Microbiological aspects of fish handling and processing in the Norwegian pelagic sector

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Scientific environment

The work with this thesis has been performed at the National Institute of Nutrition and Seafood Research (NIFES) and the Faculty of Mathematics and Natural Sciences at the University of Bergen.

NIFES

NATIONAL INSTITUTE
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Bergen, January 2015
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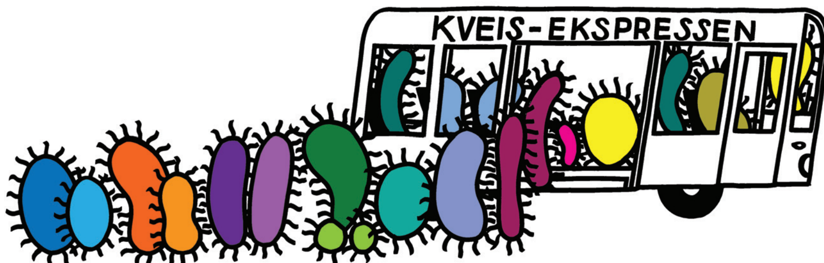

A special thanks to Trude Mette Savland who made this nice illustration of the *Anisakis*-bus getting ready for a fishilicious journey.

Abstract

Most global marine fish resources seem to have reached their limit of sustainable exploitation, whereas the demand for high quality fish products is still increasing. The fish sector needs to comply with the growing market by optimising the utilization of fish that have been harvested already, as approx. 10 - 25% of the total global wild-captured fish are rejected from the market. Microorganisms play an important role in product quality reduction, and an increased knowledge of the bacteria associated with fish, is needed. The overall aim of the work included in this thesis was to describe the bacteria associated with pelagic fish, to better utilise the fish and ensure quality and safety.

The indigenous bacterial biota of gills, skin and intestinal content from Atlantic mackerel (*Scomber scombrus*), as well as in the intestinal content of blue whiting (*Micromesistius poutassou*), were described by cultivation and PCR-DGGE methods. These samples were dominated by Gram-negative bacteria. The frequently occurring parasitic nematode larva of the genera *Anisakis* enters the fish through the intestine. The bacterial biota of these larvae was therefore expected to reflect the bacterial biota found in the fish intestine, and the shared bacterial genera comprised of *Pseudoalteromonas*, *Psychrobacter*, *Photobacterium*, and *Pseudomonas*. The latter two are known to cause spoilage in fresh fish.

During capture, landing and processing, the fish become exposed to extrinsic factors in the production environment that might affect the bacterial

biota and possibly the quality of the fish. A spot sampling programme was performed at fishing vessels and fish processing factories over a ten years period (2005-2014). The microbiological conditions of fish, surfaces of equipment and water samples was analysed by quality, hygiene and safety parameters. Samples were assessed according to a proposed assessment scheme, and good hygiene and safety conditions were found at 19 of 41 samplings. Contamination by the human pathogen *Listeria monocytogenes* was detected in fish from three samplings, and on equipment at nine samplings. One sampling on board a fishing vessel showed contamination by *E. coli* in the refrigerated sea water (RSW) tanks and on the fish, and this is of concern, since any contamination that occur early in the production chain might follow the fish throughout the process.

The effect of pumping of fish on board vessels was examined by comparing samples of Atlantic mackerel collected from the purse seine and the RSW tanks, 12 hour post capture. During the pumping intestinal content is forced out of the fish, contaminating the outer surfaces such as gills and skin of the surrounding fish. This is shown by the increased bacterial load on Iron Agar Lyngby (IAL) found in gill- and skin samples of fish collected from the RSW tank. Additionally, the genera *Vibrio* and *Oceanisphaera* were only identified in intestine samples from fish collected from the purse seine, but in all samples from fish collected from the RSW tank.

The muscle-invading *Anisakis* larvae were suspected to carry bacteria into the assumed sterile fish muscle. The presence of bacteria in larvae excised from fish muscle, was confirmed by Gram-stained histology sections. Samples from blue whiting muscle, with and without *Anisakis* larvae, were cultured and showed that the parasite acts as a contaminating vector, increasing the bacterial load in the fish muscle. However, these bacteria are not likely to be spread within the fish muscle unless the fish is processed in a way that disrupt

the capsule and the parasite, as for instance during fish mincing. A follow-up study included a 15-days long storage trial with fish mince inoculated with an *Anisakis* homogenate. The study gave surprisingly positive results, as the heterotropic plate count (HPC) were lower and the shelf-life was prolonged when the fish mince was added the *Anisakis*-homogenate. These results show that fish that are infected with parasite larvae, should not necessarily be excluded as a food resource.

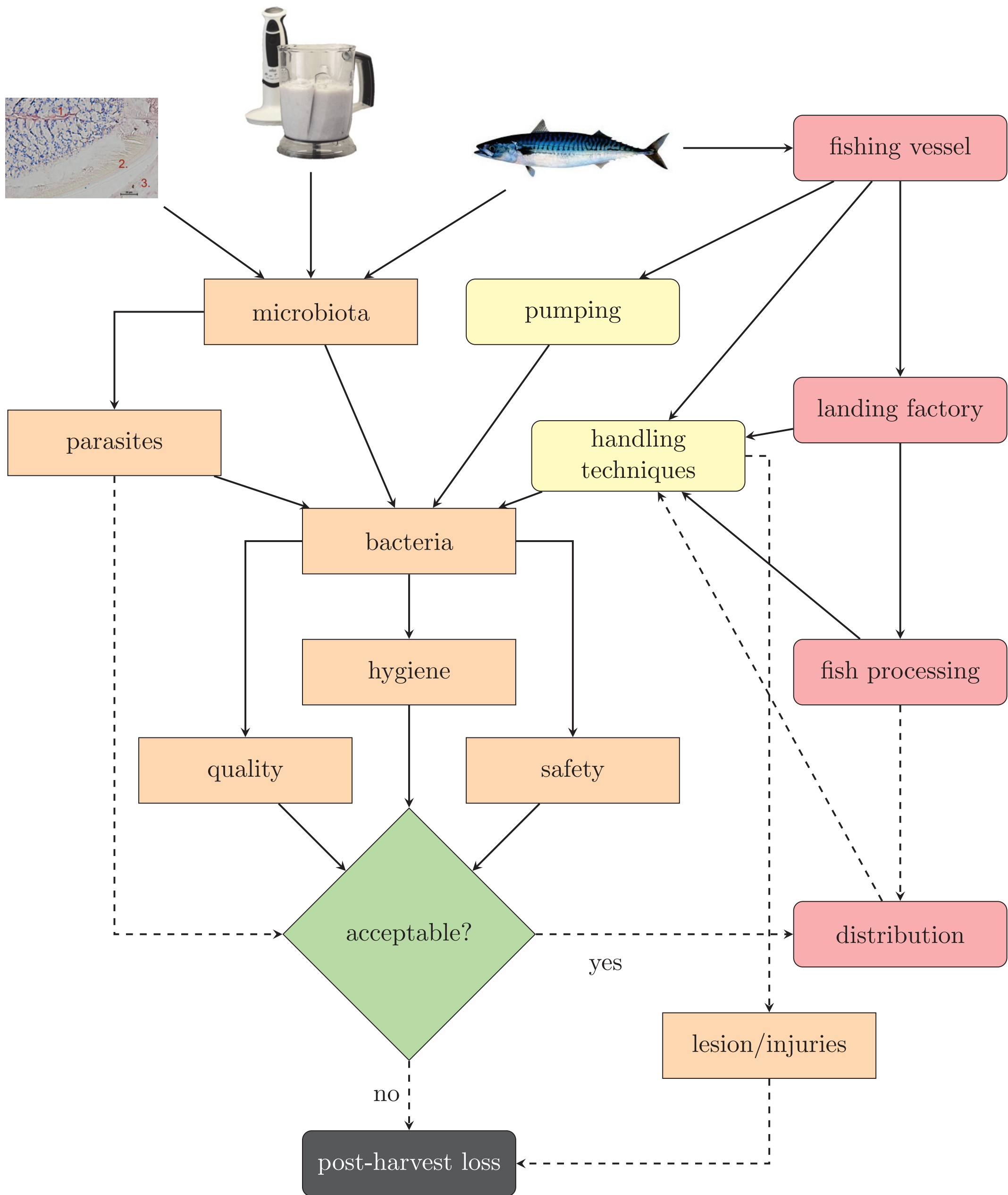

Figure A: Flowchart with some examined parameters for samples in this thesis, including Atlantic mackerel, *Anisakis*-infected fish muscle and fish mince. Pink boxes represent the production chain, from capture to end products, and the yellow boxes represent handling actions, as pumping and other handling techniques, which might affect the fish. Orange boxes represent parameters that determine if the fish product is acceptable (green box) for consumption. The black box represent the rejected fish. Dashed arrows indicates important steps or parameters that were not examined in this work.

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Glossary

16*S* rRNA gene 16*S* subunit ribosomal RNA gene.

BLAST Basic Local Alignment Search Tool.

CCP Critical Control Points.

CFU Colony Forming Units.

ddNTP Dideoxyribonucleotide Triphosphate.

DGGE Denaturing Gradient Gel Electrophoresis.

DNA Deoxyribonucleic Acid.

dNTP Deoxyribonucleotide Triphosphate.

FeS Iron Sulphide.

H_2S Hydrogen Sulphide.

HACCP Hazard Analysis and Critical Control Points.

HPC Heterotrophic Plate Count.

IAL Iron Agar Lyngby.

LAB Lactic Acid Bacteria.

MAP Modified Atmosphere Packaging.

NPN Non-Protein Nitrogen.

PCR Polymerase Chain Reaction.

RASFF The Rapid Alert System for Food and Feed.

RNA Ribonucleic Acid.

RSW Refrigerated Sea Water.

SSB Specific Spoilage Bacteria.

TMA Trimethylamine.

TMAO Trimethylamine Oxide.

TPC Total Plate Count.

Aims of the study

The main aim of this work was to increase the knowledge of the microbiology associated with the pelagic fish sector. This included both indigenous bacteria of fish, human pathogens and critical routes of contamination. The experiments were associated with two ongoing projects at the National Institute of Nutrition and Seafood Research (NIFES) concerning the pelagic sector.

Fish from fishing vessels and fish processing factories, as well as samples from the production environment, including surfaces of equipment and water samples, have been collected annually by the institute since 2005. These samples were analysed for different microbiological indicator organisms of quality and hygiene, as well as some important safety parameters. The goal was to identify critical points of handling which could be improved, and the results from these samplings, up until 2014, were the background for **Paper I**. Furthermore, a field experiment was performed on the most valuable pelagic species (NOK/kg), Atlantic mackerel (*Scomber scombrus*), where samples of fish collected from a purse seine were used to describe the indigenous microbiota. Samples of fish collected from the refrigerated sea water (RSW) storage tanks, 12 hour post harvest, were used to examine how the pumping of fish on board fishing vessels and the transportation to these tanks affect the microbiota. These findings are published in **Paper II**.

A surveillance programme on the presence of the parasitic nematode lar-

vae *Anisakis simplex* in pelagic fish species, showed that there were encapsulated larvae in the fish muscle at time of capture, confirming that these muscle-invading larvae not only enter the fish flesh post harvest. This raised questions about the nature of the microbiota of these larvae, and the possibility of these larvae to act as vectors transporting bacteria into the sterile fish muscle. An innovative hypothesis was proposed; The *Anisakis* larvae contain an indigenous microbiota in their own intestinal tract, and bring along bacteria from the fish intestine into the fish muscle. Furthermore, assuming that no *Anisakis*-induced bacterial contamination was spread within the fish muscle until the capsule and larvae were disrupted and thereby distributed during fish processing, *e.g.* production of fish mince, a second hypothesis was raised; The *Anisakis*-induced bacterial contamination of fish muscle will have negative effect on the shelf-life of a fish mince product. These hypotheses are outlined in **Paper III** and **Paper IV**.

List of publications

Paper I

Svanevik, C.S., Roiha, I.S., Lunestad, B.T. and Levsen, A. (*Submitted*). Microbiological assessment along the fish production chain of the Norwegian pelagic fisheries sector - results from a spot sampling programme.

Paper II

Svanevik, C.S. and Lunestad, B.T. (2011). Characterisation of the microbiota of Atlantic mackerel (*Scomber scombrus*). *International Journal of Food Microbiology*, 151 (2):164-170.

Paper III

Svanevik, C.S., Levsen, A. and Lunestad, B.T. (2013). The role of muscle-invading anisakid larvae on bacterial contamination of the flesh of post-harvest blue whiting (*Micromesistius poutassou*). *Food Control*, 30 (2):526-530.

Paper IV

Svanevik, C.S., Lunestad, B.T. and Levsen, A. (2014). Effect of *Anisakis simplex* (sl) larvae on the spoilage rate and shelf-life of fish mince products under laboratory conditions *Food Control*, 46 (12):121-126.

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Introduction

1.1 Marine fisheries

1.1.1 Global and pelagic fisheries

Most global marine fish resources seem to have reached their sustainable limit of exploitation. However, a growing and generally wealthier human population demands high quality food products, including fish, leading to an increase in global fish trade and higher fish prices (Food and Agriculture Organization, 2014c). The modern and specialised fishing vessels in today's global fishing fleet are so efficient that some estimates states that without proper regulation, all fish stocks that are utilised today, will be depleted within 2048 (Worm *et al.*, 2006). Though these predictions are somewhat pessimistic, the SOFIA report (The State of World Fisheries and Aquaculture, 2012) defines approximately 90% of all fish stocks harvested today as either fully-exploited, over-exploited or depleted. In 2011, 28.8% of all harvested fish stocks were fished at biological unsustainable levels. Fortunately, this portion has been decreasing since 2008 (Food and Agriculture Organization, 2014c). To ensure fair and sustainable captures, the volume of landed fish is regulated by fishing quotas set by national, and for some species, international governmental bodies. If wild capture fisheries should comply with the increasing demand for fish proteins and still provide stable catch volumes, an increase in edible fish products has to come from improved utilisation of every capture, by increased fish quality, extended shelf-life and reduced post-harvest loss.

