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Preface

The greatest adventure and the biggest challenge of my life thus far has come to an end. I have finished this master's thesis with joy, relief and indeed, with tears in my eyes. It was indescribably pleasant to be able to plunge into this research field and look closely at the work performed at sea and more precisely, on boats, and to get to know a lot of nice and helpful people.

I wish to thank my supervisors; scientist/researcher Trond K. Løvdal and senior researcher Bjørn Roth. Thank you very much to Trond for good guidance, patience and all the time you took to answer my questions. Thank you very much to Bjørn for the opportunity to participate in my first sampling trip with you, as well as the good advice. I also wish to thank engineer Leena N. Shinde for answering my questions at the laboratory.

Thank you to my husband, family and friends for their support, encouraging words, understanding and all of the motivation they gave me.

Sandra Zaremba

Stavanger, 2018

Abstract

The Norwegian food industry is best known for salmon production and distribution to over 70 countries. Salmon processing factories are susceptible to the establishment of the pathogen bacterium *Listeria monocytogenes*, which may cause the life-threating infection listeriosis. Due to its ubiquitous nature, it is a great challenge for companies to curtail the spread of *L. monocytogenes* in the processing environment of the company. The purpose of this master thesis is to investigate the prevalence of bacteria in five Norwegian fish farms for Atlantic salmon (*Salmo salar*). Sampling was performed from September 2017 to January 2018. The samples were taken from fish and seawater. The total number of tested samples was 126. For *Listeria* detection, the sensitive NMKL 136 method with a detection limit of 2 -20 cfu/L was used. Suspected *Listeria* colonies were examined using an API *Listeria* kit. The results were negative for the presence of *L. monocytogenes* for all examined samples. Despite this, the raw fish entering the processing plants are possible risk sources for finished product contamination with the bacterium *L. monocytogenes*. Based on the results produced by the present study, seawater and fish raw material do not pose a high contamination risk for food processing factories, taking into consideration that sampling was performed during the autumn and winter seasons. However, the undetectable amounts of *L. monocytogenes* in fish raw material and in seawater establish the niches in the processing environment which may lead to finished product contamination during processing and thus pose a danger to the life and health of consumers. The risk factors, the route of bacterial transmission and the control methods are discussed.

Contents

1. Introduction

1.1 Background

Norway is the biggest producer of Atlantic salmon (*Salmo salar*). In 2017, Norway exported 1 million tonnes of salmon valued at 64.7 billion NOK (Anonym 2018a). This represents an increase of 5 percent or 3.4 billion NOK relative to 2016. It was a record year for salmon exports. About 73 % of all salmon exports went to EU members states. Poland and France were the largest markets for Norwegian salmon (Anonym 2018a). At the present time it is common to lead an active lifestyle based on a healthy diet. This trend has led to an increased demand for seafood.

Products like smoked or "gravad" salmon, soft cheese, raw milk, and meat products, which do not require heat treatment before eating, may contain the foodborne pathogenic bacterium *Listeria monocytogenes*. The consumption of food contaminated with the bacteria, may give rise to the life-threatening infection listeriosis (EFSA 2013). Raw or smoked salmon, which belongs to the ready-to-eat (RTE) category of products, may be a potential source of contamination with this pathogen.

L. monocytogenes is a ubiquitous organism which occurs in the natural environment. The major reservoirs of *Listeria* are soil, forage, and surface water (EFSA 2013). *L. monocytogenes* may invade the slaughter house and process factory. The salmon may be contaminated with the bacteria during processing and be transferred to the final product. Disposal of *Listeria* from industry facilities is very costly. A recall of the product from the market can lead to a large loss of money. The total cost estimates associated with Listeria infections in the USA, for 20 years ago, was in range from 230 to 265 million USD per year (Buzby 1996). In 2000, the United States Department of Agriculture updated the cost estimates based on 2,493 listeriosis cases, to $2.3 billion per year (Crutchfield and Roberts 2000).

The most important consideration is to take care that *Listeria* does not get into the processing environment and contaminate the final product. It is also necessary to discover and find the most effective way to implement an effective disinfection method to be used at the processing factory. The purpose of many studies was to find the source of cross contamination of the pathogenic bacterium. All process steps were studied from raw material to finished product. It was observed very rarely or at a low occurrence level of *L. monocytogenes* in raw fish and in seawater. These results contrasted sharply with the high number of pathogenic bacteria in the

processing environment, like slaughterhouses and smokehouses (Ben Embarek 1994; Dillon et al. 1994; Heinitz and Johnson 1998).

