod	+	+	+	O	25	PCA	45	Pseudomonas species	Saddletail
Q261	-	rod	+	+	+	F	15	LH	8	Pseudomonas species (chlororophis/grimonti/brassicacearum)	Saddletail
E238	-	rod	+	+	+	O	15	LH	1	Pseudomonas stutzeri	Goldband
E329	-	rod	+	+	+	O	30	PCA	55	Pseudomonas stutzeri	Goldband
Q202	-	rod	+	+	+	O	25	PCA	24	Pseudomonas syringae	Saddletail
Q192	-	rod	+	+	+	O	30	PCA	41	Pseuodomonas species	Saddletail
E067	-	rod	+	+	+	O	30	PCA	1	Psuedomonas putida	Goldband
E121	-	rod	+	+	+	O	30	IA	1	Psuedomonas putida	Saddletail
E145	-	rod	+	+	+	O	25	IA	1	Psuedomonas putida	Saddletail
E146	-	rod	+	+	+	O	25	IA	1	Psuedomonas putida	Saddletail
E306	-	rod	+	+	+	O	30	PCA	54	Psuedomonas putida	Saddletail
E352	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida	Goldband
E356	-	rod	+	+	+	O	30	LH	1	Psuedomonas putida	Goldband
E368	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida	Saddletail
E394	-	rod	+	+	+	O	30	LH	1	Psuedomonas putida	Saddletail
E396	-	rod	+	+	+	O	30	LH	1	Psuedomonas putida	Goldband
E397	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida	Goldband

E400	-	rod	+	+	+	O	30	LH	1	Psuedomonas putida			Saddletail
E401	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida			Saddletail
E433	-	rod	+	+	+	o	30	IA	1	Psuedomonas putida			Saddletail
E437	-	rod	+	+	+	O	25	IA	1	Psuedomonas putida			Goldband
E439	-	rod	+	+	+	o	25	IA	1	Psuedomonas putida			Goldband
E462	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida			Saddletail
E467	-	cocci	+	+	+	O	25	LH	1	Psuedomonas putida			Goldband
E470	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida			Goldband
E473	-	rod	+	+	+	O	30	LH	1	Psuedomonas putida			Saddletail
E481	-	rod	+	+	+	O	30	LH	1	Psuedomonas putida			Goldband
E486	-	rod	+	+	+	O	30	LH	1	Psuedomonas putida			Goldband
E487	-	rod	+	+	+	O	30	LH	1	Psuedomonas putida			Goldband
E492	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida			Goldband
E493	-	rod	+	+	+	O	30	LH	1	Psuedomonas putida			Goldband
E495	-	rod	+	+	+	O	30	LH	1	Psuedomonas putida			Goldband
E499	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida			Saddletail
E561	-	rod	+	+	+	O	30	LH	1	Psuedomonas putida			Saddletail
E568	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida			Saddletail
E570	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida			Saddletail
E571	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida			Saddletail
E574	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida			Goldband
E620	-	rod	+	+	+	o	30	IA	1	Psuedomonas putida			Goldband
E621	-	rod	+	+	+	O	30	IA	1	Psuedomonas putida			Goldband
E622	-	rod	+	+	+	O	25	IA	1	Psuedomonas putida			Goldband
E624	-	rod	+	+	+	O	30	IA	1	Psuedomonas putida			Saddletail
E655	-	rod	+	+	+	O	30	LH	1	Psuedomonas putida			Saddletail
Q017	-	rod	+	+	+	O	25	PCA	1	Psuedomonas putida			Saddletail

Q019	-	rod	+	+	+	O	30	PCA	1	Psuedomonas putida	Saddletail
Q038	-	rod	+	+	+	O	25	IA	1	Psuedomonas putida	Saddletail
Q065	-	rod	+	+	+	O	15	LH	1	Psuedomonas putida	Saddletail
Q078	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida	Saddletail
Q081	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida	Saddletail
Q082	-	rod	+	+	+	O	30	LH	1	Psuedomonas putida	Saddletail
Q089	-	rod	+	+	+	O	25	IA	1	Psuedomonas putida	Saddletail
Q120	-	rod	+	+	+	N	30	PCA	1	Psuedomonas putida	Saddletail
Q142	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida	Saddletail
Q164	-	rod	+	+	+	O	30	LH	1	Psuedomonas putida	Saddletail
Q165	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida	Saddletail
Q166	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida	Saddletail
Q173	-	rod	+	+	+	O	15	LH	1	Psuedomonas putida	Saddletail
Q174	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida	Saddletail
Q222	-	rod	+	+	+	O	25	IA	1	Psuedomonas putida	Saddletail
Q223	-	rod	+	+	+	O	25	IA	1	Psuedomonas putida	Saddletail
Q227	-	rod	+	+	+	O	25	IA	1	Psuedomonas putida	Saddletail
Q229	-	rod	+	+	+	O	25	IA	1	Psuedomonas putida	Saddletail
Q233	-	rod	+	+	+	O	25	IA	1	Psuedomonas putida	Saddletail
Q240	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida	Saddletail
Q370	-	rod	+	+	+	O	15	IA	1	Psuedomonas putida	Saddletail
Q389							15	IA	1	Psuedomonas putida	Saddletail
Q451	-	rod	+	+	+	O	25	LH	1	Psuedomonas putida	Saddletail
Q502	-	rod	+	+	+	O	25	IA	1	Psuedomonas putida	Saddletail
E116	+	cocci	-	+	+	O	25			Psuedomonas putida / fluorescens/ syringae	Saddletail
E115	-	cocci	-	+	+	O	25	IA	1	Psychrobacter cibarius	Saddletail

E182	+	cocci	-	+	+	O	30	PCA	4	Psychrobacter cibarius	Saddletail
Q339	-	rod	-	+	+	F	25	PCA	3	Psychrobacter glacincola	Saddletail
2351	-	Coccobacilli	-	+	+	O	25	LH		Psychrobacter immobilis	Saddletail
2472	-	coccobacilli	-	+	+	O	25	LH		Psychrobacter immobilis	Saddletail
E003	-	cocci	-	+	+	O	25	PCA	7	Psychrobacter immobilis	Goldband
E114	-	cocci	-	+	+	No change	25	IA	1	Psychrobacter immobilis	Goldband
E135	-	cocci	-	+	+	No change	25	IA	1	Psychrobacter immobilis	Saddletail
E203	-	cocci	-	+	+	No change	25	LH	1	Psychrobacter immobilis	Goldband
E210	-	cocci	-	+	+	O	25	LH	2	Psychrobacter immobilis	Goldband
E224	-	cocci	-	+	+	No change	30			Psychrobacter immobilis	Goldband
E235	-	cocci	-	+	+	No change	30	LH	2	Psychrobacter immobilis	Goldband
E273	-	cocci	-	+	+	No change	25			Psychrobacter immobilis	Saddletail
E290	+	cocci	-	+	+	O	30	LH	10	Psychrobacter immobilis	Saddletail
Q002	+	cocci	-	+	+	O	25	PCA	1	Psychrobacter immobilis	Saddletail
Q003	+	cocci	-	+	+	O	25	PCA	9	Psychrobacter immobilis	Saddletail
Q004	-	cocci	-	+	+	No change	30	PCA	4	Psychrobacter immobilis	Saddletail
Q007	+	rod	-	+	+	O	25		1	Psychrobacter immobilis	Saddletail
Q197	+	cocci	-	+	+	O	25	PCA	1	Psychrobacter immobilis	Saddletail
Q267	+	cocci	-	+	+	O	15	LH	7	Psychrobacter immobilis	Saddletail
Q286	+	rod	-	+	+	No change		PCA	3	Psychrobacter immobilis	Saddletail
Q324	-	cocci	-	+	+	No change	25	PCA	5	Psychrobacter immobilis	Saddletail