One of the most harvested group of wild marine fish, are the pelagic species, which are found in the free water column in the ocean. The commercially exploited species are found in high volumes in nutritious areas over the continental shelf and in upwelling zones, *e.g.* the North Sea, the Barents Sea and the west coast of North and South America (Brockmann *et al.*, 1990; Moyle and Cech, 2004). Even though the pelagic zone is low in biological diversity, representing about 2% of known marine fish species (Moyle and Cech, 2004) the biomass of each species may be enormous. The fish feeds on zooplankton or smaller fish, and have typically silver coloured, streamlined body with a heavily forked tail adapted for high speed swimming. The characteristic schooling behaviour seen in many species, have important anti-predator advantages in the open oceans, but is an important factor allowing the specialised fishing vessels to capture large quantities of the same fish species at one position.

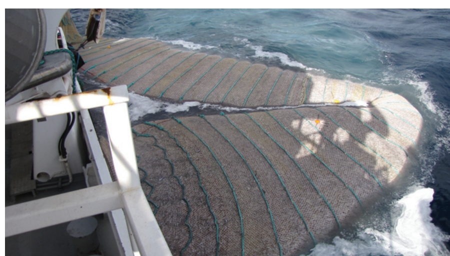
1.1.2 Volumes and values

Globally, the anchoveta (*Engraulis ringens*), sardinellas nei (*Sardinella spp.*), Atlantic herring (*Clupea harengus*) and chub mackerel (*Scomber japonicus*), are the most harvested pelagic species, accounting for 10.5 of the total 82.6 million tonnes of marine captures (Food and Agriculture Organization, 2014c). In Norway, the pelagic fish sector has experienced a significant increase in product prices the last decade, due to some fish species being limited and strictly regulated. Additionally, some species that commonly were used as ingredients in fish meal and fish oil production, have been captured for direct human consumption and therefore sold at higher prices. According to volume of capture, this sector comprise the largest fisheries in Norway, where the fishing fleet with more than 100 highly specialised ocean-going vessels, and approximately 150 registered fish landing factory operators, have a large production capacity (The Norwegian Directorate of Fisheries, 2015a,b). An

average of 1.4 million metric tonnes each year were landed through 2010-2013, which included Atlantic herring, Atlantic mackerel (*Scomber scombrus*), blue whiting (*Micromesistius poutassou*), capelin (*Mallotus villosus*), Norway pout (*Trisopterus esmarkii*), Sand lances (Ammodytidae), Atlantic horse mackerel (*Trachurus trachurus*) and European sprat (*Sprattus sprattus*). This gave an average first hand value of 5.8 billion NOK (900 million USD). Atlantic mackerel is the most valuable pelagic species in terms of NOK per kilo, and did during these four years, amount for 30% of the total pelagic income, but only 14% of the catch volume (The Norwegian Directorate of Fisheries, 2014). This species, together whit Atlantic herring, are important sources of marine fatty acids and proteins, and are therefore highly appreciated, also outside Norway. Concerning the 670.000 metric tonnes of landed Atlantic herring and Atlantic mackerel (2013), 85% was exported for approx. 6 billion NOK (920 million USD), where Russia (148't), Denmark (97't), China (69't) and Japan (64't) were the largest markets (Norwegian Seafood Council, 2014).

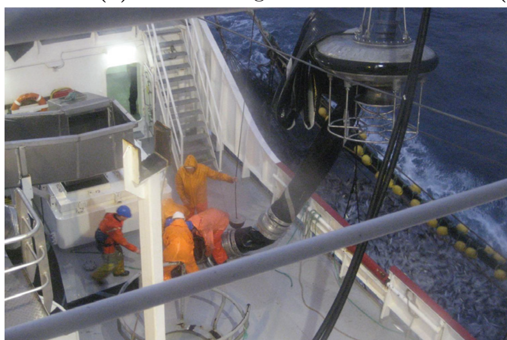
1.1.3 Catch and processing practises

The catching methods for pelagic fish are based on trawling with a cone shaped net dragged through the water column by a fishing vessel (Fig.1.1a), or by a purse seiner, where the fish are encircled by a large net with a closing mechanism at the bottom (Fig.1.1b). In both cases the catch are transferred on board into storage tanks by either a pumping device ($\approx$ 85 bar) (Fig.1.1c), or by a vacuum suction pump ($\approx$ 0.5 bar) (Fig.1.1d). The catch passes through a sift box to separate the fish from the sea water (Fig.1.1e), and through different sorting chambers (Fig.1.1f), to guide the fish into storage tanks (Fig.1.1g) which contains refrigerated sea water (RSW) at chilled temperatures ($-2\,^{\circ}$C).

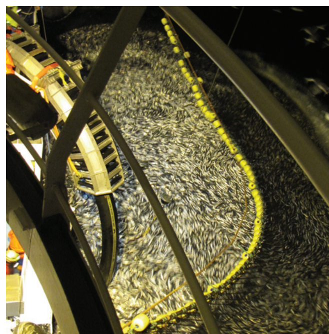


(a) Blue whiting in trawl

(b) Atlantic mackerel in purse seine



(c) On-board pumping by pressure

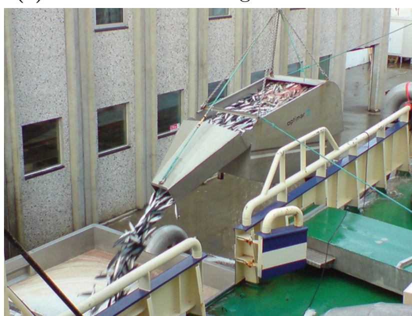


(d) On-board vacuum suction

(e) Sift box

(f) Sift box and sorting chamber

(g) Atlantic herring in RSW-tank



(h) From RSW-tank to landing tank

Figure 1.1: Catching and handling on board different Norwegian fishing vessels. Foto:NIFES

To maintain high fish quality during on-board storage and transportation, and to delay bacterial spoilage, the fish have to be transferred into the RSW storage tanks shortly after capture. The high velocity of fish during on-board pumping, could influence the fish quality by causing physically lesions in the skin and flesh (SINTEF, 2006), or by crowding and stress-related intestinal emissions contaminating the external surfaces of the fish with intestinal content (Svanevik and Lunestad, 2011). All steps during catch, on-board pumping, and storage introduces critical points of bacterial contamination before the fish enters the factory. During delivery and landing, the fish are either pumped into landing tanks (Fig.1.1h), or directly transferred by vacuum suction. In the factories, the fish are transported on conveyor belts, washed with potable water, sorted through sorting machines, processed though filleting or trimming machines, before packing and finalising of the product. In addition to the fish becoming exposed to surfaces and production waters along the production line, some manual steps are operated by trained workers. However, all these steps are critical points which introduces new risks of bacterial contamination and growth, also affecting the fish quality (Svanevik *et al.*, *Submitted*).

The current legislations, which applies for the fish industry, includes the "Law of Food Production and Food Safety (Food act)" (LOV-2003-12-19-124), the "Quality Regulations for Fish and Fish products" and the "Food Hygiene Regulations" (FOR-2008-12-22-1623; FOR-2013-06-28-844), which requires that all fish processing factories have internal controls based on a Hazard Analysis and Critical Control Points (HACCP) plan. Such a plan is not required from the fishing vessels, as they are defined as primary producers, thus all vessels must provide appropriate equipment for gentle and safe handling during capture and storage, as well as at delivery. It is important to underline that quality reduction is an irreversible process, where quality reducing factors adds up in the product during production. Handling techniques, han-

dling time and temperature during storage and production might have a large impact on the end-product, thus an improvement in one of these steps might have an important effect on the fish quality.

1.2 Bacteria in marine fish

1.2.1 Indigenous bacteria

The water temperature in the pelagic zone of northern temperate seas, range between −2 and +20 °C, and the bacterial biota is dominated of psychrothrophic and psychrophilic Gram-negative rod-shaped bacteria, representing members of the geni *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Shewanella* and *Flavobacterium*, as well as members of the families Vibrionaceae and Aeromonadaceae. Gram-positive bacteria of the geni *Bacillus*, *Micrococcus*, *Clostridium*, *Lactobacillius* and *Corynebacterium* also occur in variable proportions (Gram *et al.*, 1987; Gram and Huss, 1996). Most of these bacteria have a growth optimum at approximately 18 °C. Poikilotherm fish do not provide a temperature barrier against bacteria, thus the indigenous bacterial biota is influenced by the surrounding environmental biota, including any present contamination (Huss, 1997). The fish skin, with the overlying mucus layer, plays an important role in the immune system, protecting against microbial infection. However, immediately after death of the fish, the immune system is inactivated. At this point the internal organs and the muscles of healthy fish is considered sterile (Cahill, 1990; Nieto *et al.*, 1984), whereas the skin, the gills and the digestive tract, often contains high numbers of bacteria (Cahill, 1990; Gjerde, 1976; Herbert *et al.*, 1971; Huss, 1995). The indigenous bacteria of the fish intestine were examined for different purposes in this theses, including the examination of how intestinal emission might contaminate the surface of surrounding fish during pumping (Svanevik and Lunestad, 2011), and how the admission of intestinal

bacteria by muscle-invading *Anisakis* larvae subsequently contaminates the fish muscle post migration (Svanevik *et al.*, 2013, 2014).

1.2.2 Human pathogenic bacteria

The occurrence of human pathogenic bacteria originating from freshly caught marine fish is rarely reported, and most of the known bacteria that are present in fish have little influence on healthy people. In the majority of cases where fish have been the vehicle for the causative agent, fish and fish products have been cross-contaminated during handling and processing, followed by improper storage, preparation or reheating of the product. *Staphylococcus aureus*, *Salmonella enterica*, *Escherichia coli* and *Campylobacter jejuni* are all non-marine species that might cause infections when consuming cross-contaminated fish products. Some species, however, as *Aeromonas hydrophila*, *Clostridium botulinum*, *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *Mycobacterium marinum*, *Photobacterium damselae*, *Erysipelothrix rhusiopathiae* and *Listeria monocytogenes* are found in the marine environment and have been recognised as the causative human pathogens where fish were the vehicle (Novotny *et al.*, 2004). *A. hydrophila* and *V.alginolyticus*, together with several other species, *e.g.* *Morganella morganii Proteus vulgaris* and *Photobacterium phosphoreum*, have the ability to decarboxylate the amino acid histidine into histamine, giving histamine poisoning (Kanki *et al.*, 2004; Shalaby, 1996; Emborg and Dalgaard, 2008). This is refereed to as scombrotoxin poisoning, when associated with fish, as species of the Scombroid family *e.g.* mackerel and tuna, often are involved.

The low risk that fish represents with respect to human bacterial pathogens, was confirmed by a ten-year study of all registered foodborne illnesses and hospitalisations in the United States of America (1998-2008), where only 0.4% of the cases were related to the ingestion of fish products. However, 60% of all

illnesses caused by chemical substances were due to scombrotoxin (Painter *et al.*, 2013). In 2013, The Rapid Alert System for Food and Feed (RASFF) concerning imported food in EU/EØS countries, reported 31 cases for fish and fish producst, where *Salmonella* and *Listeria monocytogenes* were found in 3 and 27 cases, respectively, in addition to one larger outbreak of histamine poisoning (European Commission, 2013). In the Norwegian Zoonosis report for 2013, marine fish were not involved in any reported cases, though the presence of *Listeria monocytogenes* in rakfisk (limnic *Salmo trutta*), resulted in one death (Norwegian Veterinary Institute, Norwegian Food Safety Authority, Norwegian Institute of Public Health, 2013). The world wide implications of fish as vehicle for causative agent varies according to local traditions of seafood consumption, where in Japan 70% of foodborne illnesses have been related to seafood, compared to 20% in Australia (Butt *et al.*, 2004b).

1.2.3 Hygiene aspects

During capture and landing, the fish are exposed to surfaces of different handling equipment and processing water, which all represents critical points for bacterial contamination. On board fishing vessels some of the equipments are exposed to the open air (*e.g.* the pump nozzle and the sift box), which increases the possibility of contamination from seawater and droppings from sea birds. At the factories, the fish are exposed to surfaces of *e.g.* conveyor belts and filleting machines, but also workers operating the machines. The industry must meet these challenges with adequate cleaning and processing regimes, to maintain high quality and to ensure food safety, as some microbial species are able to colonise and survive in the production environment (Gram *et al.*, 2007; Kusumaningrum *et al.*, 2003). Since many foodborne pathogens are transmitted through the faecal-oral route, a possible cross-contamination might occur during handling in the production environment. This is assessed

by analyses for specific indicator organisms of faecal contamination, including coliforms, thermo-tolerant coliforms, and presumptive *E. coli*, as well as enterococci, which have a longer survival outside the mammalian intestine (Noble *et al.*, 2004). The enterotoxin producing *Staphylococcus aureus*, originating from skin and nasopharynx of humans, is sensitive to microbial competition, and are therefore of more concern when cross-contamination occurs in heat treated products (Le Loir *et al.*, 2003).

Listeria monocytogenes occur naturally in the aquatic environment and in fish raw material, and is of great concern when found in the production environment. This is related to the biofilm forming ability of this bacterium, which allows settlement even on stainless steel and other smooth surfaces. *L. monocytogenes* is therefore difficult to eliminate, once it has established and several studies have documented persistent strains of *L. monocytogenes* within the same factory, *i.e* house strains (Bagge-Ravn *et al.*, 2003; Leong *et al.*, 2014; Holch *et al.*, 2013). The spot sampling programme described in **Paper I** found contamination by *Listeria monocytogenes* and *E. coli*, in the production environment and on fish products. Despite that *Salmonella* are not considered problematic in the Norwegian pealgic sector, the pathogen is of great concern in the countries to where most of the fish are exported (Amagliani *et al.*, 2012), and *Salmonella* analyses were therefore included.