Taking into consideration the present study, as well as other studies (Ben Embarek 1994; Johansson et al. 1999), *L. monocytogenes* is seldom detected in the raw fish and in seawater. Johansson et al. (1999) and Autio et al. (1999) reported about 0-1%-10% in raw fish, while Jin et al. (1994) found no positive results for the 60 Japanese salmon samples, but 16% of the smoked salmon tested positive for *L. monocytogenes*. The percentage of *Listeria* occurrence increases with the degree of processing (Ben Embarek 1994; Dillon et al. 1994; Heinitz and Johnson 1998; Fonnesbech Vogel et al. 2001). The quantity of salmon samples infected with the pathogen increased significantly after cold smoking (Ben Embarek 1994; Jin et al. 1994). The amount of *Listeria* positive samples after cold smoking ranged from 0 to 100%, where the typical prevalence is between 7 to 40% (Rørvik and Yndestad 1991; Ben Embarek 1994; Heinitz and Johnson 1998; Norton et al. 2000a; Jemmi et al. 2002). To find the source of contamination, genotypes of *L. monocytogenes* isolates from raw fish, throughout the process line, to finished products were compared (Rørvik et al. 1995). Based on the identical clones of *L. monocytogenes*, the location of product contamination has been determined. From 475 samples taken from raw fish, water, products, and the environment of a cold-smoked salmon processing plant, no *L. monocytogenes* in all 50 raw fish samples was detected. While approximately one third of samples from smokehouse products and the environment tested positive (Rørvik et al. 1995). In many studies, the samples from raw fish tested negative for *L. monocytogenes*, while the samples of finished product tested positive for the pathogen (Rørvik et al. 1995). The DNA from *L. monocytogenes* isolated from the finished product and from the processing plant that the product came from, were compared, and determined to be identical (Norton et al. 2000b). It is noteworthy that different genotypes of *L. monocytogenes* strains were found in products from different smoke houses, but the same genotype was detected in products from the same smoke house over a longer period of time (Vogel et al. 2001). This indicates that there are home strains of specific strains of *L. monocytogenes* in every smoke house or processing plant contaminated with these bacteria (Vogel et al. 2001). Five smoke houses in the United States were visited by Norton et al. (2000a) over a 6-month period and it was found that each smoke house had an individual *L. monocytogenes* strain. A slicer machine provides a good niche for the establishment of *Listeria* biofilm. The machine is almost never dry. There are perfect conditions, humidity and sufficient organic material to

form a biofilm. The same genotype was isolated from finished products and from the slicer machine, while the raw fish was free from the pathogen (Thimothe et al. 2004).

Listeria has been found in surface water and in salmon from surface water near the coast (Huss et al. 1995). Studies show that *Listeria* present in surface water may be linked to water contaminated by humans and animals (Ryser and Marth 1999). *Listeria* is easiest to detect in the spring, when the level of runoff from agriculture and fertilizer to seawater reaches its peak (Ryser and Marth 1999).

The example of these studies show, that the main source of contamination is the process environment and equipment (Ben Embarek 1994; Rørvik et al. 1995). *L. monocytogenes* can enter to the processing plant with raw fish or seawater in a very low concentration (Autio et al. 1999). The pathogen can also be introduced by staff, who may be healthy carriers of the bacteria (Rocourt et al. 2000). The bacteria can build up over time in the processing plant in niches by forming a biofilm. It is difficult to decide on the primary source of contamination; raw material originating from nature, or the processing environment with domesticated *L. monocytogenes*. Raw fish and seawater cannot be excluded as one of the sources of *L. monocytogenes* which end up in finished products.

In the food industry, efficient production hygiene is a key measure for avoiding the accumulation of spoilage bacteria and eliminating pathogens. The persistence of bacteria is a longstanding problem in food processing environments. Environmental bacteria can survive foam cleaning and disinfection at user concentrations in the industrial environment (Camargo et al. 2017). Some important characteristics of persisting bacteria were a high growth rate at low temperature, tolerance to the cleaning agent and the ability to form a biofilm (Camargo et al. 2017). Camargo et al. (2017) suggested that strain-to-strain variation cannot explain why certain subtypes of *Listeria monocytogenes* persist in food processing environments while others are found only sporadically.