Q492	-	cocci	-	+	+	O	25	PCA	5	Psychrobacter immobilis	Saddletail
Q494	-	rod	-	+	+	O	25	PCA	5	Psychrobacter immobilis	Saddletail
E058	-	cocci	-	+	+	O	25	PCA	1	Psychrobacter sp.	Goldband
2347	-	coccobacilli	-	+	+	O	25	LH		Psychrobacter species	Saddletail
2388	-	coccobacilli	-	+	+	O	25	LH		Psychrobacter species	Saddletail
2466	-	coccobacilli	-	+	+	O	25	LH		Psychrobacter species	Saddletail
2468	-	coccobacilli	-	+	+	O	15	LH		Psychrobacter species	Saddletail
2477	-	cocci	-	+	+	No change	25	LH		Psychrobacter species	Saddletail
E016	+	rod	-	+	-	O	30			Psychrobacter species	Goldband
E056	+	rod	-	+	-	O				Psychrobacter species	Goldband
E117	-	cocci	-	+	+	O	25	IA	1	Psychrobacter species	Saddletail
E153	-	cocci	-	+	+	O	25	PCA	1	Psychrobacter species	Saddletail
E173	-	cocci	-	+	+	O	30			Psychrobacter species	Saddletail
E207	-	cocci	-	+	+	O	25	LH	1	Psychrobacter species	Goldband
E213	-	cocci	-	+	+	O	25			Psychrobacter species	Goldband
E217	-	cocci	-	+	+	O	25	LH	1	Psychrobacter species	Goldband
E338	+	rod	-	+	-	O	30			Psychrobacter species	Saddletail
E348	-	rod	-	+	+	No change	30	PCA	7	Psychrobacter species	Saddletail
E416	-	rod	-	+	+	No change	15			Psychrobacter species	Saddletail
E489	-	rod	-	+	+	O	15			Psychrobacter species	Goldband
E538	+	rod	-	+	-	O	30			Psychrobacter species	Saddletail
E630	-	rod	-	+	+	No change	25			Psychrobacter species	Saddletail
E634	-	cocci	-	+	+	O	25			Psychrobacter species	Saddletail
E654	+	rod	-	+	-	O	30			Psychrobacter species	Saddletail

E656	+	rod	+	+	+	O	25			Psychrobacter species	Saddletail
E657	-	cocci	-	+	+	O	25	LH	8	Psychrobacter species	Saddletail
E665	-	cocci	-	+	+	O	25			Psychrobacter species	Saddletail
Q058	+	cocci	-	+	+	O	25	LH	10	Psychrobacter species	Saddletail
Q484	-	cocci	-	+	+	No change	25	LH	3	Psychrobacter urativorans	Saddletail
E193	-	cocci	-	+	+	O	30	PCA	3	Psychrobater immobilis	Saddletail
E316	+	rod	-	+	-	O	30	PCA	23	Psychrobater species	Goldband
E136	-	rod	+	+	-	F	25	IA	3	Rhodococcus- erythropolis	Saddletail
E612	-	rod	+	+	+	O	0	IA	7	Salmonella typhimurium	Saddletail
2214	-	rod	+	+	+	O	30	PCA		Shewanella putrefaciens	Goldband
2413	-	rod	+	+	+	O	30	IA		Shewanella putrefaciens	Saddletail
2415	-	rod	+	+	+	O	25	IA		Shewanella putrefaciens	Saddletail
2417	-	rod	+	+	+	O	25	IA		Shewanella putrefaciens	Saddletail
2418	-	rod	+	+	+	O	30	IA		Shewanella putrefaciens	Saddletail
2467	-	rod	+	+	+	O	25	LH		Shewanella putrefaciens	Saddletail
2470	-	rod	+	+	+	O	25	LH		Shewanella putrefaciens	Saddletail
2473	-	rod	+	+	+	O	25	LH		Shewanella putrefaciens	Saddletail
2474	-	rod	+	+	+	O	25	LH		Shewanella putrefaciens	Saddletail
2475	-	rod	+	+	+	No change	25	LH		Shewanella putrefaciens	Saddletail
2476	-	rod	+	+	+	O	25	LH		Shewanella putrefaciens	Saddletail
2528	-	rod	+	+	+	O	25	LH		Shewanella putrefaciens	Saddletail
E050	-	rod	+	+	+	F	30	PCA	7	Shewanella putrefaciens	Goldband
E072	-	rod	+	+	+	O	25	IA	7	Shewanella putrefaciens	Saddletail
E074	-	rod	+	+	+	O	25	IA	7	Shewanella putrefaciens	Saddletail
E075	-	rod	+	+	+	O	30	IA	7	Shewanella putrefaciens	Saddletail

E087	-	rod	+	+	+	F	25	IA	7	Shewanella putrefaciens	Saddletail
E090	-	cocci	-	+	+	F	25			Shewanella putrefaciens	Saddletail
E095	-	rod	+	+	+	O	30	IA	30	Shewanella putrefaciens	Saddletail
E096	-	rod	+	+	+	No change	30	IA	4	Shewanella putrefaciens	Saddletail
E097	-	rod	+	+	+	O	30	IA	7	Shewanella putrefaciens	Saddletail
E098	-	rod	+	+	+	O	30	IA	30	Shewanella putrefaciens	Saddletail
E099	-	rod	+	+	+	O	30	IA	30	Shewanella putrefaciens	Saddletail
E100	-	rod	+	+	+	O	25	IA	7	Shewanella putrefaciens	Saddletail
E109	-	rod	+	+	+	O	25	IA	21	Shewanella putrefaciens	Goldband
E119	-	rod	+	+	+	O	25	IA	7	Shewanella putrefaciens	Saddletail
E122	-	rod	+	+	+	O	25	IA	25	Shewanella putrefaciens	Saddletail
E123	-	rod	+	+	+	O	25			Shewanella putrefaciens	Saddletail
E124	-	rod	+	+	+	O	25	IA	43	Shewanella putrefaciens	Saddletail
E125	-	rod	+	+	+	O	30	IA	3	Shewanella putrefaciens	Saddletail
E126	-	rod	+	+	+	No change	25	IA	25	Shewanella putrefaciens	Saddletail
E127	-	rod	+	+	+	O	25	IA	22	Shewanella putrefaciens	Saddletail
E128	-	rod	+	+	+	O	30	IA	22	Shewanella putrefaciens	Saddletail
E129	-	rod	+	+	+	O	25	IA	7	Shewanella putrefaciens	Saddletail
E130	-	rod	+	+	+	O	25	IA	7	Shewanella putrefaciens	Saddletail
E131	-	rod	+	+	+	O	25	IA	22	Shewanella putrefaciens	Saddletail
E132	-	rod	+	+	+	O	25	IA	7	Shewanella putrefaciens	Saddletail
E133	-	rod	+	+	+	No change	25	IA	3	Shewanella putrefaciens	Saddletail
E137	-	rod	+	+	+	O	25	IA	7	Shewanella putrefaciens	Saddletail
E140	-	rod	+	+	+	O	25	IA	22	Shewanella putrefaciens	Saddletail
E141	-	rod	+	+	+	O	25	IA	27	Shewanella putrefaciens	Saddletail