1.2.4 Quality assessment

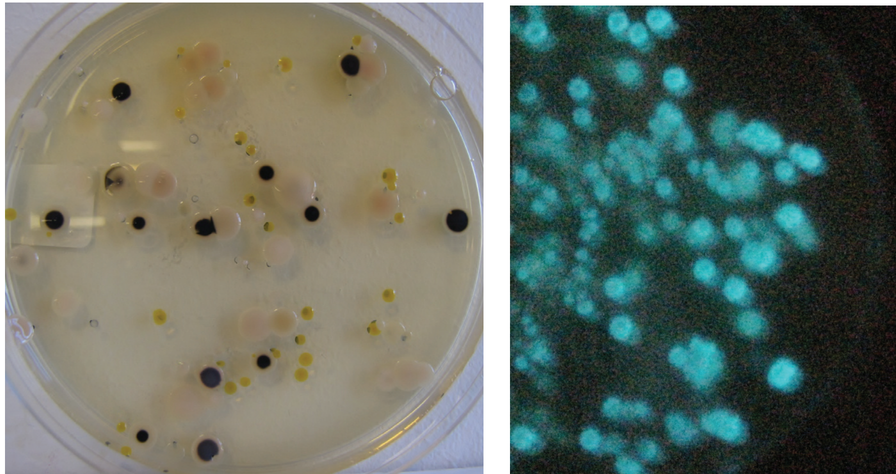
The term fish quality refers to the properties of a fish product, which should appear appetizing as well as nutritious and safe for the consumer. Quality is however a subjective term, as the different quality requirements apply for different products. For instance, fish used for fish mince production are often considered to have lower quality compared to fish used for fillet production, but both end products could still be of high quality. The bacterial growth,

recorded as colony forming units (CFU) per gram product, does not necessarily reflect the product quality, and there are currently no legislation setting the CFU limits for what is good or bad quality. However, the former Norwegian quality regulation for fish and fishery products (FOR-1996-06-14-667) presented some guidelines for fresh fish stored aerobically, where together with sensory evaluations, the CFU on IAL should not exceed 6.7 log per gram sample, though other growth media as Long and Hammer, and Marine agar could give higher CFU numbers (Broekaert *et al.*, 2011; Dalgaard *et al.*, 1997).

Personal taste and marked availability may also affect the consumers perception of a product, and it is, in the end, the customers evaluation of the product and product quality that are of importance, and the customers are often more strict than the given legislation. Fish of lower quality, which is rejected either from the marked, or at the fish processing plant, accounts for 10 - 25% of the total global wild-captured fisheries (Food and Agriculture Organization, 2014a,b). This post-harvest loss is mainly due to mechanical inflicted injuries on the fish as skin lesions, the presence of visual parasites, or bacterial deterioration and spoilage. This means that to ensure high product quality, all steps from capture to landing has to be performed in the best way possible, having quality in focus all the way.

1.2.5 Specific spoilage bacteria (SSB)

All kind of food stuff have a bacterial biota, which specialise in the breakdown and decomposition of its nutrients, and these are referred to as specific spoilage bacteria (SSB). Fish muscles serves as a rich growth substrate to some psychrotrophic bacteria, due to the low levels of carbohydrates, relatively high pH (6.2 - 6.5), high water activity ($a_w \approx 0.99$), and high amounts of non-protein nitrogen (NPN), *e.g.* trimethylamine oxide (TMAO) (Gram and Huss, 1996; Nychas *et al.*, 2007). The specific spoilage bacterias (SSBs) utilise



(a) H_2S-producing bacteria (black center).

(b) Fluorescent bacteria.

Figure 1.2: Bacteria grown on IAL (20 °C for 72 h) collected from; (a) the gills of Atlantic mackerel (**Paper II**) where black colonies are H_2S-producing bacteria with iron sulphide precipitation, (b) the intestine of blue whiting (**Paper III**) where blue colonies are fluorescent bacteria emitting light.

amino acids, carbohydrates and other organic compounds in different steps of their metabolism, which in turn produces off-odours and off-flavours (Huss, 1995). The appearance of different spoilage compounds in different foods and food products, which are bacterial induced, depends on the storage and temperature conditions of the products. For marine fish from temperate waters stored aerobically under refrigerated conditions, the reduction of TMAO to trimethylamine (TMA) and the production of hydrogen sulphide (H_2S) from sulphur (S) containing substrates, are the most recognised spoilage products, giving the foul smell of rotten fish and rotten eggs, respectively (Gram, 1992; Nychas *et al.*, 2007).

The TMA concentration in a fish product could be measured by the titration method (Conway and Byrne, 1936), and should for instance not exceed 35 mg of nitrogen/100 g sample for fish from the family Gadidae (EC - No

2074/2005). The fish mince in **Paper IV** was made of Atlantic cod (*Gadus morhua*), and the highest measurement recorded was about 7 mg of nitrogen/100 g (Svanevik *et al.*, 2014). The presence of H_2S-producing bacteria could be measured by the indicative Iron Agar Lyngby (IAL) medium containing cysteine, which some bacteria utilize to make pyruvat. H_2S is a biproduct of this reaction, and when released into the IAL medium, ferric iron, Fe(III), is reduced into ferrous iron, Fe(II), and iron sulphide (FeS) is formed as black precipitation. The H_2S-producing bacteria are recognised as colonies with black center (Fig.1.2a). The observed shelf-life of fish may vary between fish species, storage temperatures, atmosphere conditions and initial bacterial load and composition (Huss, 1994; Adams and Moss, 2008). Fish stored aerobically on ice are, in general, rejected when the bacterial numbers of SSBs reaches $10^8 - 10^9$ CFU per gram on IAL (Gram and Huss, 1996). However, most fish products are rejected after sensory evaluation by the consumer before the SSBs reaches these values.

One of the most common spoilage bacterium in fish stored at low temperatures, is *Shewanella putrefaciens*, a rod-shaped, Gram-negative, γ-Proteobacteria. Members of the family Shewanellaceae are highly specialised in anaerobic respiration, though they also grow very well in the presence of oxygen. Producing both H_2S and TMA (Ringø *et al.*, 1984; Perry *et al.*, 1993; Gram, 1992; Gram and Dalgaard, 2002), *Shewanella* species have been isolated and characterised as the domination spoiler of chilled fish stored aerobically, as Atlantic cod (*Gadus morhua*), plaice (*Pleuronectes platessa*), anglerfish (*Lophius piscatorius*), common sole (*Solea solea*) and Atlantic mackerel (*Scomber scombrus*) (Tab.1.1), but also in smaller concentrations in modified atmosphere packed (MAP) fish as farmed Atlantic cod (Hovda *et al.*, 2007b) and farmed Atlantic halibut (*Hippoglossus hippoglossus*) (Hovda *et al.*, 2007c).

The halophilic, rod-shaped, Gram-negative, vibrio-like *Photobacterium phos-*

phoreum, is naturally found in the marine environment of both sediments and seawater, often recognised by its fluorescent abilities (Fig.1.2b). The bacterium is able to grow in air, but fermentative respiration is preferred, as the bacterium have a high tolerance for CO_2 (Dalgaard *et al.*, 1997; Farmer and Hickman-Brenner, 2006). This bacterium is able to produce high amounts of TMA, due to the large cell size with high metabolic activity. It is therefore often associated with spoiled modified atmosphere packed (MAP) products, which are also found to spoil at lower SSBs numbers (10^7 CFU per gram) (Dalgaard, 1995). *P. phosphoreum* has been found to spoil several fish species, *e.g.* Atlantic cod, plaice and saithe (Dalgaard, 1995; Dalgaard *et al.*, 1997; Hovda *et al.*, 2007b; Debevere and Boskou, 1996) (Tab. 1.1), and was found as the dominating spoiler of fish mince made from *Anisakis*-infected fish (Svanevik *et al.*, 2014).

Several members of *Pseudomonas* are also found to spoil fish, in addition to other proteinous food stuff (Gram and Huss, 1996; Ercolini *et al.*, 2010). The most common species are *Pseudomonas fragi*, *P. putida* and *P. fluorescens*, which are often associated with milk, giving the sweet rotten odour from ethyl ester production. These bacteria do not produce H_2S, and only some strains produce TMA, but they are still found to dominate the spoilage bacteria, when present, due to enhanced competitive advantages by the production of siderophors (Gram and Melchiorsen, 1996; Gram *et al.*, 2002). *P. fluorescens* has been associated with the production of mehtyl mercaptane and dimethyl disulfid, both adding to the foul smelling of deteriorated food (Miller *et al.*, 1973b). Even though members of *Pseudomonas* not necessarily are airophilic, they are sensitive to increased CO_2 concentrations, and therefore not associated with MAP treated products (Zhao *et al.*, 1992).

Additionally, some other species have also been found as active SSBs in fish. This includes the marine vibrios, which have been associated with spoiled

seafood after temperature abuse (*e.g.* 20 °C for some time), as most vibrios favour higher temperatures for growth (Gram *et al.*, 1987; Adams and Moss, 2008). Members of *Psychrobacter* have been identified as active spoilers in rehydrated salt-cured Atlantic cod, producing a musty odour, probably due to the breakdown of phenylalanine (Juni and Heym, 1986; Bjørkevoll *et al.*, 2003). *Brochothrix thermosphacta* are most often associated with the spoilage of fresh meat, but have in some cases been associated with MAP treated meat and fish (Adams and Moss, 2008; López-Gálvez *et al.*, 1998). Similarly, members of the lactic acid bacteria (LAB) could play an important role in spoilage of lightly preserved MAP treated fish, where the combination of low pH and low oxygen levels favours the growth of LABs (Françoise, 2010; Nychas *et al.*, 2007). Since many of the known SSBs of fish are found among the indigenous biota, on the skin and in the intestine of the fish, as well as in the associated seawater (Cahill, 1990; Austin, 2006; Nayak, 2010), the development of spoilage will depend on the extrinsic factors such as handling and storage conditions.

Table 1.1: The specific spoilage bacteria associated with some marine fish species of Northern temperate waters.

	Atmosphere:Air		Atmosphere:MAP	
Fish species	Bacteria species	Sources	Bacteria species	Sources
Atlantic cod *Gadus morhua*	*Ph. phosphoreum* *Sh. frigidimarina* *Sh. baltica* *Sh. putrefaciens* *Ps. putida*	Broekaert *et al.* (2011) Vogel *et al.* (2005)	*Ph. phosphoreum* *Pseudomonas* sp.	Dalgaard (1995); Debevere and Boskou (1996) Dalgaard *et al.* (1997) Hovda *et al.* (2007b)
Plaice *Pleuronectes platessa*	*Ph. phosphoreum* *Sh. frigidimarina* *Sh. baltica*	Broekaert *et al.* (2011) Vogel *et al.* (2005)	*Ph. phosphoreum*	Dalgaard *et al.* (1997)
Angler fish *Lophius piscatorius*	*Ps. fragi* *Sh. frigidimarina*	Broekaert *et al.* (2011)		
Saithe *Pollachius virens*	*Ph. phosphoreum*	Broekaert *et al.* (2011)	*Ph. phosphoreum*	Dalgaard *et al.* (1997)
Common sole *Solea solea*	*Br. thermosphacta* *Sh. frigidimarina* *Shewanella* sp.	Broekaert *et al.* (2011) López-Gálvez *et al.* (1998)	*Br. thermosphacta* *Lactobacillus* sp.	López-Gálvez *et al.* (1998)
Atlantic mackerel *Scomber scombrus*	*Ps. fragi* *Sh. frigidimarina*	Broekaert *et al.* (2011)		
Haddock *Melanogrammus aeglefinus*	*Ph. phosphoreum* *Psychrobacter* sp. *Pseudomonas* sp.	Reynisson *et al.* (2010) Olafsdottir *et al.* (2006)		

1.2.6 Abiotic quality parameters

At quality inspections of fish products, several abiotic measurements are included. For example, the pH could change during storage and bacterial growth, and then affect the bacterial community in the samples. The presence of different biogenic amines, and some other chemical compounds, could also reflect the quality and the bacterial activity, as some of these are bi-products from bacterial metabolism, including TMA and H_2S. Spoilage compounds produced by bacteria should not be confused with spoilage compounds produced during autolysis or chemical degradation (Fig.1.3). Autolysis is the self-digestion of a food product and is the first degradation to start in foods, since some enzymes are still active for some time after harvesting. There are also compounds associated with chemical spoilage, where the products are degraded chemically rather than by bacterial activity. A common chemical degradation process is the oxidation of fat, rancidification, which is often related to improper storage conditions.

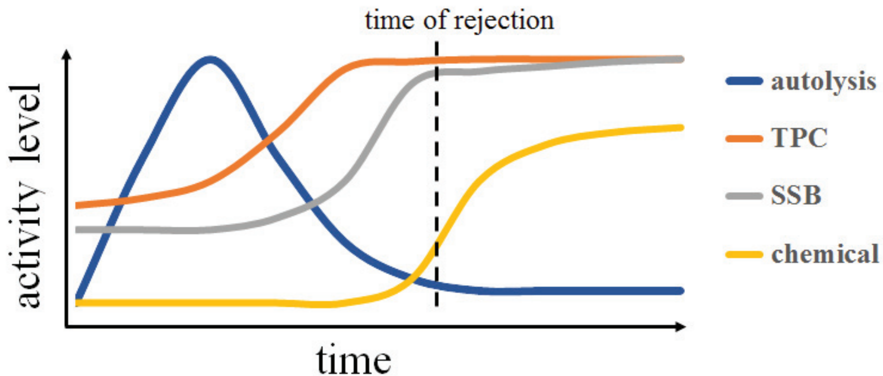


Figure 1.3: Sketch of the activity level of different spoiling processes, in time, starting with autolytic degradation, followed by bacterial growth (TPC) and increased SSBs and ended by chemical degradation. Modified after Gram and Huss (1996).