1.2 The *Listeria* genus

Traditionally, the *Listeria* genus contains six species, but more new species and subtypes have been described (den Bakker et al. 2014). Figure 1.2 shows a phylogenetic tree with eight *Listeria*-species: *L. monocytogenes*, *L. welshimeri*, *L. innocua*, *L. ivanovii*, *L. grayi*, *L. seeligeri* (widely recognizable), *L. rocourtiae*, and *L. marthii* (Cossart 2011). Among them, *L. monocytogenes* and *L. ivanovii* are pathogenic in mice, but only *L. monocytogenes* is supposed to be pathogenic in humans (Seeliger 1981). Three human listeriosis cases caused by *L. ivanovii*, and one by *L. seeligeri* (Jay 2005) have been noted. *L. monocytogenes* has been divided into 13 serotypes, based on H- and O-antigen (Seeliger 1981). Three serotypes mainly cause human infections: 1/2a, 1/2b and 4b (Ward et al. 2004). Serotype 1/2a is mainly responsible for human disease cases in Scandinavia (McLauchlin 1990; Ward et al. 2004). Recent studies showed that serotype 1/2a was isolated most frequently in Japan, reflecting a change in the predominant serotype in pork from 1/2c to 1/2a (Yoshikawa et al. 2018). Al-Ali et al. (2018) concluded that the presence of 1/2a serotype in gallbladder from cattle and sheep in Iraq indicates public health risk through cross-contamination of meat at slaughterhouses. Latorre et al. (2007) analysed 5,788 samples. 121 (2.1%) samples were contaminated with *L. monocytogenes*. The highest prevalence was found in smoked salmon (10.6%). The most common serotypes were 1/2a.

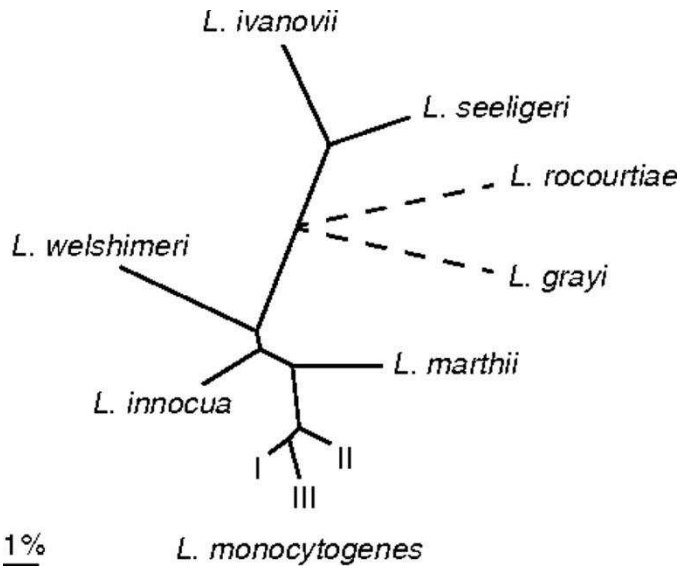

Figure 1.2 Listeria-genus. Phylogenetic tree of over eight Listeria species. The location and length of the lines indicate the relationship between Listeria species. The striped line indicates that the distance and location of L. rocourtiae and L. grayi is undefined. Used with permission from article author.

L. monocytogenes was first described by E.G.D. Murray in 1926. He observed the elevated production of monocytes in the blood of rabbits exposed to these bacteria (Murray E.G.D. 1926). Therefore, he suggested the name *Bacterium monocytogenes* (Murray E.G.D. 1926). One year later, Pirie proposed the name *Listerella*, but the name had already been given to another bacterium. In the end, in 1940, it was decided to use the name *Listeria monocytogenes* (Gray and Killinger 1966). Primarily, *Listeria* was associated with infections in domestic

animals. In the 1980s, after an epidemic of human listeriosis, it was recognized from laboratory results that *L. monocytogenes* was a foodborne pathogen (Schuchat et al. 1991).