E142	-	rod	+	+	+	O	25	IA	27	Shewanella putrefaciens	Saddletail
E143	-	rod	+	+	+	O	25	IA	30	Shewanella putrefaciens	Saddletail
E144	-	rod	+	+	+	O	25	IA	30	Shewanella putrefaciens	Saddletail
E147	-	rod	+	+	+	O	25	IA	22	Shewanella putrefaciens	Saddletail
E148	-	rod	+	+	+	O	25	IA	3	Shewanella putrefaciens	Saddletail
E149	-	rod	+	+	+	No change	25	IA	4	Shewanella putrefaciens	Saddletail
E178	+	rod	+	+	+	O	30			Shewanella putrefaciens	Saddletail
E200	-	rod	+	+	-	O	30			Shewanella putrefaciens	Goldband
E206	-	rod	+	+	+	O	25	LH	3	Shewanella putrefaciens	Goldband
E219	-	rod	+	+	+	O	30	LH	20	Shewanella putrefaciens	Goldband
E221	-	rod	+	+	+	O	25			Shewanella putrefaciens	Goldband
E227	-	rod	+	+	+	O	25			Shewanella putrefaciens	Goldband
E229	-	rod	+	+	+	O	25	LH	39	Shewanella putrefaciens	Goldband
E239	-	rod	+	+	+	O	15	LH	21	Shewanella putrefaciens	Goldband
E241	-	rod	+	+	+	O	25	LH	59	Shewanella putrefaciens	Goldband
E248	-	rod	+	+	+	O	25	LH	4	Shewanella putrefaciens	Goldband
E261	-	rod	+	+	+	O	25	LH	28	Shewanella putrefaciens	Saddletail
E268	-	rod	+	+	+	O	30	LH	3	Shewanella putrefaciens	Saddletail
E276	-	rod	+	+	+	O	30	LH	29	Shewanella putrefaciens	Saddletail
E283	-	rod	+	+	+	F	25	LH	2	Shewanella putrefaciens	Saddletail
E285	-	rod	+	+	+	O	25	LH	7	Shewanella putrefaciens	Saddletail
E286	-	rod	+	+	+	O	25	LH	7	Shewanella putrefaciens	Saddletail
E287	-	rod	+	+	+	O	25	LH	20	Shewanella putrefaciens	Saddletail
E288	-	rod	+	+	+	O	25	LH	20	Shewanella putrefaciens	Saddletail
E298	-	rod	+	+	+	O	25	LH	20	Shewanella putrefaciens	Saddletail
E299	-	rod	+	+	+	O	25			Shewanella putrefaciens	Saddletail

E350	-	rod	+	+	+	O	25			Shewanella putrefaciens	Goldband
E353	-	rod	+	+	+	O	30	LH	20	Shewanella putrefaciens	Goldband
E354	-	rod	+	+	+	O	25	LH	25	Shewanella putrefaciens	Goldband
E358	-	rod	+	+	+	No change	25	LH	29	Shewanella putrefaciens	Goldband
E366	-	rod	+	+	+	O	25	LH	3	Shewanella putrefaciens	Saddletail
E375	-	rod	+	+	+	O	25	LH	20	Shewanella putrefaciens	Saddletail
E383	-	rod	+	+	+	O	25			Shewanella putrefaciens	Saddletail
E390	-	rod	+	+	+	O	25	LH	27	Shewanella putrefaciens	Goldband
E399	+	rod	+	+	+	O	25			Shewanella putrefaciens	Saddletail
E410	-	rod	+	+	+	O	25	LH	39	Shewanella putrefaciens	Saddletail
E412	-	rod	+	+	+	O	30	LH	3	Shewanella putrefaciens	Saddletail
E415	-	rod	+	+	+	O	25	LH	25	Shewanella putrefaciens	Saddletail
E420	-	rod	+	+	+	O	30	LH	60	Shewanella putrefaciens	Goldband
E422	-	rod	+	+	+	O	25	LH	39	Shewanella putrefaciens	Goldband
E434	-	rod	+	+	+	No change	25	IA	5	Shewanella putrefaciens	Saddletail
E436	-	rod	+	+	+	O	25	IA	22	Shewanella putrefaciens	Goldband
E438	-	rod	+	+	+	O	25	IA	20	Shewanella putrefaciens	Goldband
E450	-	rod	+	+	+	O	25	IA	22	Shewanella putrefaciens	Goldband
E451	-	rod	+	+	+	O	25			Shewanella putrefaciens	Goldband
E452	-	rod	+	+	+	No change	25	IA	3	Shewanella putrefaciens	Goldband
E453	-	rod	+	+	+	O	25	IA	22	Shewanella putrefaciens	Goldband
E456	-	rod	+	+	+	No change	25	PCA	4	Shewanella putrefaciens	Goldband
E461	-	rod	+	+	+	O	30	LH	20	Shewanella putrefaciens	Saddletail
E472	-	rod	+	+	+	O	25	LH	27	Shewanella putrefaciens	Saddletail

E475	-	rod	+	+	+	O	25	LH	20	Shewanella putrefaciens	Saddletail
E478	-	rod	+	+	+	O	30	LH	3	Shewanella putrefaciens	Saddletail
E485	-	rod	+	+	+	O	25	LH	28	Shewanella putrefaciens	Goldband
E497	-	rod	+	+	+	O	25	LH	30	Shewanella putrefaciens	Saddletail
E498	-	rod	+	+	+	O	30	LH	20	Shewanella putrefaciens	Saddletail
E523	-	rod	+	+	+	O	25	PCA	49	Shewanella putrefaciens	Saddletail
E525	-	rod	+	+	+	O	25			Shewanella putrefaciens	Goldband
E526	-	rod	+	+	+	O	30			Shewanella putrefaciens	Goldband
E540	-	rod	+	+	+	O	25	IA	27	Shewanella putrefaciens	Saddletail
E541	-	rod	+	+	+	O	25	IA	22	Shewanella putrefaciens	Saddletail
E542	-	rod	+	+	+	O	25	IA	22	Shewanella putrefaciens	Saddletail
E543	-	rod	+	+	+	O	25			Shewanella putrefaciens	Saddletail
E544	-	rod	+	+	+	O	25			Shewanella putrefaciens	Goldband
E545	-	rod	+	+	+	O	25			Shewanella putrefaciens	Goldband
E546	-	rod	+	+	+	O	25			Shewanella putrefaciens	Saddletail
E547	-	rod	+	+	+	O	25			Shewanella putrefaciens	Saddletail
E548	-	rod	+	+	+	O	25			Shewanella putrefaciens	Saddletail
E550	-	rod	+	+	+	O	25			Shewanella putrefaciens	Goldband
E551	-	rod	+	+	+	O	25	IA	30	Shewanella putrefaciens	Goldband
E552	-	rod	+	+	+	O	25	IA	30	Shewanella putrefaciens	Goldband
E553	-	rod	+	+	+	O	25	IA	21	Shewanella putrefaciens	Goldband
E555	-	rod	+	+	+	O	25	IA	30	Shewanella putrefaciens	Goldband
E556	-	rod	+	+	+	O	25	IA	27	Shewanella putrefaciens	Goldband
E557	-	rod	+	+	+	O	25	IA	7	Shewanella putrefaciens	Goldband
E558	-	rod	+	+	+	O	30	IA	43	Shewanella putrefaciens	Goldband
E564	-	rod	+	+	+	O	30	LH	22	Shewanella putrefaciens	Saddletail
E569	-	rod	+	+	+	O	30	LH	22	Shewanella putrefaciens	Saddletail