One of the most important actions against bacterial growth during storage and processing of fresh fish, is temperature control (Gram and Huss, 1996). On board fishing vessels, storage tanks with RSW ($-2\,^{\circ}$C) will, in addition to inhibit the activity of some autolytic enzymes, reduce the bacterial growth for even the psychrotrophic and psychrophilic bacteria. However, the survival of these bacteria will be good. During landing and processing the temperature should also be kept low, including inside the production facilities, to avoid escalated growth of these bacteria.

1.3 Parasitic nematodes in marine fish

Even though flesh of newly caught and healthy fish is considered to be sterile (Cahill, 1990; Huss, 1995), many of the commercially important pelagic fish species from Northeast Atlantic fishing grounds, *e.g.* blue whiting (*Micromesistius poutassou*), Atlantic herring (*Clupea harengus*) and Atlantic mackerel (*Scomber scombrus*), are commonly infected with the larvae of parasitic nematodes, with *Anisakis simplex* as the most prominent species (Llarena-Reino *et al.*, 2012; Levsen and Lunestad, 2010; Levsen *et al.*, 2005; Llarena-Reino *et al.*, 2012; Butt *et al.*, 2004a). The fish act as transport hosts, transferring the larvae to the definitive host, which for the *Anisakis* spp. are various cetacean species. The parasite reaches the fish host when the fish feeds on *Anisakis*-infected planctonic crustaceans (Fig.1.4). After ingested by a fish, the parasite usually penetrates the intestinal wall and enters into the visceral cavity, where they subsequently encapsulate on internal organs, or further migrate into the fish muscle. This behaviour is the reason for the attention parasites receive from food safety authorities and consumers, since accidental ingestion of live larvae may cause anisakiasis, *i.e.* infection with larval anisakid nematodes. The infection is characterised by abdominal pain, diarrhoea and

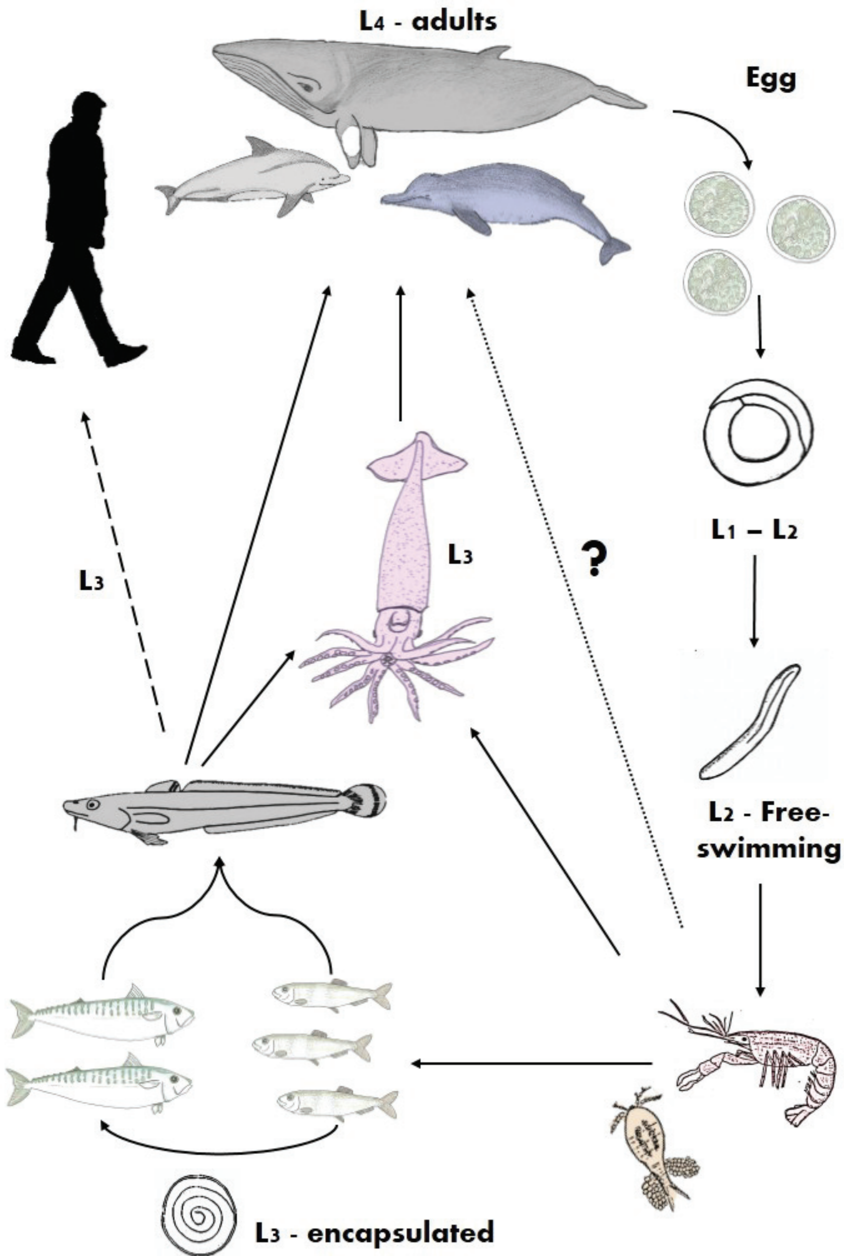


Figure 1.4: The life cycle of *Anisakis simplex*. At the third larval stage (L3), the parasite may pass through several trophic levels, until maturing into adults in different whale species. Humans are not a natural host, and the parasite will not develop here. Modified after Huss and Embarek (2004).

nausea, and in some cases allergic reactions (Jay *et al.*, 2005; Valls *et al.*, 2005; Caballero *et al.*, 2011). The current EU and Codex regulations specify that any fish product originating from wild stocks, and is to be consumed raw or almost raw, has to undergo freezing at a core temperature of −20 °C for at least 24 hours in order to destroy any nematode larvae possibly present (EC - No 853/2004; CODEX STAN 244-2004). These parasites are, therefore, of most concern when consuming raw, semi-raw or lightly preserved fish products (Jay *et al.*, 2005).

Some other nematodes are found in fish of Northern marine waters, and includes *Pseudoterranova decipiens* (Bristow and Berland, 1992),*Contracaecum osculatum* (Køie and Fagerholm, 1995) and *Hysterothylacium aduncum* (Klimpel and Rückert, 2005). Even though anisakidosis most often is caused by *A. simplex*, infections caused by *Pseudoterranova decipiens* has been reported (Mehrdana *et al.*, 2014). The liver worm, *Contracaecum osculatum*, has been found to cause infections in humans in some few cases (Schaum and Müler, 1967), whereas *Hysterothylacium aduncum*, also present in many commonly consumed marine fish species, matures in fish and is not able to cause infections in humans (Klimpel and Rückert, 2005). The nematode larvae examined in the work included in this thesis, are most likely to be the species *A. simplex*. However, since no molecular identification was performed, the larvae are referred to as *Anisakis* larvae in the upcoming text.

1.4 Parasite-induced bacterial contaminations

Studies of the symbiosis between bacteria and nematodes have been done for several environments, such as sediments and deep-sea habitats (Polz *et al.*, 1992), where the bacterivourous nematodes feeds on bacteria. Little is, however, known about the larval stage of these nematodes, including the fish

parasite *Anisakis simplex*, which has been in focus in **Paper III** and **Paper IV**. These larvae are found in the intestine of the fish, and are likely to get coated with intestinal bacteria on their surface before migrating into the visceral cavity of the fish, and further into the flesh. This could lead to a bacterial contamination of the fish flesh, where the parasite act as a vector for transmitting these bacteria. Additionally, the *Anisakis* larvae may contain an internal microbiota of their own, in their own intestinal tract, either established in the early larval stage, or obtained after feeding on faecal matter in the intestine of the fish (host) (Svanevik *et al.*, 2013).

If parasites can bring along bacteria into the fish, the presumption that fish muscle of freshly caught fish is sterile and free of any bacteria, is no longer legitimate. And, in those cases where the fish is processed in such way that the capsule between the parasite and the fish (host), or even the whole parasite, is disrupted, *e.g.* as in fish mince products, the bacterial contamination of the flesh increases the bacterial load throughout the product, and might effect the degradation of the product. This is not necessarily in a negative way, as the composition and the synergy of the introduced bacteria affects the bacterial behaviour and development in the fish product. The experiment described in **Paper IV** found that a change in the bacterial composition of the fish mince introduced by the *Anisakis* larvae, changed the bacterial growth and spoilage rate (Svanevik *et al.*, 2014). This could be related to the ability of some bacteria to affect the growth of other bacteria, either by metabiosis, where growth is enhanced, or by antagonisms *e.g.*, the production of antibacterial substances as toxic biogenic amines. Some bacteria produce siderophores, which enhances the iron uptake, giving competitive advantages (Gram *et al.*, 2002; Gram, 1993; Gram and Melchiorsen, 1996), which might have been the case for the fish mince samples without *Anisakis* larvae, where *Pseudomonas* was identified as the dominant spoiler.

1.5 Identification of bacteria

1.5.1 Conventional microbiology

The traditional way to classify bacteria has been based on fenotypic characteristics, similar to the system used in eukaryote systematics. The bacteria are divided into two major groups, Gram-positive and Gram-negative bacteria, depended on their cell wall composition (Buck, 1982; Gram, 1884). Further classification is performed by morphological features such as shape (*e.g.* cocci, rod or corkscrew), cell chains, motility, pigmentation and endospores. Additional characteristics includes biochemical properties, such as the ability to utilise oxygen as terminal electron acceptor, production of specific enzyme (*e.g.* catalase or oxidase), toxin production, sugar fermentation, and degradation of amino acids and other organic molecules. These features could be tested by selective growth media or by biochemical identification kits *e.g.* the API (bioMerieux) used in **Paper II**. Conventional microbiology require the bacteria to be able to grow under artificial growth condition. This could exclude a broad range of the bacteria that are found in samples with complex bacterial communities, such as in fish (Vartoukian *et al.*, 2010; Wilson *et al.*, 2008; Spanggaard *et al.*, 2000). However, many pathogens, as well as the majority of SSBs, could be retrieved by cultivation and these methods are therefore still highly applicable for many microbiological experiments (Cambon-Bonavita *et al.*, 2001; Dalgaard *et al.*, 1993).

1.5.2 The 16S rRNA gene

Modern gene based technology enables us to classify bacteria and study their phylogeny by comparing the similarity of selected parts of the genome, by culture-independent methods. The determination of the complete nucleotide sequence of the 16S subunit ribosomal RNA gene (16S rRNA gene) (Brosius

et al., 1978) (Fig. 1.5), and the development of the polymerase chain reaction (PCR) method (Saiki *et al.*, 1985, 1988) allowing *in vitro* amplification of the deoxyribonucleic acid (DNA), made it possible to develop primers that could bind specific parts of the DNA, coding for the corresponding ribonucleic acid (RNA).

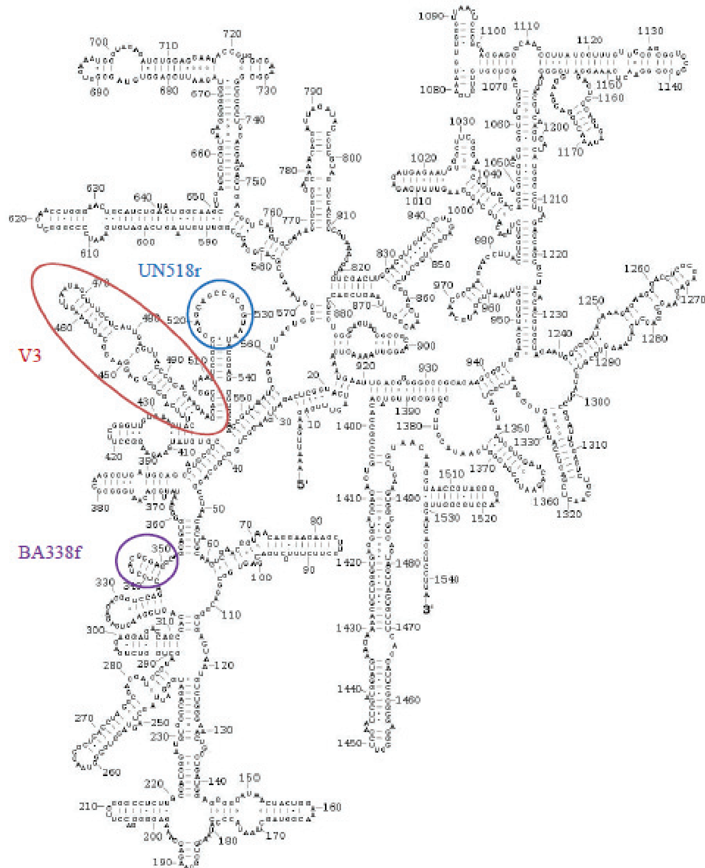
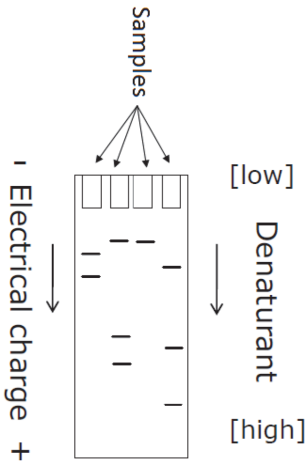


Figure 1.5: The complete 16S rRNA gene of *Escherichia coli*. The purple and the blue circles indicate, respectively, where the forward primer BA338f and the reverse primer UN518r will attach to the 16S rRNA gene. The red circle marks the hyper variable region V3, which is a common target region.