L. monocytogenes is a gram-positive and non-sporeforming bacterium. It is a short (about 1-2 µm in length) rod-shaped organism (Rocourt 1999). *L. monocytogenes* is facultative anaerobic; capable of survival and growth in the presence or absence of oxygen. The pathogen bacterium is catalase positive and oxidase negative (Meloni 2015). It expresses a beta hemolysin, which causes the destruction of red blood cells in the blood agar. The presence of the hemolysin is a major virulence factor of *L. monocytogenes* (Swaminathan et al. 2001) This property helps to distinguish between this bacterium and other nonpathogenic *Listeria* species (Swaminathan et al. 2001). When the bacterium is cultured at temperatures between 20 ºC and 25 ºC, it may become motile via peritrichous flagella (Farber and Peterkin 1991). The bacterium is able to multiply at temperatures between 0 ºC and 45 ºC, but the optimum growth temperature is between 30 ºC and 37 ºC (Walker et al. 1990). *L. monocytogenes* is capable of growing at pH between 4.4 and 9.6, but the optimum pH is approximately 7 (Thevenot et al. 2006). *L. monocytogenes* also has a high salt tolerance and can multiply in up to 10% NaCl (Rørvik and Yndestad 1991). The survival rate at low pH and high salt concentrations is dependent on temperatures (Cole et al. 1990). Perfect conditions exist for *L. monocytogenes* development in vacuum packed products such as smoked salmon. Salting can inhibit normal flora and allow *L. monocytogenes* to multiply without competing with other bacteria.

1.3 Listeriosis

The pathogenic bacterium *L. monocytogenes* may give rise to the illness of listeriosis. The disease can occur in humans and animals. Listeriosis was initially associated with sick sheep and called The Circling Disease or Silage Disease (ADDL 2007). Epidemiological evidence and multiple outbreaks have shown that epidemic listeriosis is a foodborne illness (Bula et al. 1995). The development of listeriosis occurs through the intake of contaminated food. The risk of infection increases with the number of bacteria consumed, and the consumption of contaminated food over several days produces an increased chance of infection. The infectious dose depends on many factors such as the immunological status of the host, type of food, pathogenicity and virulence of the *Listeria* strains (Jemmi and Stephan 2006). Listeriosis is a rare disease in humans, but with high morbidity, hospitalization (>92%), and lethality (25-30%) (Swaminathan 2001). In 2010 alone, based on the collected data, it has

been estimated and recorded that listeriosis resulted in 23 150 illnesses, 5 463 deaths, and 172 823 disability-adjusted life years globally (de Noordhout et al. 2014). In the EU states, about 1,470 human listeriosis cases were noted in 2011, with a mortality rate of 12.7% (EFSA 2013). Due to the high mortality rate, *L. monocytogenes* is a leading cause amongst the fatalities of foodborne bacterial pathogens (Paoli 2005). The illness is a zoonotic disease, and therefore able to spread from animals to humans (EFSA 2007). It was the fifth most common zoonotic infection in Europe, after *Campylobacter*, *Salmonella*, *Yersinia*, and VTEC (Verotoxigenic *Escherichia coli*) infections (EFSA 2008). Human listeriosis is usually caused by *Listeria monocytogenes* (EFSA 2013). The high risk groups are; the elderly, pregnant woman, newborn babies, and people with impaired immune systems, such as HIV patients (Farber and Peterkin 1991). An increasing incidence of the disease has been observed in people over 60 years old (Gillespie et al. 2010). *L. monocytogenes* isolated from normal sterile samples, such as blood, is a sign of clinical listeriosis. The incubation time is typically 2-3 weeks, and up to three months (Dawson et al. 2006). The initial symptoms in healthy people often resemble the common flu. The infection may cause a fever, headache, muscle aches, nausea, vomiting, diarrhea, abdominal pain, or weight loss. Other symptoms such as a stiff neck, confusion, loss of balance and convulsions may occur if the infection spreads to the central nervous system. The most serious symptoms of listeriosis in humans are encephalitis, septicemia, and meningitis, which may lead to death (Disson and Lecuit 2013). Listeriosis is treated with ampicillin and gentamicin, because these drugs seem to be synergistic. The disease may occur at any time during pregnancy (Swaminathan 2001). Most pregnant women experienced a flu-like illness and gastrointestinal symptoms (Swaminathan 2001). Listeriosis in pregnant women may result in spontaneous abortion, stillbirth, premature delivery, and early onset neonatal infection (Slutsker and Schuchat 1999). In EU Members States and Norway, an increasing trend of listeriosis cases was observed in the years between 2002 and 2006, but no significant increasing or decreasing trend in human listeriosis was noted from 2008 to 2011 (EFSA 2013). In 2006, listeriosis was noted in 23 EU member states (EFSA 2013).