E582	-	rod	+	+	+	O	25	LH	7	Shewanella putrefaciens	Saddletail
E586	-	rod	+	+	+	No change	25	LH	17	Shewanella putrefaciens	Goldband
E610	-	rod	+	+	+	O	25	IA	58	Shewanella putrefaciens	Saddletail
E611	-	rod	+	+	+	O	25	IA	25	Shewanella putrefaciens	Saddletail
E613	-	rod	+	+	+	O	30	IA	25	Shewanella putrefaciens	Saddletail
E616	-	rod	+	+	+	O	25	IA	58	Shewanella putrefaciens	Saddletail
E617	-	rod	+	+	+	No change	25	IA	15	Shewanella putrefaciens	Saddletail
E619	-	rod	+	+	+	O	25	IA	25	Shewanella putrefaciens	Saddletail
E623	-	rod	+	+	+	O	25	IA	7	Shewanella putrefaciens	Saddletail
E625	-	rod	+	+	+	O	25	IA	58	Shewanella putrefaciens	Saddletail
E626	-	rod	+	+	+	O	25	IA	22	Shewanella putrefaciens	Saddletail
E627	-	rod	+	+	+	O	25	IA	22	Shewanella putrefaciens	Saddletail
Q016	-	rod	+	+	+	O	25	PCA	62	Shewanella putrefaciens	Saddletail
Q026	-	rod	+	+	+	O	30	PCA	3	Shewanella putrefaciens	Saddletail
Q029	-	rod	+	+	+	No change	25	IA	4	Shewanella putrefaciens	Saddletail
Q030	-	rod	+	+	+	O	30	IA	7	Shewanella putrefaciens	Saddletail
Q031	-	rod	+	+	+	O	30	IA	25	Shewanella putrefaciens	Saddletail
Q032	-	rod	+	+	+	O	15	IA	3	Shewanella putrefaciens	Saddletail
Q033	-	rod	+	+	+	O	25	IA	3	Shewanella putrefaciens	Saddletail
Q034	-	rod	+	+	+	O	30	IA	3	Shewanella putrefaciens	Saddletail
Q036	-	rod	+	+	+	No change	25	IA	5	Shewanella putrefaciens	Saddletail
Q037	-	rod	+	+	+	O	25	IA	25	Shewanella putrefaciens	Saddletail
Q039	-	rod	+	+	+	O	25	IA	3	Shewanella putrefaciens	Saddletail
Q040	-	rod	+	+	+	O	30	IA	25	Shewanella putrefaciens	Saddletail

Q041	-	rod	+	+	+	No change	15	IA	4	Shewanella putrefaciens	Saddletail
Q042	-	rod	+	+	+	O	25	IA	25	Shewanella putrefaciens	Saddletail
Q043		rod	+	+	+	No change	25	IA	12	Shewanella putrefaciens	Saddletail
Q044	-	rod	+	+	+	O	25	IA	3	Shewanella putrefaciens	Saddletail
Q045	-	rod	+	+	+	O	25	IA	36	Shewanella putrefaciens	Saddletail
Q046	-	rod	+	+	+	No change	25	IA	5	Shewanella putrefaciens	Saddletail
Q048	-	rod	+	+	+	No change	15	LH	4	Shewanella putrefaciens	Saddletail
Q049	-	rod	+	+	+	O	15	LH	2	Shewanella putrefaciens	Saddletail
Q050	-	rod	+	+	+	No change	25	LH	3	Shewanella putrefaciens	Saddletail
Q053	-	rod	+	+	+	O	25	LH	7	Shewanella putrefaciens	Saddletail
Q055	-	rod	+	+	+	No change	25	LH	30	Shewanella putrefaciens	Saddletail
Q059	-	rod	+	+	+	O	25	LH	21	Shewanella putrefaciens	Saddletail
Q060	-	rod	+	+	+	O	25	LH	21	Shewanella putrefaciens	Saddletail
Q064	-	rod	+	+	+	No change	25	LH	30	Shewanella putrefaciens	Saddletail
Q067	-	rod	+	+	+	No change	15	LH	5	Shewanella putrefaciens	Saddletail
Q072	-	rod	+	+	+	O	25	LH	21	Shewanella putrefaciens	Saddletail
Q074	-	rod	+	+	+	O	30	LH	3	Shewanella putrefaciens	Saddletail
Q075	-	rod	+	+	+	O	25	LH	4	Shewanella putrefaciens	Saddletail
Q076	-	rod	+	+	+	No change	25	LH	26	Shewanella putrefaciens	Saddletail
Q077	-	rod	+	+	+	No	25	LH	5	Shewanella putrefaciens	Saddletail

Q080	-	rod	+	+	+	change O	30	LH	22	Shewanella putrefaciens		Saddletail
Q087	-	rod	+	+	+	O	30	IA	23	Shewanella putrefaciens		Saddletail
Q088	-	rod	+	+	+	O	30	IA	3	Shewanella putrefaciens		Saddletail
Q090	-	rod	+	+	+	O	25	IA	24	Shewanella putrefaciens		Saddletail
Q091	-	rod	+	+	+	O	30	IA	25	Shewanella putrefaciens		Saddletail
Q096	-	rod	+	+	+	O	30			Shewanella putrefaciens		Saddletail
Q097	-	rod	+	+	+	O	25	IA	25	Shewanella putrefaciens		Saddletail
Q098	-	rod	+	+	+	O	25	IA	20	Shewanella putrefaciens		Saddletail
Q099	-	rod	+	+	+	O	30			Shewanella putrefaciens		Saddletail
Q100	-	rod	+	+	+	O	25	PCA	4	Shewanella putrefaciens		Saddletail
Q102	-	rod	+	+	+	N	25	PCA	10	Shewanella putrefaciens		Saddletail
Q118	-	rod	+	+	+	O	30	PCA	4	Shewanella putrefaciens		Saddletail
Q119	-	rod	+	+	+	N	30	PCA	13	Shewanella putrefaciens		Saddletail
Q127	-	rod	+	+	+	O	30	PCA	10	Shewanella putrefaciens		Saddletail
Q144	-	rod	+	+	+	O	25	LH	3	Shewanella putrefaciens		Saddletail
Q146	-	rod	+	+	+	No change	25	LH	27	Shewanella putrefaciens		Saddletail
Q148	-	rod	+	+	+	No cahnge	25	LH	5	Shewanella putrefaciens		Saddletail
Q149	-	rod	+	+	+	F	25	LH	3	Shewanella putrefaciens		Saddletail
Q150	-	rod	+	+	+	No change	25	LH	27	Shewanella putrefaciens		Saddletail
Q151	-	rod	+	+	+	O	25	LH	20	Shewanella putrefaciens		Saddletail
Q152	-	rod	+	+	+	O	25	LH	20	Shewanella putrefaciens		Saddletail
Q154	-	rod	+	+	+	No change	25	LH	5	Shewanella putrefaciens		Saddletail
Q155	-	rod	+	+	+	F	25	LH	3	Shewanella putrefaciens		Saddletail