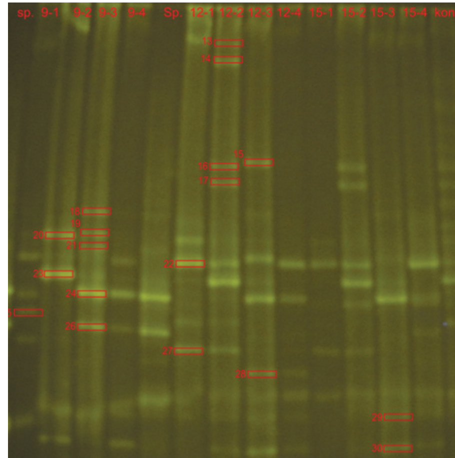
This DNA sequence is often referred to as $16S$ rDNA or $16S$ rRNA gene. In the following text it will consequently be referred to as $16S$ rRNA gene. This gene is the most commonly used when examining bacterial diversity because it consists of both non-variable conserved regions, and regions that are highly variable among the different bacterial species. Woese (1987) described the $16S$ rRNA gene as the most suitable molecular chronometer because it consists of many domains with positions that change at different rates. The $16S$ rRNA gene exist in all bacteria, thus this gene is used to examine the phylogeny of different species based on the percentage of common sequences in a specific region.

1.5.3 Denaturing gradient gel electrophoresis (DGGE)

The denaturing gradient gel electrophoresis (DGGE) is applied on complex samples containing several bacterial species that have to be separated before sequencing and identification. The principles are based on the ability of double-stranded DNA fragments of the same length, but different in sequence, to dissociate, *i.e* denaturate or melt, into a single stranded form (Fischer and Lerman, 1983). In a polyacrylamide gel with increasing concentration of a denaturing agent *e.g.* a mixture of urea and formamide, a constant high temperature and an electric current (DC), the DNA fragments will appear as bands at different positions. A DNA fragment consists of "melting domains" characterized as stretches of base-pairs with an identical melting temperature, which is sequence specific (Muyzer and Smalla, 1998). The different DNA fragments will therefore dissociate at different positions through the gel towards the positively charged end, depending on the A-T (adenine-thymine) and the G-C (guanine-cytocine) ratio of the sequence. This is because the G-C base-pairs form triple bonds and need a higher concentration of denaturing agents to dissociate, compared to the A-T base-pairs, which form double



(a) Sketch of the main pricipals of a DGGE gel.



(b) Picture of a DGGE gel stained with SYBR®Gold and exposed to UV-light.

Figure 1.6: Principals of DGGE. Parallel bands found at different positions down the gel may represent different bacterial species found in the same sample.

bonds. When the temperature and concentration for the lowest melting domain is reached, the molecule will dissociate and becomes branched. This will slow down the migration speed and can be seen as bands when examining the gel. Parallel fragment bands will spread through the gel as the concentration of the denaturant increases, and the fragments with highest G-C ratio will migrate the furthest (Fig.1.6). The denaturing gradient, the running time and the running voltage will affect the quality of band separation throughout the gel.

1.5.4 Sequencing and data processing

Sanger sequencing (Sanger *et al.*, 1977) has been an important method for identifying the nucleotide sequences of purified DNA fragments. In a later modified method, a PCR with a nucleotide mix of deoxyribonucleotide triphosphate (dNTP) and the fluorescent dideoxyribonucleotide triphosphate (ddNTP)

(one fluorescent color for each nucleotide) is most common. The ddNTP contains a H atom at the 3' end instead of an OH group, which will stop the polymerase to copy the fragment. The chance of a ddNTP to be inserted at one position in each copy during the PCR run, results in different sized fragments with a fluorescent nucleotide at the end. The fragments are separated by size by electrophoresis and the excited light at each position gives the order of the nucleotides (Rosenblum *et al.*, 1997).

Obtained sequences are searched for similarity by the Basic Local Alignment Search Tool (BLAST), using blastn query with the nucleotide collection (nr/nt) database and the megablast program selection. Venter *et al.* (2004) recommends that only sequences with match $< 97\%$ in BLAST are recognised as identical.

Methodological considerations

2.1 Research cruises and locations

An important prerequisite for samples used in this thesis, was that they all should be collected during research cruises with fishing vessels during authentic commercial catches, or from fish processing factories during production. The results from the work presented could therefore be applied directly for the fish sector, as all samples represents fish that were intended for human consumption.

The research cruises were organised by the National Institute of Nutrition and Seafood Research (NIFES) during the actual catch season of the fish species of interest. Samples included in **Paper I** were collected during several cruises in the Atlantic Ocean, the North Sea, the Norwegian Sea and the Barents Sea. Additionally, some vessels were examined when docked for delivery, and samplings at fish processing factories were included as well. These samplings were conducted during the years 2005-2014. The sampling for **Paper II** was performed in the North sea in 2009, whereas the samplings for **Paper III** and **Paper IV** were performed west of Ireland, in the Atlantic Ocean, in 2011 and 2012. Those samplings that were performed at sea are included in Fig.2.1.

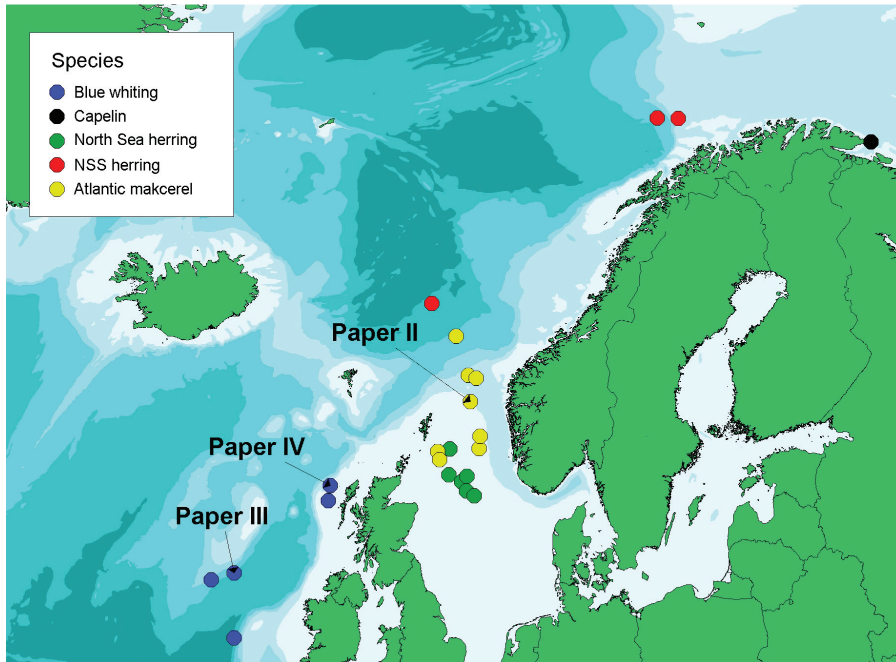


Figure 2.1: The position for samplings included in this thesis. Samplings for **Paper II, III** and **IV** are marked, whereas all the other points are samplings for **Paper IV**.

2.2 Sampling

All fish collected for paper **Paper I**, **Paper II** and **Paper III**, were from authentic capture- and landings. For **Paper II** and **Paper III**, the fish samples were collected using sterile techniques, which involved lowering an autoclaved perforated metal bucket into the purse seine and the RSW storage tank, to collect the fish. The fish in **Paper I** were aseptically collected, either from the RSW tanks prior to landing, or at different sites during the production line at the fish processing factory. All fish were handled, using glows washed in 70 % ethanol, before transferring into sterile plastic bags for transportation on ice, to the laboratory. Samples from surfaces of equipments (**Paper I**) were collected by different commercial kits *i.e.* Hygicult Dip-slides (TPC

and E/*beta*-GUR) and PathCheck swabs (*Salmonella* and *Listeria*), or sterile hydrated swabs. In **Paper IV**, only the *Anisakis* larvae were collected from freshly caught blue whiting (*Micromesistius poutassou*), whereas the fish mince which was made as surimi substitute, was made of farmed cod (*Gadus morhua*) prior to the experiment start. Farmed cod, belong to the same family as blue whiting, are fed formulated feed and are not likely to have any *Anisakis* infections in the body muscle, thus these were chosen as raw material for fish mince without *Anisakis* larvae. The pre-made cod mince was aliquoted in sterile bags with 100 grams in each, and subsequently frozen at $-20\,^{\circ}\mathrm{C}$ for about two months. The fish mince bags were slowly thawed at $4\,^{\circ}\mathrm{C}$ the night before the experiment started.

2.3 Cultivation

Samples in **Paper II** and **Paper III** were prepared for cultivation based analyses in a provisional laboratory on board the fishing vessel. This had some disadvantages, as the experiments are more to be considered as field studies, which introduces some biases compared to a controlled laboratory experiment. However, in these experiments the preparation of the samples shortly after capture were evaluated as the most important action against other biases, as the time from the fish dies until it is analysed, will affect the bacterial number and composition in the samples. The laboratory was thoroughly washed and disinfected before start. Hygicult Dip-slides and PahtCheck swabs in **Paper I** were incubated during the cruises in small travel incubators at temperatures according to the protocol. All the cultivation steps that were done in **Paper IV**, and of fish and hydrated swab samples in **Paper I**, were performed in a laboratory on shore at NIFES.

In all examination of fish, the Iron Agar Lyngby (IAL) growth medium was

chosen. The reason for this choice was that in addition to be a general medium for bacteria from temperate waters, this medium expresses the presence of H_2S producing SSBs as black colonies, after incubation at 20 °C for 72 hours (Gram *et al.*, 1987; Gram, 1992). A minor modification from the original protocol was made, where 1 % NaCl was added to increase the recovery of some halophilic Vibrionaceae species (Baumann *et al.*, 1984; Farmer and Hickman-Brenner, 2006). Despite the obvious advantage of this media, where the H_2S producing SSBs are expressed, the heterotropic plate count (HPC) on IAL are often lower than parallels grown on Marine agar or Long & Hammer agar (Broekaert *et al.*, 2011). Culture-based analyses will always be restricted to those bacteria that are able to grow in or on artificial growth media, and will not necessarily reflect the bacterial diversity. On the other hand, bacteria have to grow to give spoilage, thus most spoilage bacteria are quantified and identified by cultivation methods (Gram and Dalgaard, 2002). In addition to the morphological characterisations of the isolates obtained on IAL, the API® identification kits was applied, including including API®20E, API®20NE and API®50CHB. These kits are, however, developed for bacterial species with optimum temperatures of 30 − 37 °C, hence they failed to identify several isolates.

2.4 Molecular identification

For identifying bacterial species in mixed cultures from IAL plates, *i.e* bulk cells, the PCR-DGGE approach were applied for **Paper II**, **Paper III** and **Paper IV**, where a high amount of bacterial DNA was obtained and several different species were identified. When examining the non-culturable species, by extracting DNA directly from the sample tissue, a lower diversity was obtained. Especially concerning the skin samples. The main challenge was

the lack of proper 16*S* rRNA gene PCR products, despite a high DNA yield after DNA extraction. The DNA yield consisted probably of high amounts of eukaryotic fish DNA, which might have concealed the bacterial DNA that were present in the samples. This could be the case in **Paper II**, which complies with other studies (Bakke *et al.*, 2011; Hovda *et al.*, 2007a) where the same primers gave bands in the DGGE that were identified as teleost DNA. The search for sequence matches in BLAST was done according to Venter *et al.* (2004), where only sequences with match < 97 % were recognised as identical. The 16*S* rRNA gene is, however, not as variable within all bacterial families, whereas members of the Vibrionaceae could be difficult to differentiate. The use of other protein-encoding genes as *rpoD* and *toxR* have been suggested as alternative targets for identifying different vibrios (Pascual *et al.*, 2010). This was not included in this work, as many species still are differentiated by the 16*S* rRNA gene.

Results and discussion

The overall goal of this thesis was to increase the knowledge of bacteria associated with the Norwegian pelagic sector, by identifying critical points during fish handling that could be improved for better utilisation of the pelagic fish species. The highlights of results from the four written papers included in this thesis, are presented and discussed in the following sections. It is important to underline that the presence of muscle-invading *Anisakis* larvae (**Paper III and IV**) is not a quality parameter that the sector can easily control, and the aim of the work presented was to achieve knowledge about the bacteria found in these larvae and how these bacteria might affect the degradation and shelf-life of fish mince products.

3.1 Indigenous marine bacteria

In the work presented, the indigenous bacteria of Atlantic mackerel (*Scomber scombrus*) were characterised. Additionally the bacteria found in the intestine of blue whiting (*Micromesistius poutassou*) and the bacteria associated with *Anisakis* larvae, were described.

3.1.1 Atlantic mackerel

The experiment presented in **Paper II** identified the indigenous micorbiota of Atlantic mackerel, where samples from gills, skin and intestinal content were withdrawn from fish collected in the purse seine. The heterotropic plate count (HPC) were log 3.7 $\pm$ 0.6, log 2.4 $\pm$ 0.4, and log 6.2 $\pm$ 0.6 CFU per gram, for

gills, skin and intestinal content, respectively. The number of H_2S-producing bacteria were log 1.9 ± 0.5, log 2.6 ± 0.1, and log 3.9 ± 0.6 CFU per gram. The intestinal content had the highest number of HPC per gram, and also the highest number of H_2S-producing bacteria *e.g.* specific spoilage bacterias (SSBs), which indicates that any contamination by the intestinal content will increase the number of spoilage bacteria.