1.4 Contamination of food products

Food is the main route of transmission of listeriosis to humans (WHO 2004). The highest risk group is food that has not been heated before consumption. Examples include ready-to-eat products, soft cheese such as Brie, and Camembert, smoked vacuum packed salmon, raw fish

and meat in general, raw milk, fruits and vegetables (Farber and Peterkin 1991). Since *L. monocytogenes* is able to survive and multiply at low temperatures, food stored in refrigerators for long periods of time poses a high risk of *L. monocytogenes* presence. Pasteurization (heat treatment at 72 ^{o}C for 15 min) can remove such contamination and reduce bacterial numbers to below detection levels (Jay 2005). The RASFF (Rapid Alert System for Food and Feed) reported in 2014 that *L. monocytogenes* tended to occur in smoked fish from Poland and cheese from France and Italy (Baele 2015).

The safe limit of the amount of *L. monocytogenes* is set to 100 cfu per gram at the end of the stated shelf-life. This limit applies in the EU, Norway, Switzerland, Australia, Canada, and New Zealand. In the USA, there is no *L. monocytogenes* allowed at the latest eat-by date (Løvdal et al. 2015). It is important to maintain low initial numbers of *L. monocytogenes* in fresh products, to limit the amount of *L. monocytogenes* at the end of shelf-life. Numbers of *L. monocytogenes* below 100 cfu/g, were also below 100 cfu/g after 3 weeks of storage at 5 ^{o}C. If the initial concentration of *L. monocytogenes* was about 300-400 cfu/g, *L. monocytogenes* grew to 3 x 10^4 cfu/g during the same storage time (Rørvik 2000).

1.5 *Listeria* in the processing environment

L. monocytogenes has a widespread distribution. The organism is found in soil, on plants, in freshwater and seawater, in silage, in sewage, and in fecal materials (Ryser and Marth 1999). *L. monocytogenes* is a hygiene problem in food companies. Bacteria can enter into food processing factories through raw foods of animal origin, raw plants, soil on workers' shoes and clothing, transport equipment, and through healthy human carriers (Swaminathan et al. 2001). Within the facility, bacteria can become established in processing machinery with poor designs, in locations that are difficult to clean. *Listeria* thrives in humid environments with residues of organic matter. The presence of nutrients generates beneficial surroundings for bacterial growth. It has been noted that *Listeria* was detected in moist areas such as floors, floor drains, condensed and stagnant water, product residues, processing equipment, tube systems, screws, cracks in conveyor belts, and wheels in trolleys. *Listeria* can adhere to cast iron, stainless steel, glass, plastic, Teflon, and nitrile (Ferreira et al. 2014).

Listeria can form a homogenous biofilm or other bacteria may be involved (Ferreira et al. 2014). A biofilm is a collection of bacteria that are attached to a surface and to each other. They are surrounded with self-made mucus that gives them protection and increased survival rates. Biofilms are not removed by daily routine cleaning (Langsrud et al. 2016). Fagerlund et

al. (2017) observed that biofilms exposed to daily cleaning and disinfection cycles, were dominated by *Pseudomonas putida* (65-76%), *Pseudomonas fluorescens* (11-15%), and *Listeria monocytogenes* (3-11%). In single-species biofilms, *L. monocytogenes* developed a higher tolerance to cleaning and disinfection over time both for peracetic acid and quaternary ammonium disinfectant, indicating that a broad-spectrum mechanism was involved (Fagerlund et al. 2017). Biofilms are preferentially established in the surface irregularities of conveyor belts, potentially constituting harborage sites for persistent contamination (Fagerlund et al. 2017). Biofilms are considered to be significant reservoirs which contaminate food products during processing (Paoli 2005). Studies show that some *Listeria* strains belong to a specific environment. *Listeria* strains have been established in one or more locations over a longer period. The reason for this may be a poor washing procedure, strains resistant to detergent or disinfection, or non-hygienic machine design which does not allow for thorough cleaning (Rørvik 2000; Giaouris et al. 2015). Specific strains are recognizable within a specific production location. Upon finding *Listeria* at a specific location, one can trace it back to the original location of occurrence based on the genotype of the *Listeria* bacterium.