Q157	-	rod	+	+	+	O	15	LH	4	Shewanella putrefaciens	Saddletail
Q161	-	rod	+	+	+	O	30	LH	30	Shewanella putrefaciens	Saddletail
Q168	-	rod	+	+	+	O	15	LH	29	Shewanella putrefaciens	Saddletail
Q198	-	rod	+	+	+	O	25	PCA	53	Shewanella putrefaciens	Saddletail
Q205	-	rod	+	+	+	O	30	PCA	5	Shewanella putrefaciens	Saddletail
Q210	-	rod	+	+	+	O	25			Shewanella putrefaciens	Saddletail
Q221	-	rod	+	+	+	No change	25	IA	12	Shewanella putrefaciens	Saddletail
Q224	-	cocci	+	+	+	O	25	IA	3	Shewanella putrefaciens	Saddletail
Q225	+	rod	+	+	+	No change				Shewanella putrefaciens	Saddletail
Q226	+	rod	+	+	+	O	30			Shewanella putrefaciens	Saddletail
Q231	-	rod	+	+	+	O	25			Shewanella putrefaciens	Saddletail
Q232	-	rod	+	+	+	O	25	IA	3	Shewanella putrefaciens	Saddletail
Q234	-	rod	+	+	+	No change	25	IA	25	Shewanella putrefaciens	Saddletail
Q236	-	rod	+	+	+	O	25	IA	3	Shewanella putrefaciens	Saddletail
Q237	-	rod	+	+	+	O	25	IA	36	Shewanella putrefaciens	Saddletail
Q238	-	rod	+	+	+	No change	25	IA	25	Shewanella putrefaciens	Saddletail
Q241	-	rod	+	+	+	O	25	LH	7	Shewanella putrefaciens	Saddletail
Q243	-	rod	+	+	+	No change	15	LH	22	Shewanella putrefaciens	Saddletail
Q246	-	rod	+	+	+	O	25	LH	4	Shewanella putrefaciens	Saddletail
Q248	-	rod	+	+	+	No change	25	LH	26	Shewanella putrefaciens	Saddletail
Q252	-	rod	+	+	+	No change	25	LH	5	Shewanella putrefaciens	Saddletail
Q257	-	rod	+	+	+	No	25	LH	5	Shewanella putrefaciens	Saddletail

						change					
						No					
Q258	-	rod	+	+	+	change	15	LH	22	Shewanella putrefaciens	Saddletail
Q263	-	rod	+	+	+	O	25	LH	39	Shewanella putrefaciens	Saddletail
Q264	-	rod	+	+	+	O	25	LH	39	Shewanella putrefaciens	Saddletail
Q265	-	rod	+	+	+	O	25	LH	20	Shewanella putrefaciens	Saddletail
Q266	-	rod	+	+	+	O	25	LH	30	Shewanella putrefaciens	Saddletail
Q271	-	rod	+	+	+	O	15	LH	7	Shewanella putrefaciens	Saddletail
Q273	-	rod	+	+	+	O	25	LH	7	Shewanella putrefaciens	Saddletail
						No					
Q275	-	rod	+	+	+	change	15	LH	5	Shewanella putrefaciens	Saddletail
Q358	-	rod	+	+	+	O	25			Shewanella putrefaciens	Saddletail
Q360	-	rod	+	+	+	O	25	IA	21	Shewanella putrefaciens	Saddletaill
Q362	-	rod	+	+	+	O	30	IA	3	Shewanella putrefaciens	Saddletail
						No					
Q363	-	rod	+	+	+	change	25	IA	5	Shewanella putrefaciens	Saddletail
						No					
Q365	-	rod	+	+	+	change	30	IA	27	Shewanella putrefaciens	Saddletail
						No					
Q367	-	rod	+	+	+	change	25	IA	5	Shewanella putrefaciens	Saddletail
Q373	-	rod	+	+	+	O	25	IA	7	Shewanella putrefaciens	Saddletail
Q375	-	rod	+	+	+	O	25	IA	3	Shewanella putrefaciens	Saddletail
Q376	-	rod	+	+	+	O	30	IA	30	Shewanella putrefaciens	Saddletail
Q377	-	rod	+	+	+	O	25	IA	3	Shewanella putrefaciens	Saddletail
Q378	-	rod	+	+	+	O	25	IA	3	Shewanella putrefaciens	Saddletail
Q379	-	rod	+	+	+	O	25			Shewanella putrefaciens	Saddletail
						No					
Q381	-	rod	+	+	+	change	15	IA	5	Shewanella putrefaciens	Saddletail

Q382	-	rod	+	+	+	No change	15	IA	5	Shewanella putrefaciens	Saddletail
Q386	-	rod	+	+	+	O	25	IA	7	Shewanella putrefaciens	Saddletail
Q387	-	rod	+	+	+	O	30	IA	30	Shewanella putrefaciens	Saddletail
Q396	-	rod	+	+	+	O	30			Shewanella putrefaciens	Saddletail
Q403	-	rod	+	+	+	O	25	LH	3	Shewanella putrefaciens	Saddletail
Q404	-	rod	+	+	+	O	25	LH	4	Shewanella putrefaciens	Saddletail
Q405	-	rod	+	+	+	No change	25	LH	5	Shewanella putrefaciens	Saddletail
Q412	-	rod	+	+	+	O	25	LH	5	Shewanella putrefaciens	Saddletail
Q434	-	rod	+	+	+	No change	25	LH	30	Shewanella putrefaciens	Saddletail
Q449	-	rod	+	+	+	O	15	LH	7	Shewanella putrefaciens	Saddletail
Q454	-	rod	+	+	+	O	25	LH	25	Shewanella putrefaciens	Saddletail
Q461	-	rod	+	+	+	No change	25	LH	5	Shewanella putrefaciens	Saddletail
Q467	-	rod	+	+	+	O	25	LH	7	Shewanella putrefaciens	Saddletail
Q468	-	rod	+	+	+	O	30	LH	7	Shewanella putrefaciens	Saddletail
Q472	-	rod	+	+	+	O	25	LH	3	Shewanella putrefaciens	Saddletail
Q473	-	rod	+	+	+	O	25	LH	4	Shewanella putrefaciens	Saddletail
Q474	-	rod	+	+	+	O	25	LH	3	Shewanella putrefaciens	Saddletail
Q480	-	rod	+	+	+	O	15	LH	20	Shewanella putrefaciens	Saddletail
Q482	-	rod	+	+	+	No change	30	LH	5	Shewanella putrefaciens	Saddletail
Q497	-	rod	+	+	+	F	25	IA	11	Shewanella putrefaciens	Saddletail
Q498	-	rod	+	+	+	No change	25	IA	5	Shewanella putrefaciens	Saddletail
Q499	-	rod	+	+	+	O	25	IA	3	Shewanella putrefaciens	Saddletail

Q500	-	rod	+	+	+	O	15	IA	7	Shewanella putrefaciens	Saddletail
Q503	-	rod	+	+	+	O	25	IA	8	Shewanella putrefaciens	Saddletail
Q507	-	rod	+	+	+	O	15	IA	3	Shewanella putrefaciens	Saddletail
Q508	-	rod	+	+	+	No change	30	IA	32	Shewanella putrefaciens	Saddletail
Q509	-	rod	+	+	+	O	30			Shewanella putrefaciens	Saddletail
Q511	-	rod	+	+	+	O	25	IA	3	Shewanella putrefaciens	Saddletail
2205	-	rod	+	+	+	O	15	LH		Shewanella putrefaciens/ algae	Saddletail
2242	-	rod	+	+	+	O	30	LH		Shewanella putrefaciens/ algae	Saddletail
2243	-	rod	+	+	+	O	25	LH		Shewanella putrefaciens/ algae	Saddletail
2247	-	rod	+	+	+	O	30	LH		Shewanella putrefaciens/ algae	Goldband
2283	-	rod	-	+	+	F	30	PCA		Shingobacterium multivorum	Saddletail
2203	-	rod	+	+	+	O	25	LH		Sphingomonas paucimobilis	Saddletail
E001	+	cocci	-	+	-	F	30	PCA	1	Sphingomonas paucimobilis	Goldband
E302	-	rod	+	+	+	F	30	PCA	4	Sphingomonas paucimobilis	Saddletail
E331	-	rod	-	+	-	No change	30	PCA	2	Sphingomonas paucimobilis	Goldband
2169	+	cocci	-	+	-	F	30	PCA		Staph. Auricularis	Saddletail
2188	+	cocci	-	+	-	F	30	PCA		Staph. Auricularis	Saddletail
2167	+	cocci	-	+	-	F	30	PCA		Staph. Kloosii	Saddletail
2172	+	cocci	-	+	-	F	30	PCA		Staph. Xylosus	Saddletail
Q436	+	rod	-	+	-	F	25			Staphyloccus sciuri	Saddletail
E011	+	cocci	-	+	-	F	30	PCA	3	Staphylococcus aureus	Goldband
E018	+	cocci	-	+	-	F	30	PCA	1	Staphylococcus aureus	Goldband
E606	+	rod	-	-	+	F	30			Staphylococcus aureus	Saddletail
E024	+	cocci	-	+	-	F	30	PCA	5	Staphylococcus cohnii	Goldband
E025	+	cocci	-	+	-	F	30	PCA	5	Staphylococcus epidermidis	Goldband