The bacterial profile complied with what the literature describes for marine fish species (Gjerde, 1976; Gram and Huss, 1996; Austin, 2006), and was dominated by Gram-negative taxa, which have all been previously isolated from marine samples. This included the genera *Bizonia*, *Oceanisphaera*, *Photobacterium*, *Proteus*, *Pseudoalteromonas*, *Psychrobacter*, *Shewanella*, *Synechococcus* and *Vibrio* (Juni and Heym, 1986; Waterbury and Rippka, 1989; Spanggaard *et al.*, 2000; Gram and Dalgaard, 2002; Austin, 2006; Waterbury, 2006; Wilson *et al.*, 2008; Srinivas *et al.*, 2011). The identified Gram-positive bacteria were members of *Bacillus*, Clostridiales, *Vagococcus*, and *Mycobacterium*, also found in other marine samples (Matches *et al.*, 1974; Huss and Pedersen, 1979; Gonzlez *et al.*, 2000; Hartmans *et al.*, 2006; Françoise, 2010; Austin, 2006). *Staphylococcus sciuri* have previously been isolated from terrestrial animals (Stepanovic *et al.*, 2001), and the identified *Mycoplasma iguanae* have, as the name implies, formally been isolated from the green iguana (*Iguana iguana*) (Brown *et al.*, 2006). Several of these taxa were sheared between gill-, skin- and intestine samples, though *Bacillus* was identified in skin samples only. Members of *Bizonia* and *Pseudoalteromonas* were found in gill- and skin samples exclusively, whereas *Oceanisphaera* and a Clostridiales bacterium, were identified from the intestine samples only. Several different *Vibrio* species were also detected in the intestine samples. The identification of *Photobacterium* sp. and *Shewanella putrefaciens* in all samples are of particular interest, as this confirms the presence of SSBs among the indigenous

bacteria of fish.

The bacterial DNA from each sample was extracted from three different parallels; directly from the tissue samples, from mixed cultures on IAL plates (*i.e.* bulk cells), and from pure cultures obtained on IAL. The identified bacterial taxa, where they were present and by which method they were obtained, are listed in Tab. 3.1.

Table 3.1: Table from **Paper II**, Svanevik and Lunestad (2011). Overview of the different bacterial taxa found in gills, skin and intestinal content (gut) samples collected from det purse seine (PS) and the RSW-tank. PCR-DGGE and sequencing were performed on bacterial DNA extracted from bulk cells, directly from fish tissue and intestinal content (directly) and pure cultures.

Sequence results	Bulk cells								Directly						Pure cultures					
	PS				RSW				PS			RSW			PS			RSW		
	Gills	Skin	Gut	Gut[a]	Gills	Skin	Gut	Gut[a]	Gills	Skin	Gut	Gills	Skin	Gut	Gills	Skin	Gut	Gills	Skin	Gut
γ-proteobacterium						x			x											
Bizonia sp.																x			x	
B. paragorgia															x					
Oceanisphaera sp.			x	x	x												x			x
Photobacterium sp.	x	x	x																	
P. phosphoreum																			x	
Proteus sp.	x	x	x		x															
P. vulgaris	x	x	x		x										x	x	x	x		x
Pseudoalteromonas sp.															x	x				x
P. tetradonis															x	x			x	
Psychrobacter sp.	x	x	x		x	x	x							x						
P. immobilis			x		x		x	x												
P. marincola																		x		
P. cibarius																			x	
P. faecalis															x					
Shewanella sp.			x	x		x	x	x							x		x	x		x
S. putrefaciens	x	x	x	x	x	x	x	x	x		x	x		x						
Vibrio sp.			x	x	x	x	x	x									x			x
V. kanaloae															x	x				
V. splendidus																	x		x	x
V. pomeroyi																	x			
Uncultured bacterium	x	x	x		x															
Bacillus sp.																x				
Clostridiales											x									
Mycobacterium sp.					x															
Mycoplasma iguanae						x			x											
Staphylococcus sciuri									x	x	x		x	x						
Synechococcus sp.									x		x	x		x						
Vagococcus sp.				x				x												
V. carniphilus	x	x	x		x	x	x	x												
Teleost isolate 18*S*												x	x	x						

PS=purse seine, RSW=refrigerated sea water tank, species above the line = Gram-negative, species below the line = Gram-positive, [a] were incubated anaerobically.

3.1.2 Blue whiting intestine

The intestine of blue whiting (*Micromesistius poutassou*) were examined in **Paper III**, and all the identified taxa are known from marine samples, including *Oceanisphaera* sp. (Srinivas *et al.*, 2011), *Psychrobacter* sp. (Lee *et al.*, 2004; Romanenko *et al.*, 2001), *Pseudoalteromonas* sp. (Eilers *et al.*, 2000), *Photobacterium phosphoreum* (Dalgaard *et al.*, 1997; Olafsdottir *et al.*, 2006), *Psychrobacter jeotgali* (Yoon *et al.*, 2003), *Shewanella* sp. (Svanevik and Lunestad, 2011), *Arthrobacter* sp., *Exiguobacterium* sp., and *Micrococcus* sp. (Austin, 2006). For the purpose of this experiment, the presence of *Shewanella* and *P. phosphoreum*, are of most interest, as these are known to cause spoilage in fish *i.e.* being SSBs. As the nematode larvae enter the fish through the gastro-intestinal tract, the larvae would be exposed to these bacteria, and possibly bring them along into to the body cavity and the fish muscle.

3.1.3 *Anisakis* larvae

The bacteria found in the *Anisakis* larvae were examined when preparing the *Anisakis*-homogenate for the fish mince experiment in **Paper IV**, and were expected to reflect the bacteria found in the intestine of the fish host. These larvae were collected from the body cavity of blue whiting, where they had established capsules on the visceral organs, after migrating from the fish intestine. Some sequences were identified as Bacteroidetes bacterium, whereas others were further identified as members of the Flavobacteiaceae family, including *Aequorivita* sp., *Arenibacter latericius*, and *Gelidibacter mesophilus*, all being important components of the microbial biomass of the pelagic zone (Bowman, 2006). The *Pseudomonas*-like bacterium *Pusillimonas* sp., are sometimes found in sea water (Cao *et al.*, 2011), whereas the terrestrial Microbacteriaceae bacterium is found in soil (Evtushenko and Takeuchi, 2006)

and not commonly associated with marine fish. However, the identified *Photobacterium phosphoreum*, *Pseudoalteromonas* sp., *Pseudomonas* sp., *P. fluorescens*, *Psychrobacter* sp., *P. celer*, are all known from fish samples *i.e.* Atlantic mackerel (Svanevik and Lunestad, 2011; Broekaert *et al.*, 2011), Atlantic cod (Broekaert *et al.*, 2011; Vogel *et al.*, 2005) and haddock (Reynisson *et al.*, 2010; Olafsdottir *et al.*, 2006), supporting that these larvae assimilate some bacteria from the host.

3.1.4 The shared bacterial taxa

By comparing the bacteria identified from the intestine of Atlantic mackerel and blue whiting with the bacteria from the *Anisakis* larvae, some of the shared bacterial taxa stands out (Tab.3.2). The samples of *Anisakis*-infected blue whiting muscle (**Paper III**) are included in the table as well, as they should reflect the identified bacteria found in the *Anisakis* larvae (**Paper IV**). Though, being aware that these samples were collected one year apart.

All the compared samples had bacteria identified as *Psychrobacter* sp., where some were identified as *P. celer* and *P. immobilis.* Members of this genus are recognised from the marine environment, including sea water (Romanenko *et al.*, 2001), the Arctics (Lee *et al.*, 2004) and fish (Bagge-Ravn *et al.*, 2003), but also pathological specimens from humans and animals (Juni and Heym, 1986). Despite being found in several of the samples included in this work, and that members of *Psychobacter* are sometimes found as food spoiler, *e.g* in salted and rehydrated cod (Bjørkevoll *et al.*, 2003), they have little spoilage potential in fresh fish (Gennari *et al.*, 1999). Members of *Photobacterium* and *Pseudomonas* were found in the intestine of both Atlantic mackerel and blue whiting, as well as in the *Anisakis* larvae. These two species, in addition to *Shewanella* sp., which was found in the intestine samples only, have been identified as the dominant spoiling bacteria in fresh fish stored aerobically or

in MAP (Dalgaard, 1995; Dalgaard *et al.*, 1993, 1997; Gram and Dalgaard, 2002; Jørgensen and Huss, 1989; Olafsdottir *et al.*, 2006; Vogel *et al.*, 2005).

Table 3.2: The sheared bacterial taxa found in the intestine of Atlantic mackerel and blue whiting, in the *Anisakis* larvae, and in the muscle of *Anisakis*-infected (+) blue whiting.

Bacteria / Samples	**Paper II** A. mackerel intestine	**Paper III** Blue whiting intestine	**Paper IV** *Anisakis* larvae	**Paper III** *Anisakis*(+) blue whiting muscle
Oceanisphaera sp.	x	x		
Photobacterium sp.	x	x	x	
Pseudoalteromonas sp.	x		x	x
Pseudomonas sp.	x	x	x	
Psychrobacter sp.	x	x	x	x
Shewanella sp.	x	x		
Vibrio sp.	x			x

3.2 Routes of contamination

The limited availability of fish resources, and the still growing demand for fish proteins, challenge the fish sector for more and better products. The identification of handling processes that might affect the product conditions, are important measures for improving the utilisation of every capture and landing. The work included in this these examines some microbiological parameters throughout the production chain (**Paper I**), as well as one particular handling step, the on-board pumping of fish (**Paper II**).

3.2.1 Handling conditions

During 41 samplings at fishing vessels and fish processing factories, 1161 samples associated with capture, storage, landing and production of pelagic fish, were collected for microbiological examination of quality, hygiene and safety, as described in **Paper I**. Fish were collected from vessels before landing, from the landing tank and during production. Different critical contact points that the

Table 3.3: Tentative assessment scheme for fish-, surfaces-, and water samples. These guidelines are based on contemporary legislations for food products, and are set for quality, hygiene, and safety by limits of HPC, the faecal indicator organisms thermo-tolerant (t.t.) coliforms and enterococci, and *L. monocytogenes.* All samples are evaluated according good conditions ($<m$), acceptable conditions (between m and M), and not acceptable conditions ($>M$). The number of fish and surface samples with values between m and M should not exceed 60 % and 40 %, for HPC and faecal indicator organisms, respectively. Water used in production should hold potable quality, with no faecal indicator organisms, and HPC should not exceed m. No sample should be positive for *L. monocytogenes.*

Sample	**Quality**		**Hygiene**				**Safety**	
	HPC		Faecal indicator organisms				*Listeria monocytogenes*	
			t.t. coliform		enterococci			
	m	M	m	M	m	M	m	M
Fish (log CFU g-1)	5.7	6.7	0.6	1.3	2.7	3.1	neg	neg
Surface (log CFU/cm2)	0.8	1.7	0.3	0.8			neg	neg
Water (log CFU 100 ml-1)	2 log CFU/ml		0	0	0	0	neg	neg

fish were exposed to during the production chain, *i.e.* surfaces of equipment and water used during storage, washing and production, were also included. To assess the microbiological conditions of these samplings, an assessment scheme was proposed with guidelines for good ($<m$), acceptable ($>m$ -$<M$), and not acceptable ($>M$) conditions, based on contemporary legislations (Tab.3.3). Concerning hygiene and safety parameters, good conditions were found in 19 of 41 samplings. However, the presence of faecal indicator organisms and *L. monocytogenes* at 22 and 9 samplings, respectively, indicates that both vessels and factories have a potential to improve their cleaning and handling regimes to better ensure appropriate hygienic and safety conditions during capture and production.

At three particular samplings, a connection between contaminated contact point samples and contaminated fish, was found. In two of these *Listeria monocytogenes* was found, and these samplings were interrelated, where one was from a fishing vessel, and the other was from fish processing factory re-

ceiving the fish. The *L. monocytogenes* positive samples found on the vessel included the pump nozzle, the sift box, the sorting chamber, water from the RSW tank and fish. In the factory, this bacterium was found in fish and on a swab sample of the conveyor belt. This indicates that contamination that occur early in the in the production chain, could follow the product throughout the production. The transfer of *L. monocytogenes* from vessels to production facilities is of concern, as this pathogen is able to colonise in food-processing environments. The bacteria produce and establish in biofilms, which may become persistent in the production environment and subsequently contaminate food products (Carpentier and Cerf, 2011; Cruz and Fletcher, 2011; Jensen *et al.*, 2008; Møretrø and Langsrud, 2004; Pérez-Rodríguez *et al.*, 2008). Additionally, *L. monocytogenes* is known to be tolerant of low temperatures, including freezing, which reduces the chance of eliminating this bacteria from the product (Rocourt *et al.*, 2000). Collectively, the occurrence of *L. monocytogenes* in fish from vessels and factories were less than 0.8 %, which is lower than reported for fish by other studies, where 1.6 % and 2.2 % are reported by the Irish food safety department and EFSA, respectively (Leong *et al.*, 2014; EFSA, 2013).

The third sampling had high numbers of *E. coli* detected in water samples (<1.7 log CFU per ml) collected from the RSW tank on board a vessel, and on fish (<2.4 log CFU per gram) collected from the RSW tank at time of delivery. This contamination of faecal bacteria was later related to the area where the vessel had filled the RSW tanks, being too near the shore and holding water of poor hygienic quality. Additionally, four more samplings had samples positive for *E.coli*, where it was detected on fish, conveyor belts and water samples from the landing tank and the sorting machine. As an indicator for faecal contamination, the *E.coli* originate from the intestine of warm-blooded animals, and its presence reveals contamination from sources

of the same origin. The routes of contamination could have been through contaminated seawater or other water sources, through bird dropping around the vessels, and improper hygienic conditions among workers at processing plants. Though *E.coli* are sensitive to several disinfectants, adequate cleaning regimes would eliminate the problem (Rice *et al.*, 1999; Singh *et al.*, 2002).