1.6 Methods used for Listeria detection

During this study, the traditional microbiological methods for the detection of *L. monocytogenes* were used. The catalase test was performed. Catalase is an enzyme found in aerobic and facultative anaerobic bacteria. It deactivates poisonous oxygen compounds such as radicals and hydrogen peroxide. The latter property was used for catalase testing. One drop of hydrogen peroxide was dripped onto one bacterial colony. The catalase converts H_2O_2 to oxygen and water, and gas bubbles were formed.

Virulent strains like *L. monocytogenes* and *L. ivanovii* produce a beta hemolysin, while *L. innocua* and *L. welshimeri* are avirulent strains and do not produce a hemolysin. This property is often used for the CAMP (Christie-Atkins-Munch-Petersen) test, as the final test for *L. monocytogenes* detection (Jay 2005). In this study Listeria Brilliance™ (Oxoid, Basingstoke, United Kingdom) selective agar, containing substances that the inhibit growth of other bacteria was used. The use of selective agar plates allowed for the separation of the desired bacteria. On the Listeria Brilliance agar plates, colonies of *L. monocytogenes* were revealed with a light, misty halo around the colony. This is due to the enzyme lecithinase, which

hydrolyses lecithin in the medium (Anonym 2015). The selective Listeria Brilliance agar have both a good sensitivity and specificity (Park et al. 2014).

To detect *L. monocytogenes* at spp. level, API® Listeria (BioMerieux, Brussel, Belgium) was used. This is a set of ten biochemical tests. Based on the results, the probability of which *Listeria* species it is, is calculated. Rebuffo et al. (2006) reports that the API Listeria kit was able to identify 88% of the test isolates and 93% of *L. monocytogenes* strains of 277-strain test set. The biochemical reactions were interpreted based on the positive or negative colour of the reactions. The API kit results of two *Listeria* species is shown in the table below.

Table 1.6 API Listeria kit for Listeria spp. identification. Biochemical reactions and test results for L. monocytogenes and L. innocua is shown (Bille et al. 1992).

Biochemical wells		**Test results**	
Test	Reaction	*L. monocytogenes*	*L. innocua*
DIM	Presence of aryl amidase	-	+
ESC	Hydrolysis of esculin	+	+
α-MAN	Presence of α-mannosidase	+	+
DARL	Acid formation of D-arabitol	+	+
XYL	Acid formation of D-xylose	-	-
RHA	Acid formation of L-rhamnose	+	+
MDG	Acid formation of methyl-αD-glucopyranoside	+	+
RIB	Acid formation of D-ribose	-	-
GIP	Acid formation of glucose-1-phosphate	-	-
TAG	Acid formation of D-tagatose	-	-

Only the DIM-test, discriminates between *L. monocytogenes* and *L. innocua*. The DIM-test confirms the absence of the enzymatic substrate aryl amidase. The API Listeria kit also detects the hydrolysis of esculin, the presence of α-mannosidase, and the acid formation of arabitol, D-xylose, L-rhamnose, methyl-αD-glucopyranoside, D-ribose, glucose-1-phosphat, and D-tagatose. Received results were entered into a computer program, which contained the different profiles of many species, the obtained profiles are compared with other known profiles based on an algorithm (%ID) and similarity to the most typical profile within a species (T-index) is established. Identification is based on the %ID and T-index values. See Appendix for more information.

CFU provides an overview of how many cultured heterotrophic bacteria are found in the sample. The CFU number may be treated as a hygiene indicator. Bacteria in samples grow in agar until they become visible. One bacterial colony is counted as one bacterium, one CFU (Colony Forming Units). The CFU number is used to calculate bacterial concentration in the sample. In this study, aerobic incubation was performed.

1.7 Aim

The purpose of this study was to investigate the prevalence of the pathogenic bacterium *L. monocytogenes* in salmon farms. Particular fish farms were chosen in cooperation with staff of the fish processing company responsible for product quality and included fish farms with assumed *L. monocytogenes* problems, due to increased *L. monocytogenes* positive samples in the factory after the processing of fish coming from these salmon farms. The overall bacterial level was checked in:

- Seawater
- Well boat
- Fish

The water samples and the muscle and skin samples were examined for total aerobic bacterial count.