E049	+	cocci	-	+	-	F	30	PCA	5	Staphylococcus epidermidis		Goldband
E162	+	cocci	-	+	-	F	30	PCA	5	Staphylococcus epidermidis		Saddletail
E181	+	cocci	-	+	-	F	30	PCA	9	Staphylococcus epidermidis		Saddletail
E575	+	cocci	-	+	+	F	25	LH	2	Staphylococcus epidermidis		Saddletail
E002	+	cocci	-	+	-	F	30	PCA	2	Staphylococcus hominis		Goldband
E020	+	cocci	-	+	-	F	30	PCA	4	Staphylococcus hominis		Goldband
E030	+	cocci	-	+	-	F	30	PCA	2	Staphylococcus hominis		Goldband
E150	-	cocci	N	+	-	F	30	PCA	2	Staphylococcus hominis		Saddletail
E513	+	cocci	-	+	-	F	30	PCA	2	Staphylococcus hominis		Goldband
E009	+	cocci	-	+	-	F	30			Staphylococcus saprophyticus		Goldband
2209	+	cocci	-	+	-	F	30	PCA		Staphylococcus species		Goldband
E021	+	rod	-	+	-	F	30			Staphylococcus species		Goldband
E048	+	rod	-	+	-	F	30			Staphylococcus species		Goldband
E063	+	cocci	-	+	-	F	30	PCA	15	Staphylococcus species		Goldband
E102	+	cocci	-	+	-	F	30	IA	8	Staphylococcus species		Saddletail
E185	+	cocci	-	+	-	F	30			Staphylococcus species		Saddletail
E359	+	cocci	-	+	-	F	30			Staphylococcus species		Goldband
E435	+	cocci	-	+	-	F	30			Staphylococcus species		Saddletail
E047	+	rod	+	+	-	O	30		0	Staphylococcus warneri	4	Goldband
E155	+	cocci	-	+	-	F	30	PCA		Staphylococcus warneri	7	Saddletail
E365	-	rod	+	+	+	F	15	LH		Stentrophomonas maltophilia	1	Saddletail
E101	-	rod	+	+	+	F	30	IA		vibrio alginolyticus	1	Saddletail
E103	-	rod	+	+	+	F	30			vibrio alginolyticus		Saddletail
E214	-	rod	+	+	+	F	25			vibrio alginolyticus		Goldband
E232	-	rod	+	+	+	F	25	LH		Vibrio alginolyticus	1	Goldband
E234	-	rod	+	+	+	F	25	LH		vibrio alginolyticus	3	Goldband
E259	-	rod	+	+	+	F	25	LH		vibrio alginolyticus	1	Saddletail

E272	-	rod	+	+	+	F	25	LH	3	vibrio alginolyticus	Saddletail
E278	-	rod	+	+	+	F	25	LH	1	vibrio alginolyticus	Saddletail
E297	-	rod	+	+	+	F	25	LH	1	vibrio alginolyticus	Saddletail
E373	-	rod	+	+	+	F	25	LH	1	vibrio alginolyticus	Saddletail
Q071	-	rod	+	+	+	F	25	LH	1	vibrio alginolyticus	Saddletail
Q421	-	rod	+	+	+	F	25	LH	1	vibrio alginolyticus	
Q424	-	rod	+	+	+	F	30	LH	1	vibrio alginolyticus	Saddletail
Q455	-	rod	+	+	+	F	25	LH	1	vibrio alginolyticus	Saddletail
E211	+	rod	+	+	+	O	25			Vibrio sp.	Goldband
2348	-	rod	+	+	+	a	25	LH		Vibrio species	Saddletail
E157	+	rod	+	+	-	F	30			Vibrio species	Saddletail
E168	+	rod	+	+	+	F	30			vibrio species	Saddletail
E208	+	rod	+	+	+	O	25			Vibrio species	Goldband
E209	-	rod	-	+	+	No change	25	LH	7	Vibrio species	Goldband
E246	+	rod	+	+	+	O	15			vibrio species	Goldband
E269	+	rod	+	+	+	F	30			Vibrio species	Saddletail
E275	-	rod	-	+	+	O	25	LH	4	Vibrio species	Saddletail
E336	+	rod	+	+	-	F	30			Vibrio species	Saddletail
E379	-	rod	+	+	+		25			Vibrio species	Saddletail
E427	-	rod	-	+	+	O	30	LH	9	Vibrio species	Goldband
E522	-	rod	+	+	+	No change	30	PCA	17	Vibrio species	Saddletail
E633	+	rod	+	+	+	F	30			Vibrio species	Saddletail
E245	+	rod	+	+	+	O	15			Vibrio vulnificus	Goldband

E642	-	rod	-	+	+		25			Arenibacter species	Saddletail
E648	-	rod	-	+	+	No change	30			Arenibacter species	Saddletail
E243	-	rod	-	+	+	F	30	LH	2	Arthrobacter agilis	Goldband
E518	-	rod	-	+	+	F	30	PCA	2	Arthrobacter agilis	Goldband
Q134	+	rod	+	+	+	F	25	PCA	5	Arthrobacter agilis	Saddletail
Q135	+	rod	+	+	-	F				Arthrobacter agilis	Saddletail
Q147	+	rod	+	+	-	F				Arthrobacter agilis	Saddletail
Q183	+	rod	-	-	-	F	25	PCA		Arthrobacter agilis	Saddletail
Q310	+	rod	-	+	-	F	30			Arthrobacter agilis	Saddletail
Q325	+	rod	-	+	+	O	25			Arthrobacter bergerei	Saddletail
Q489	+	rod	-	+	+	O	30			Arthrobacter bergerei	Saddletail
Q129	+	rod	+	+	-	F	30	PCA		Arthrobacter globiformis	Saddletail
2193	+	rod	+	+	+	F	30	PCA		Bacillus cereus	Goldband
2187	+	rod	+	+	+	F	30	PCA		Bacillus cereus group	Saddletail
E034	+	rod	+	+	+	F	30			Bacillus cereus group	Goldband
E134	-	rod	+	+	+	O	25	IA	7	Bacillus cereus group	Saddletail
E156	+	rod	+	+	+	F	30			Bacillus cereus group	Saddletail
E169	+	rod	+	+	+	F	30			Bacillus cereus group	Saddletail
E184	+	rod	+	+	+	F	30			Bacillus cereus group	Saddletail
E310	+	rod	+	+	+	F	30			Bacillus cereus group	Saddletail
E312	-	rod	+	+	+	F	30	PCA	5	Bacillus cereus group	Saddletail
E321	+	rod	+	+	-	F	30			Bacillus cereus group	Saddletail
E323	+	rod	+	+	+	F	30			Bacillus cereus group	Saddletail
E502	+	rod	+	+	+	F	0	PCA	18	Bacillus cereus group	Saddletail
E045	+	cocci	-	+	-	F	30	PCA	6	Bacillus luciferensis	Goldband
E046	+	cocci	+	+	-	F	30			Bacillus luciferensis	Goldband