Since the number of incidents, where marine fish have been the vehicle for causative agents giving illness in humans, is very low, these routes of contaminations may work for all kinds of micro-organisms, including the SSB of fish. The detection of some specific species, which should be absent, strengthens the linkage between the contamination of fish from critical contact points.

3.2.2 On-board pumping

The Atlantic mackerel that were examined in **Paper II**, were collected at two sites, the purse seine (PS) and the RSW tank. This was to see if any differences in the bacterial flora occurred after the fish were pumped on board the fishing vessel. The HPC (*i.e.* plate counts -PC- in **Paper II**) in samples from skin and gills collected from the RSW tank were higher than the samples collected from the purse seine, thus the differences were not significant as the parallel samples had a large range. Members of *Oceanisphaera* and *Vibrio* were detected in the intestine samples only, from fish collected from the purse seine. However, these genera were identified in all samples, *i.e.* gills, skin and intestine, from fish collected from the RSW tank. Due to the crowding during on-board pumping, as well as the possible stress-related intestinal release, some feacal matter from the fish seems to contaminate the outer surfaces of the surrounding fish. This is probably how the intestinal bacteria could contaminate the fish, and most likely the storage water in the RSW also. These findings indicate that pumping is an important rout of contamination on board vessels. If this is the case, the fish that were delivered to the processing factory, had already

been contaminated before landing, thus a possible enhanced quality might have been lost.

3.2.3 Muscle-invading *Anisakis* larvae

The experiment in **Paper III** was designed to reveal if *Anisakis* larvae found in pelagic fish may carry bacteria into the fish muscle, and this was first examined by excising muscle blocks with *Anisakis* larvae for fixation and preparation of histology sections. The Gram-stained sections of an *Anisakis* larva within the fish muscle tissue evidently show that the larva has a densely folded intestine holding high numbers of bacteria (Fig.3.1).

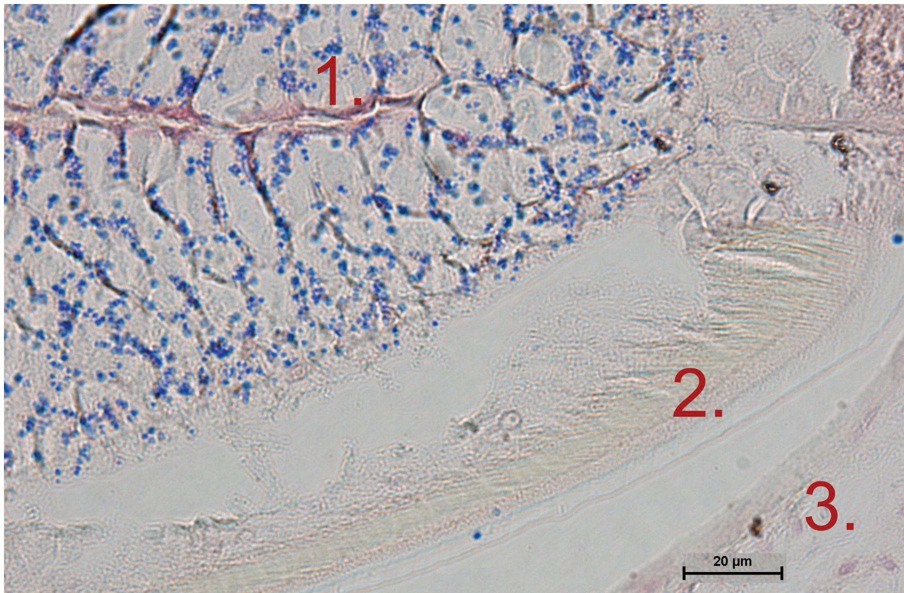


Figure 3.1: Gram-stained *Anisakis* larva in the musculature of blue whiting. Gram positive bacteria stained blue. Gram-negative bacteria stained red. 1. Larval intestine densely populated with bacterial cells. 2. Larval cuticle. 3. Host induced connective tissue capsule.

Furthermore, the bacterial contamination introduced into the fish muscle by these muscle-invading *Anisakis* larvae, was examined by a cultivation

study including muscle blocks with and without larva, *i.e. Anisakis*(+) and *Anisakis*(-), and the intestine of the blue whiting. The HPC and the number of H_2S-producing bacteria were ten times higher in the *Anisakis*(+) samples compared to the *Anisakis*(-) samples. Even though a wide spread among the parallels were found, and that the HPC and the number of H_2S-producing bacteria were inconclusive, the increased bacterial load in the *Anisakis*(+) muscle samples is probably introduced by the larvae. Still, muscle of freshly caught fish should not hold any bacteria (Cahill, 1990; Huss, 1995), and especially those not holding any *Anisakis* larvae. Despite that, bacteria were found, indicating either contamination during the homogenisation and plating, or, though very hard to control, that a *Anisakis* larvae have passed through the muscle sections, leaving bacteria within the fish muscle. None of the studies included in this these could confirm bacteria on the outside of the larvae, thus the possibility of them being there should not be ignored.

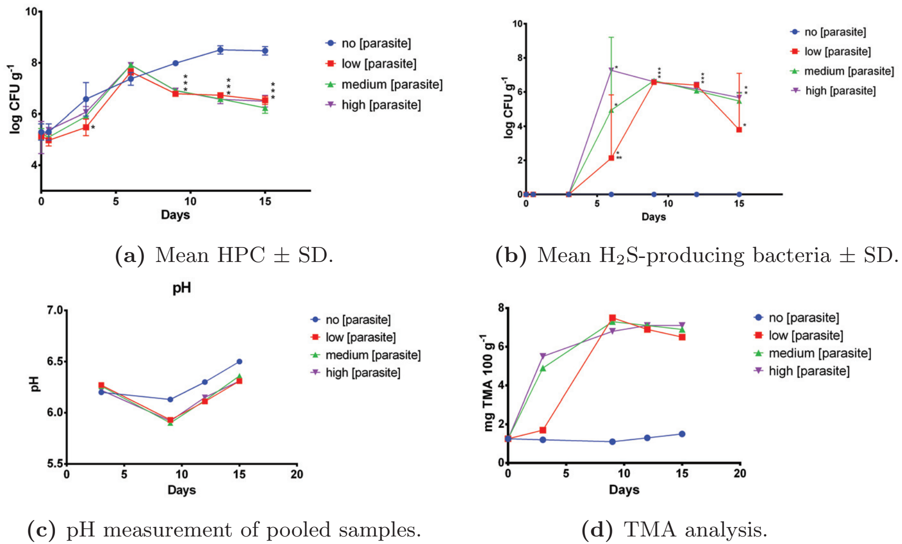
The identified bacterial taxa from the three sample types are all known from the marine environment (Austin, 2006; Cahill, 1990; Nayak, 2010; Svanevik and Lunestad, 2011). The known SSBs, *Shewanella* sp. and *Photobacterium phosphoreum* (Gram and Huss, 1996; Gram *et al.*, 1987; Hovda *et al.*, 2007b), were found in intestine samples only. Even though *Shewanella* sp. and *P. phosphoreum* were not recorded in the present *Anisakis*(+) samples, they are frequently found in the intestine of marine fish. Whenever any *Anisakis*-induced bacterial contamination of fish muscle occur, these taxa may be introduced, possibly affecting the bacterial composition and quality of certain fish products.

3.3 Fish mince with *Anisakis* larvae

The findings in **Paper II** and **Paper III** expanded the knowledge about the indigenous bacteria within the intestine of Atlantic mackerel and blue whiting, both holding several different SSBs of fish. The experiment in **Paper III** suggest that these SSBs might be found in the densely populated intestine of *Anisakis* larvae, thus the following hypothesis was proposed: The *Anisakis*-induced bacterial contamination of fish muscle will have negative effect on the shelf-life of a fish mince product.

The storage trial was performed with fish mince added a homogenate of *Anisakis* larvae at three concentrations, referred to as low-, medium- and high[parasites] samples. Additionally, no[parasite] and negative control samples, were without any parasite additives. The samples with *Anisakis*-homogenate added showed a significantly increase in the number of H_2S-producing bacteria (Fig.3.2b) and the level of TMA/N (Fig.3.2d) during storage, compared to the no[parasite] samples. However, the HPC (Fig.3.2a) of the no[parasite] samples were the highest, and these samples also seemed to spoil more rapidly as they got coated with a greenish slime and developed a foul sweetish smell. Since the number of H_2S-producing bacteria and the level of TMA/N were low, the changes had to be induced by other bacteria than those commonly associated with the production of H_2S and TMA. Noteworthy, these results indicate that the parasite-induced bacteria does not necessarily affect the shelf-life adversely, as we suggested in **Paper III** (Svanevik *et al.*, 2013).

The bacteria that were identified from the *Anisakis*-homogenate, *i.e.* the *Anisakis* larvae, were described in section 3.1.3, thus many of these species were not recovered from the parasite inoculated fish mince samples. *Psychrobacter* sp. and *Pseudomonas fragi*/*fluorescens* were found in all samples, whereas *Photobacterium phosphoreum* were found exclusively in the samples



(a) Mean HPC ± SD.

(b) Mean H_2S-producing bacteria ± SD.

(c) pH measurement of pooled samples.

(d) TMA analysis.

Figure 3.2: Measurements from no-, low-, medium- and high[parasite] smaples. Mean HPC and H_2S-producing bacteria in log(CFU g^{-1} + 1) ± SD. TMA in mg TMA/N 100 g^{-1}. *differs significantly ($p<0.05$) from no[parasite].**differs significantly ($p<0.05$) from high[parasite].

where the *Anisakis*-homogenate was added. It is well documented that *P. phosphoreum* contributes to off-odour and spoilage involving the production of TMA and other degradation products in fish from northern temperate waters stored at refrigerator temperature, and especially in fish in MAP (Dalgaard *et al.*, 1997; Gram and Dalgaard, 2002; Hovda *et al.*, 2007b). Since none of the species *P. phosphoreum*, *Pseudomonas fluorescens*, *P. fragi* and *Psychrobacter* sp. produce H_2S (Dalgaard *et al.*, 1993; Gennari *et al.*, 1999), some undetected H_2S-producing bacteria had to be present. Some sequences had the same probability of being either *Pseudomonas fluorescens* or *P. fragi*, although each species possesses different properties as active spoiler. For example, *P. fluorescens* is a commonly occurring fish spoiler, where some strains produce TMA and could be the TMA-contributor in the samples added *Anisakis*-homogenate, together with *P. phosphoreum* (Gram and Dalgaard, 2002).

P. fragi is mainly associated with a sweetish and fruity odour due to the production of ethyl esters, and could be responsible for the characteristic sweetish spoilage odour in the no[parasite] samples (Miller *et al.*, 1973a). Since the PCR-DGGE method seems unable to differentiate between the two species, probably due to sensitivity constraints, it is not possible to decide if it was the same *Pseudomonas* species in all samples. Members of the genus *Psychrobacter* are known to contribute to no or minor spoilage according to Gennari *et al.*(1999), and it seems that this species is outgrown by other stronger specific spoilers in those samples that spoiled most rapidly, *i.e.* the no[parasite] and low[parasite] samples, where the highly competitive *Pseudomonas* (Gram and Melchiorsen, 1996; Gram *et al.*, 2002; Gram, 1993), was found. *Psychrobacter* sp., *Pseudomonas fragi/fluorescens* and *Photobacterium phosphoreum* were recorded in the medium[parasite] samples at day 12, and in the high[parasite]-samples at day 9, 12 and 15, which suggests that the *Pseudomonas* were not able to outgrow *Psychrobacter* sp. in the persence of *P. phosphoreum*, and thus the bacterial growth was reduced (Fig.3.2a).

By comparing the samples added *Anisakis*-homogenate only, the amount of homogenate added, reflected a theoretical infection intensity of *Anisakis* larvae. Amonge these samples, only a weak negative effect on the spoilage activity of H_2S-producing bacteria (Fig. 3.2b) and TMA (Fig.3.2d), was seen. The conclusion for this study is that the interactions between the bacteria induced by the *Anisakis* larvae appears to be complex, and that *Anisakis*-infected fish should not necessarily be excluded as food resources. Furthermore, the suggested hypothesis about the negative effect of these bacteria, has to be rejected.

3.4 Comparison of the applied identification tools

As described in the study of Atlantic mackerel (**Paper II**), the pure cultures obtained after growth on IAL were identified by PCR-DGGE and sequencing. In addition, isolates from these pure cultures were tested for different morphological and biochemical traits, and classified by Gram-reaction and oxidase/catalase producing abilities. The isolates were identified by the commercial identification kit series API®(bioMerieux), including API®20E, API®20NE and API®50CHB. Totally 63 isolates were identified by sequencing, whereas only 33 of these were identified by the API® kits (Tab.3.4). The bacteria identified by API® represented ten different genera found in eight different families. The bacteria identified by sequencing represented 13 genera from nine different families. Ten isolates were identified as *Proteus vulgaris* by both identification methods. Additionally, four more isolates corresponded by genera, where two were identified as *Vibrio*, and two as *Shewanella*. Two isolates were identified within the same family, as the genera *Psychrobacter* and *Moraxella* are both found in the Moraxellaceae family. The identified *Grimontia hollisae*, formerly known as *Vibrio hollisae*, and *Vibrio kanaloae* are both found in the family Vibrionaceae. The remaining 19 isolates identified by the API® kits were not in compliance with the species identified by sequencing.

The API® kits are clearly not suitable for identifying psychrotrophic bacteria with low optimum temperatures, and the reliance of the identified isolates are questionable. The sequence results were different for more than half of the isolates, and some isolates not identified at all. However, these kits are available at a low price and is a rapid method for initial testing of isolates. Especially if the isolate of interest might be among the human pathogens, as these bacteria tend to thrive at those temperatures these kits are designed for.