2. Materials and methods

2.1 Sample collection

A total number of 126 samples were collected from five fish farms (A, B, C, D, E) in the period from September 2017 to January 2018. Plant B was visited twice. The five visited fish farms were located in different areas of Norway. The fish farms were chosen because of concerns about the Listeria presence by the companies owning the farms. An overview over the sampling site, sampling period, date, and number of samples collected at each farm is presented in the table below:

Table 2.1.1 The distribution of samples collected at the five fish farms and the number of collected samples.

Sampling site	Sampling period	Sampling date	Water samples	Muscle + skin samples	Skin samples	Gills samples	Pooled samples (skin + gills from 3 fish)	Total sample number
Plant A	Visit 1	27.09.2017	6	10	-	-	-	16
Plant B1	Visit 2	19.10.2017	6	-	10	10	-	26
Plant C	Visit 3	07.11.2017	4	10	10	10	-	34
Plant B2	Visit 4	30.11.2017	3	-	10	10	2	25
Plant D	Visit 5	17.01.2018	6	-	10	-	-	26
Plant E	Sent per post	04.12.2017	9	-	-	-	-	9
Total			34	20	40	30	2	126

Both water- and fish samples were collected. The number and type of samples varied from plant to plant, depending on the needs and possibilities. Water samples consisted of seawater from fish cages and well boats. The fish samples were taken as a cross-section of muscle with skin, mucus from the skin, and gills samples. From plant E, nine water samples of seawater were sent. They were taken at three different depths. Table 2.1.2 gives an overview of the samples taken at each farm, the number of samples, and the sampling method used.

Table 2.1.2 An overview of sampling on each fish farm.

PLANT	TYPE SAMPLE	SAMPLING PLACE	NUMBER OF SAMPLES
A	Water samples	Fish cage	2
		Delousing water – 36°C	2
		Well boat	2
	Fish samples	Muscle + skin	10
B1	Water samples	Fish cage	2
		Before loading	2
		Before unloading	2
	Sodibox cloths	Mucus from skin	10
	Swabs	Gills	10
C	Water samples	Fish cage	2
		Blood water	2
	Sodibox cloths	Mucus from skin	10
	Swabs	Gills	10
	Fish samples	Muscle + skin	10
B2	Water samples	Fish cage	1
		Before loading	1
		Before unloading	1
	Sodibox cloths	Mucus from skin	10
	Swabs	Gills	10
	Pooled samples from 3 fish	Mucus from skin + gills	2
D	Water samples	Fish cage 1	2
		Fish cage 2	2
		Well boat, before unloading	2
	Sodibox cloths	Mucus/ from skin	10
E	Water samples	Inside the cage 0.5 m	1
		Inside the cage 5 m	1
		Inside the cage 15 m	1
		Outside the cage 0.5 m	1
		Outside the cage 5 m	1
		Outside the cage 15 m	1
		Between the cage and land 0.5m	1
		Between the cage and land 5 m	1
		Between the cage and land 15 m	1

Water was taken from the fish cage, well boat before the fish was loaded and just before unloading, blood water, and warm water from the delousing boat. Regarding the above-mentioned samples, surface water was collected. Nine water samples of seawater from within the fish cages, outside cages and water between the land and cages were sent. All of them were gathered at three different depths: 0.5 m, 5 m, and 15 m. Figure 2.1.1 shows the water samples collected in sterile 1-liter polyethylene bottles. The examined fish were taken from the fish cage and killed by a blow to the head. In plant D, the skin samples were taken from live fish. Skin samples were taken with sterile Sodibox cloths (Sodibox, La Foret-Fouesnant, France) starting from the head, thoroughly between the fins, ending with the tail, taking into consideration the collection of as much mucus and organic material as possible. Figure 2.1.2 shows the Sodibox cloth with the gloves included in the set. The gloves were changed at each time a new sample was taken. Gill samples were taken with a sterile swab (FloqSwabs, Copan, Brescia, Italy) from the same ten examined fish. Figure 2.1.3 shows the FloqSwabs. The gill cover was lifted, and the gills were systematically swabbed. The swabs were stored in 15 ml Falcon tubes (Falcon, VWR, Leuven, Belgium) with 5 ml of buffered peptone water (107228, Merck KGaA, Darmstadt, Germany). Sodibox cloths were also used in the pooled samples of three fish, which were taken from the skin and gills. The muscle + skin samples were taken by using a sterile scalpel and stored in a sterile bag or container. The weight of the tested samples was about 9-10 g per sample. The fish was picked up from the fish cage. All samples were stored in cooling bags with several cooling elements to maintain a temperature of approx. 4 ^{o}C. The analysis was initiated within 24 hours after sample collection.