Appendix 12

HPLC results for saddletail snapper

Days	Inosine	mg/100g	IMP	mg/100g	ATP	mg/100g	AMP	mg/100g	ADP	mg/100g	Hx	mg/100g
0	464.87	42.48428			62.97	7.043477					57.68	2.827828
0	465.24	42.5181			355.68	39.7844					336.88	16.51593
0					638.02	71.36539					790.09	38.73507
0					738.74	82.63138					692.65	33.95796
0					584.83	65.41585					651.46	31.93857
7					423.69	47.39162					586.42	28.74991
7					748.15	83.68393					953.83	46.76261
7					697.88	78.061					741.05	36.33083
7	429.96	39.29387			82.92	9.274973					431.77	21.16802
7					183.44	20.51859					201.57	9.8822
14					683.53	76.45589					725.49	35.56798
14					551.71	61.71123					588.58	28.85581
14	581.54	53.14671			447.66	50.07278			669.18	38.74733	543.53	26.64718
14	364.54	33.31516	640.03	90.09739	149.74	16.74909	480.28	56.2092			623.99	30.59182
14					555.34	62.11727					553.27	27.1247
14					225.86	25.26345					230.58	11.30445
21					497.89	55.69123					837.03	41.03636
21					331.19	37.04508			30.27	1.752715	535.92	26.27409
21	377.76	34.52334	48.59	6.840042	210.82	23.58116			35.07	2.030648	867.33	42.52185
28					579.71	64.84316					711.67	34.89044
28					622.82	69.66521					620.19	30.40553
28					424.16	47.4442					556.32	27.27423
28	324.64	29.66872			212.05	23.71874	31.86	3.728711			684.44	33.55546
28			161.11	22.67955	220.26	24.63707					172.94	8.478582

Appendix 13

HPLC results for goldband snapper

	Inosine	mg/100g	IMP	mg/100g	ATP	mg/100g	AMP	mg/100g	ADP	mg/100g	Hx	mg/100g
GB2 D0	498.08	45.51933			41.81	4.676636					1089.06	53.39241
GB2 D7	962.33	87.94695			85.13	9.522172					251.299	12.32022
GB2												
D14	743.94	67.98838			65	7.270541					355.81	17.44399
GB1												
D14	947.05	86.55052			71.898	8.042113					525.81	25.77844
GB2												
D21	494.34	45.17753			82.81	9.262669					560.79	27.49337
GB1												
D21					538.91	60.2795					616.8	30.23933
GB2												
D27	583.06	53.28562			88.23	9.868921					699.95	34.31585
GB1												
D28					309.012	34.56438					336.6	16.5022
GB2												
D35	49.59	4.53201									157.78	7.735345

Appendix 14

Photos of goldband and saddletail snapper

Appendix 15

Summary of MALDI- TOF results for saddletail and goldband snapper

aquisition time	name	sample	%	family	genus	species	datacount
20 May 2011 13:51	RPH_000_0156_4G4[c]	E-coli Standard	99.9	Family I Enterobacteriaceae	Escherichia	coli	220
20 May 2011 13:51	RPH_000_0156_4H2[c]	q234_02	0				159
20 May 2011 13:51	RPH_000_0156_4H1[c]	q234	90	Family I Alteromonadaceae	Shewanella	putrefaciens	191
20 May 2011 13:51	RPH_000_0156_4G2[c]	q497_04	0				98
20 May 2011 13:51	RPH_000_0156_4G1[c]	q497_03	86.4	Family I Alteromonadaceae	Shewanella	putrefaciens	162
20 May 2011 13:51	RPH_000_0156_4F4[c]	q224_02	0				123
20 May 2011 13:51	RPH_000_0156_4F3[c]	q224	75.6	Family I Alteromonadaceae	Shewanella	putrefaciens	128
20 May 2011 13:51	RPH_000_0156_4F2[c]	e87_02	0				153
20 May 2011 13:51	RPH_000_0156_4F1[c]	e87	0				163
20 May 2011 13:51	RPH_000_0156_4E4[c]	q374_02	0				110
20 May 2011 13:51	RPH_000_0156_4E3[c]	q374	0				146
20 May 2011 13:51	RPH_000_0156_4E2[c]	e558_02	0				139
20 May 2011 13:51	RPH_000_0156_4E1[c]	e558	79.2	Family I Alteromonadaceae	Shewanella	putrefaciens	139
20 May 2011 13:51	RPH_000_0156_4D4[c]	e101_02	0				63
20 May 2011 13:51	RPH_000_0156_4D3[c]	e101	0				82
20 May 2011 13:51	RPH_000_0156_4D2[c]	q90_02	0				101
20 May 2011 13:51	RPH_000_0156_4D1[c]	q90	0				103

aquisition time	name	sample	%	family	genus	species		datacount
20 May 2011 13:51	RPH_000_0156_4C3[c]	q87	75.6	Family I Alteromonadaceae	Shewanella	putrefaciens	133	
20 May 2011 13:51	RPH_000_0156_4C2[c]	e120_02	0				152	
20 May 2011 13:51	RPH_000_0156_4C1[c]	e120	0				145	
20 May 2011 13:51	RPH_000_0156_4B4[c]	e367_02	0				174	
20 May 2011 13:51	RPH_000_0156_4B3[c]	e367	0				187	
20 May 2011 13:51	RPH_000_0156_4B2[c]	e113_04	0				196	
20 May 2011 13:51	RPH_000_0156_4B1[c]	e113_03	0				189	
20 May 2011 13:51	RPH_000_0156_4A4[c]	q508_02	75.6	Family I Alteromonadaceae	Shewanella	putrefaciens	192	
20 May 2011 13:51	RPH_000_0156_4A3[c]	q508	79.2	Family I Alteromonadaceae	Shewanella	putrefaciens	196	
20 May 2011 13:51	RPH_000_0156_4A2[c]	e363_02	0				187	
20 May 2011 13:51	RPH_000_0156_4A1[c]	e363	0				151	
20 May 2011 13:51	RPH_000_0156_4G3[c]	E-coli Standard	80.6	Family I Enterobacteriaceae	Escherichia	coli	159	
20 May 2011 13:51	RPH_000_0156_3G4[c]	E-coli Standard	0				160	
20 May 2011 13:51	RPH_000_0156_3L4[c]	q55_02	79.2	Family I Alteromonadaceae	Shewanella	putrefaciens	188	
20 May 2011 13:51	RPH_000_0156_3L3[c]	q55	0				137	
20 May 2011 13:51	RPH_000_0156_3L2[c]	e354_02	86.4	Family I Alteromonadaceae	Shewanella	putrefaciens	154	

aquisition time	name	sample	%	family	genus	species		datacount

aquisition time	name	sample	%	family	genus	species	datacount
20 May 2011 13:51	RPH_000_0156_3K4[c]	q270_02	0				73
20 May 2011 13:51	RPH_000_0156_3K3[c]	q270	0				83
20 May 2011 13:51	RPH_000_0156_3K2[c]	q163_02	0				73
20 May 2011 13:51	RPH_000_0156_3K1[c]	q163	0				116
20 May 2011 13:51	RPH_000_0156_3J4[c]	q160_02	0				73
20 May 2011 13:51	RPH_000_0156_3J3[c]	q160	0				81
20 May 2011 13:51	RPH_000_0156_3J2[c]	q203_02	80.8	Family I Pseudomonadaceae	Pseudomonas	sp.	287
20 May 2011 13:51	RPH_000_0156_3J1[c]	q203	0				265
20 May 2011 13:51	RPH_000_0156_3I4[c]	q104_02	0				218
20 May 2011 13:51	RPH_000_0156_3I3[c]	q104	0				198
20 May 2011 13:51	RPH_000_0156_3I2[c]	q43_02	75.6	Family I Alteromonadaceae	Shewanella	putrefaciens	141
20 May 2011 13:51	RPH_000_0156_3I1[c]	q43	79.2	Family I Alteromonadaceae	Shewanella	putrefaciens	132
20 May 2011 13:51	RPH_000_0156_3H4[c]	e405_02	0				117
20 May 2011 13:51	RPH_000_0156_3H3[c]	e405	0				125
20 May 2011 13:51	RPH_000_0156_3H2[c]	e465_02	0				193
20 May 2011 13:51	RPH_000_0156_3H1[c]	e465	0				168
20 May 2011 13:51	RPH_000_0156_3G2[c]	e490_02	0				146