Table 3.4: Morphology of isolates obtained after cultivation of gills, skin and intestinal content (gut), collected from Atlantic mackerel. Isolates were dentified by API ® and PCR-DGGE.

Isolate No.	Display	Colony size	Colour	Transparent	Surface	Edge	Elevation	Haemolysis	H_2S	Cell morph.	API®	DGGE
1	S	5	Y	Y	SH	NR	F	α	N	R(S)	N	*Bizionia paragorgiae*
2	S	6	W	N	SH	NR	C	α	Y	R(S)	*Proteus vulgaris*	*Proteus vulgaris*
3	C	5	P	N	SM	NR	C	α	Y	R(S)	N	*Proteus vulgaris*
4	S	5	P/W	N	SM	NR	C	α	Y	R(S)	*Proteus vulgaris*	*Proteus vulgaris*
5	C	9	P/W	N	SM	NR	F	α	N	R(SM)	N	*Shewanella* sp.
6	C	6	P/W	N	SM	NR	F	α	Y	R(S)	N	*Shewanella* sp.
7	S	S	B/Y	N/Y	SM	NR	F	α	N	R(S)	*Proteus vulgaris*	*Proteus vulgaris*
8	S	S	W	N/Y	D	NR	F	α	N	R(S)	N	*Proteus vulgaris*
9	C	5	Y	Y	SM	I	F	β	N	R(MB)	N	*Bizionia* sp./*Formosa* sp.
10	S	4	P/W	N	SM	I	C	α	Y	R(SB)	N	*Proteus vulgaris*
11	S	S	P	N	SM	I	C	α	N	R(S)	*Proteus vulgaris*	*Pseudoalteromonas tetraodonis*
12	S	S	P	N	SM	I	C	α	N	R(S)	*Pasteurella pneumotropica*	*Pseudoalteromonas* sp.
13	S	5	P/W	N	SM	I	C	α	N	R(SM)	*Proteus vulgaris*	*Proteus vulgaris*
14	C	1	P/W	N	SM	E	F	γ	N	C	N	*Photobacterium phosphoreum*
15	S	2	Y	Y	SM	E	C	α	N	R(M)	*Proteus vulgaris*	*Bizionia* sp.
16	S	2	Y	Y	SM	E	C	α	Y	R(M)	*Bizionia* sp.	
17	S	3	P	N/Y	SM	NR	F	γ	N	R(MB)	*Proteus vulgaris*	*Proteus vulgaris*
18	S	1	P	N/Y	SM	NR	F	γ	N	R(S)	*Proteus vulgaris*	*Vibrio splendidus*
19	C	3	P	N	SM	NR	C	γ	Y	R(M)	N	*Vibrio splendidus*
20	S	S	B/P	N/Y	SM	NR	F	α	N	R(L)	*Proteus vulgaris*	*Proteus vulgaris*
21	S	2	Y/B	N/Y	SM	NR	F	α	N	R(M)	N	*Proteus vulgaris*
22	S	2	Y/B	N/Y	SM	NR	F	α	N	R(S)	*Proteus vulgaris*	*Proteus vulgaris*
23	R	2	R/B	N	SM	NR	F	α	N	R(S)	*Proteus vulgaris*	*Proteus vulgaris*
24	S	S	W	Y	SM	NR	F	α	N	R(M)	N	*Proteus vulgaris*
25	S	S	W	Y	SM	NR	F	α	N	R(S)	N	*Proteus vulgaris*
26	C	2	W	N	D	I	F	β	N	R(S)	*Providencia alcalifaciens*	*Vibrio splendidus*
27	S	4	P/W	N	SM	I	C	β	N	R(SB)	*Proteus vulgaris*	*Proteus vulgaris*
28	S	2	P/W	N	SM	I	F	β	N	R(SB)	*Proteus vulgaris*	*Vibrio* sp.
29	S	9	P/W	N	SM	E	C	β	Y	R(SB)	*Proteus vulgaris*	*Proteus vulgaris*
30	C	10	P/W	N	SM	E	C	β	Y	H	*Providencia alcalifaciens*	*Vibrio splendidus*
31	C	7	P/W	N	SH		NR	α	Y	R(M)	*Empedobacter brevis*	*Pseudoalteromonas tetraodonis*
32	C	S	W	N	G	I	F	α	N	R(SB)	*Empedobacter brevis*	*Pseudoalteromonas* sp.
33	C	9	P/W	N	SH	E	C	α	N/Y	R(S)	*Empedobacter brevis*	*Pseudoalteromonas tetraodonis*
34	C	S	W	Y	SM	E	C	α	Y	R(S)	*Shewanella putrefaciens*	*Shewanella* sp.
35	C	S	W	Y	SM	E	C	α	N	R(S)	N	*Pseudoalteromonas* sp.
36	C	S	W	Y	SM		NR	α	N	R(SB)	*Grimontia hollisae*	*Vibrio kanaloae/lentus*
37	C	5	P/W	N	SM	E	C	γ	Y	R(SB)	*Moraxella* sp.	*Psychrobacter celer*
38	C	4	P	N	SM	E	F	β	N	R(S)	N	*Shewanella* sp.
39	C	S	Y/B	N/Y	SM		F	α	N	R(S)	N	*Proteus vulgaris*
40	C	2	P	N	SM	E	C	γ	N	R/C(S)	N	*Psychrobacter* sp.
41	S	4	P/W	N	SM	I	C	α	Y	R(S)	N	*Vibrio kanaloae/lentus*
42	C	S	Y	N/Y	SH	I	F	α	N	R(S)	*Vibrio kanaloae/lentus*	
43	C	2	P	N	SM	E	C	β	N	R/C	*Aeromonas salmonicida**	*Pseudoalteromonas tetraodonis*
44	C	4	P	N	SM	E	C	β	N/Y	R(SB)	N	*Shewanella* sp.
45	C	7	P/W	N	SM	I	F	α	N	C	N	*Shewanella* sp.
46	S	5	P/W	N	D	I	F	γ	N	C N	*Psychrobacter cibarius*	
47	S	6	P/W	N	SM	E	F	α	Y	R(I)	*Psychrobacter* sp.	
48	C	6	P/W	N	SM	E	F	α	N	R(I)	*Aeromonas salmonicida**	*Vibrio* sp.
49	C	3	P	N	SM	E	C	β	N	R(I)	*Vibrio vulnificus*	*Vibrio* sp.
50	C	S	W	N/Y	SM		F	α	N	R(I)	*Aeromonas salmonicida**	*Vibrio pomeroyi*
51	C	S	B/P	N/Y	SM	E	F	γ	N	R(S)	N	*Oceanisphaera* sp.
52	C	2	R/B	N	SM	E	F	α	N	R(S)	*Shewanella putrefaciens*	*Shewanella* sp.
53	C	0.5	P/W	N/Y	SM	E	F	β	N	R(M)	*Vibrio alginolyticus*	*Vibrio splendidus*
54	C	0.5	P	N	SM	E	F	β	N	R(M)	*Empedobacter brevis*	*Pseudoalteromonas* sp.
55	C	0.2	W	N	SM	E	F	β	N	R(M)	*Empedobacter brevis*	*Pseudoalteromonas* sp.
56	C	S	Y/B	N/Y	SM	NR	F	β	N	R(S)	*Oligella ureolytica*	*Aeromonas salmonicida*
57	C	S	Y/B	N/Y	SM	E	F	γ	N	R/C(S)	N	*Pseudomonas* sp.
58	S	S	Y/B	N/Y	SM	E	F	α	N	R	*Aeromonas salmonicida**	*Shewanella* sp.
59	S	S	Y/B	N/Y	SM	E	F	α	N	R(S)	*Proteus vulgaris*	
60	C	S	Y/B	N/Y	SM	E	F	γ	N	R(SK)	N	*Pseudomonas* sp.
61	C	6	P	N	SH	E	C	γ	Y	R(S)	N	*Psychrobacter faecalis*
62	C	S	W	N	G	E	F	β	N	R(L)	N	*Psychrobacter faecalis*
63	C	1	Y	N	D	I	C	γ	N	R(K)	N	*Bacillus* sp.

Abbreviation: Display (S=swarm, C=circular, R=rhizoid), Colony size (⌀ mm, S=swarm), Colour (Y=yellow, W=white, P=pink, B=brown, R=red), Transparent (Y=yes, N=no), Surface (SH=shiny, SM=smooth, D=dry, G=greasy), Edge (E=entire, I=irregular, NR=not registered), Elevation (F=flat, C=convex), Haemolysis on sheep blood (α=alpha, β=beta, γ=gamma), H_2S-production (Y=yes, N=no), Cell morphology (R=rod, C=cocci, H=helical/spiral, S=short, L=long, M=medium, B=bent, K= cluster), API® result (N=no match),*ssp. Salmonicida.

Concluding remarks

The work described in this thesis aimed to increase the knowledge of the microbiology associated with the Norwegian pelagic fish sector. A main goal was to identify some critical points for bacteria contamination that could be improved for better utilisation and reduced post-harvest loss of fish.

The indigenous bacterial biota of Atlantic mackerel (*Scomber scombrus*) were described, as well as the bacteria associated with the blue whiting (*Micromesistius poutassou*) intestine. The bacteria found in the *Anisakis* larvae were identified, and to some extent, these bacteria reflected those found in the fish intestine. The shared bacterial genera comprised of *Pseudoalteromonas*, *Psychrobacter*, *Photobacterium*, and *Pseudomonas*, where the latter two are known to be specific spoilers in fresh fish.

Further on, the bacterial biota of fish is affected and changed by handling during capture, landing and processing. During pumping on board fishing vessels, some intestinal content are forced out of the fish, contaminating the outer surfaces as gills and skin of the surrounding fish, and possibly the storage water in the refrigerated sea water (RSW) tanks. This is shown by the increased bacterial load found in gill- and skin samples of fish collected from the RSW tank. Additionally, the genera *Vibrio* and *Oceanisphaera* were only identified in intestine samples from fish collected from the purse seine, but in all samples from fish collected from the RSW tank.

The spot sampling programme revealed contamination of fish and equipments by the human pathogen *Listeria monocytogenes*, and of fish and storage water by *E. coli*. It seems like contamination that happens early in the produc-

tion chain follow the fish throughout the process. Thus, improper handling could effect the shelf-life of the end products, having impact on the post-harvest loss.

The muscle-invading *Anisakis* larvae withdrawn from the fish muscle, carried along bacteria inside its own intestine. The parasite acts as a contaminating vector, and the presence of any *Anisakis* larvae will therefore increase the initial bacterial load in the fish muscle, which previously was assumed to be sterile. However, it is unlikely that these bacteria are spread within the fish muscle unless the fish is processed in a way that disrupt the capsule and the parasite, as for instance in fish mince or surimi production. The storage trial with fish mince gave surprisingly positive results, as the shelf-life was prolonged when the mince were inoculated with a *Anisakis*-homogenate. These results show that fish infected with parasite larvae, should not necessarily be excluded as a food resource, even though the thought of it could be quite repulsive. The possibility of minced fish products made from *Aniskis*-infected fish, having increased shelf-life, could become important in the future, when looking for new protein resources for human consumption.

This thesis states that focus on proper handling of fish from capture until landing and processing, will improve the quality and shelf-life of fish products, hence the following take home messages:

- The indigenous bacterial biota of fish and associated *Anisakis* larvae contains specific spoilage bacteria (SSB).
- Contamination occurring early in the process follows the product throughout the production chain.
- On-board pumping increases the bacterial load of fish gills and skin.
- *Anisakis* larvae encapsulated within fish muscle contains bacteria.
- Fish mince seems to increase shelf-life if inoculated with bacteria from the *Anisakis* larvae.
- *Anisakis*-infected fish should not necessarily be excluded as food resources.

Future perspectives

The findings presented in this these raise new interesting questions that would be interesting to investigate. For instance, what happens with *Pseudomonas* when it is present in a fish mince with *Photobacterium.* It would also be interesting to examine the interactions between the bacteria isolated from the fish mince added the *Anisakis*-homogenate. Maybe as a co-culture study, to see if the same outcome occurs under different conditions.

As there were some problems with extracting bacterial DNA directly from fish tissue **Paper II**, an additional experiment was planed and partly conducted during the fall of 2012. The plan was to repeat the sampling as done for the Atlantic mackerel, but to use culture-independent Next Generation pyro-sequencing for bacterial identification. The samples were aseptically collected and frozen at $-20\,^{\circ}\mathrm{C}$ immediately, and the DNA was extracted shortly after thawing. However, the DNA showed as smears on the agarose gel, despite the high DNA output. Several different extraction methods were tested, but none gave good DNA quality. Despite a lot of effort put in to these challenges, involving other scientist and institutes, these samples did not give any results and the work has ended, as there are no more sample material left to test new methods. It is important to further develop culture-independent methods for fish samples, as this would give a broader background knowledge about the bacterial biota obtained by culture-dependant methods.

Furthermore, only parts of the Norwegian pelagic fish sector were examined for microbiological conditions in this work, and an expanded sampling plan would be necessary to get a more complete picture of the production chain,

where the fish is followed from capture to end product. In addition to the critical points examined in this thesis, examination of fish products at the expiration day should be included, as it is at this time the legislation for *Listeria monocytogenes* applies.

The indigenous bacteria of Atlantic herring (*Clupea harengus*) should be examined as well, as this species are landed and exported the most (volumes). In addition, dermersal and benthic species with a high turnover in Norway, as for instance Atlantic cod (*Gadus morhua*), Atlantic saith (*Pollachius virens*) and Greenland halibut (*Reinhardtius hippoglossoides*), should be examined. Fish handling and processing, as well as the increased focus on fish quality, are not only of importance for the pelagic species, but in the whole fish sector.

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