20 May 2011 13:51	RPH_000_0156_3F4[c]	e312_02	0				61
20 May 2011 13:51	RPH_000_0156_3F3[c]	e312	0				68
20 May 2011 13:51	RPH_000_0156_3F2[c]	q314_02	0				234
20 May 2011 13:51	RPH_000_0156_3F1[c]	q314	0				179
20 May 2011 13:51	RPH_000_0156_3E4[c]	e600_02	0				184
20 May 2011 13:51	RPH_000_0156_3E3[c]	e600	0				191
20 May 2011 13:51	RPH_000_0156_3E2[c]	q329_02	0				202
20 May 2011 13:51	RPH_000_0156_3E1[c]	q329	0				279
20 May 2011 13:51	RPH_000_0156_3D4[c]	q155_02	0				155
20 May 2011 13:51	RPH_000_0156_3D3[c]	q155	0				117
20 May 2011 13:51	RPH_000_0156_3D2[c]	e283_04	0				52
20 May 2011 13:51	RPH_000_0156_3D1[c]	e283_03	0				70
20 May 2011 13:51	RPH_000_0156_3C4[c]	q157_02	0				79
20 May 2011 13:51	RPH_000_0156_3C3[c]	q157	0				70
20 May 2011 13:51	RPH_000_0156_3C2[c]	q463_02	0				97
20 May 2011 13:51	RPH_000_0156_3C1[c]	q463	0				107

aquisition time	name	sample	%	family	genus	species	datacount
20 May 2011	RPH_000_0156_3B3[c]	q178	0				123

13:51							
20 May 2011 13:51	RPH_000_0156_3B1[c]	e358	0				97
20 May 2011 13:51	RPH_000_0156_3A4[c]	q146_02	0				100
20 May 2011 13:51	RPH_000_0156_3A3[c]	q146	0				163
20 May 2011 13:51	RPH_000_0156_3A2[c]	q76_02	79.2	Family I Alteromonadaceae	Shewanella	putrefaciens	161
20 May 2011 13:51	RPH_000_0156_3A1[c]	q76	0				126
20 May 2011 13:51	RPH_000_0156_3G3[c]	E-coli Standard	76.5	Family I Enterobacteriaceae	Escherichia	coli	210
20 May 2011 13:51	RPH_000_0156_2G4[c]	E-coli Standard	0				194
20 May 2011 13:51	RPH_000_0156_2L4[c]	e329_02	95.6	Family I Pseudomonadaceae	Pseudomonas	stutzeri	147
20 May 2011 13:51	RPH_000_0156_2L3[c]	e329	88.8	Family I Pseudomonadaceae	Pseudomonas	stutzeri	108
20 May 2011 13:51	RPH_000_0156_2L2[c]	e530_02	0				253
20 May 2011 13:51	RPH_000_0156_2L1[c]	e530	77.2	Family I Pseudomonadaceae	Pseudomonas	sp.	287
20 May 2011 13:51	RPH_000_0156_2K4[c]	e13_02	79.8	Family I Pseudomonadaceae	Pseudomonas	putida	242
20 May 2011 13:51	RPH_000_0156_2K3[c]	e13	99.9	Family I Pseudomonadaceae	Pseudomonas	putida	190
20 May 2011 13:51	RPH_000_0156_2K2[c]	q198_02	0				161
20 May 2011 13:51	RPH_000_0156_2K1[c]	q198	86.4	Family I Alteromonadaceae	Shewanella	putrefaciens	186

Appendix 16

Information for saddletail and goldband snapper caught in Exmouth, WA

Exmouth Trip 15th- 22nd March

Wednesday 17th

Goldband 1- blue/ 1 yellow, 138m

Goldband 2- blue/ 2 yellow, 138m

Goldband 3- blue, 138m

Saddletail 1- red, 138m

Thursday 18th

Saddletail 2- red/ 1 yellow, 100m

Saddletail 3- red/ 2 yellow, 100m

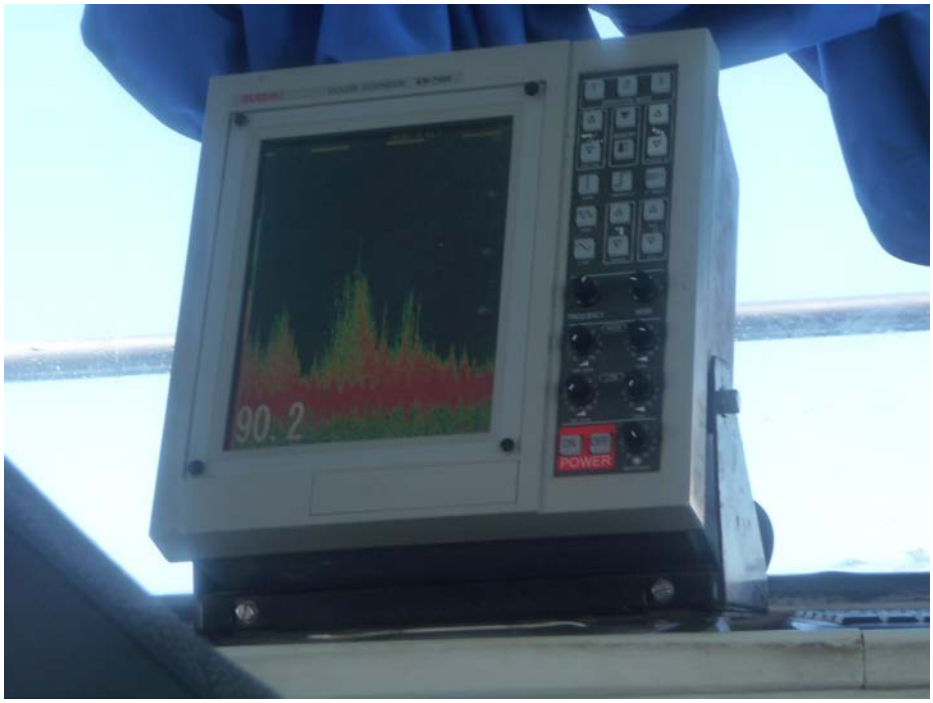
Saturday 20th

Saddletail (shallow) 1- white/ 1 green, 120m

Saddletail (shallow) 2- white/ 2 green, 120m

Saddletail (shallow) 3- white, 120m

- Fish were stored in freshwater ice brine, tummy down, head to tail. Temperature of fish stored at the top of the ice box were measured, 5.6C when ice had melted, 0.8C after ice was added.
- Co-ordinates of where each fish were caught
    - 1st spot- longitude- 18 minutes, 181 sec south, latitude- 11432337
    - 2nd spot- 2055090 south, 11453397 east
    - 3rd spot- 20˚24374 south, 11505957 east


90.2
POWER