Figure 2.1.1 The water samples in 1-liter polyethylene bottles.

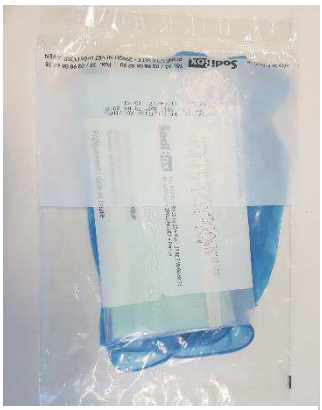

Figure 2.1.2 The Sodibox cloth set used for skin samples and pooled samples.

Figure 2.1.3 The FloqSwabs used for gill samples.

2.2 NMKL136 method

The method is used for the detection of *L. monocytogenes* in foods and feedstuffs and for the enumeration of *L. monocytogenes* in foods. In this study, the NMKL136 method was used for the detection of *L. monocytogenes*. The NMKL136 method is a two-step selective enrichment process. The primary enrichment is performed in an enrichment broth with reduced selectivity (Half-Fraser broth) at 37 ºC for 24 hours. The primary enrichment culture is further enriched in a secondary enrichment broth with full selectivity (Full-Fraser broth) at 30 ºC for 24 hours. Figures 2.2.1 and 2.2.2 show the negative (yellow) and the positive (black) reactions in Half-Fraser and Full-Fraser broth after 24 hours of incubation. The culture obtained from the secondary enrichment is plated out on the selective solid medium Listeria Brilliance or an equivalent selective medium. After incubation, the presumptive *L. monocytogenes* colonies may be confirmed by morphological and biochemical tests.

To prepare a Half Fraser and Full Fraser broth, 27.5 g of Fraser Listeria Selective Enrichment Broth (base) was suspended in 500 ml milliQ water and autoclaved for 15 min at 121 ºC and then cooled down to below 50 ºC. To make Half Fraser broth, the contents of 1 vial of ammonium iron(III) citrate and 1 vial of selective supplement were first dissolved in each original vial by adding sterile milliQ water (1 ml) and then mixed into 500 ml sterile Fraser broth base. To prepare Full Fraser broth, the content of 1 extra vial of selective supplement were added to already prepared Half Fraser broth.

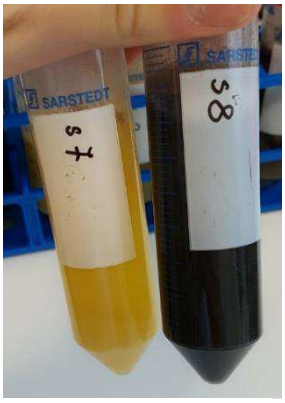

Figure 2.2.1 The negative (to the left) and the positive (to the right) reaction in Half Fraser after 24 hours of incubation at 37 ºC.

Figure 2.2.2 The negative (to the left) and the positive (to the right) reaction in Full Fraser after 24 hours of incubation at 30 ºC.

2.3 Detection limit for the Fraser method (NMKL 136) for detecting *L. monocytogenes* in sterile water

To determine the detection limit for the Fraser method, two strains of *L. monocytogenes* previously isolated from salmon slaughterhouses in 2015 were used (Eri 2016). S12 was isolated from a water tank and MB5 was isolated from a gutting machine. Two cryorings of each Microbank were transferred with a pipette tip to each of its sterile Erlenmeyer flasks. To each Erlenmeyer flask, 50 ml of Trypticase Soy Broth Yeast Extract was added. TSBYE medium was added and cultured on shaking (30 ºC, RPM 150) overnight. A 10x dilution series of both strains was made until a 10^{-12} dilution in autoclaved milliQ water was achieved. For dilutions, 15 ml Eppendorf tubes were used. Dilutions were performed in three parallels. To calculate the initial concentration of the bacterial cultures, dilutions 10^{-5} and 10^{-7} were transferred to small 1.5 ml sterile tubes and plated on TSAYE agar plates with Eddy Jet (IUL Instruments, Barcelona, Spain) spiral plater in two parallels. The plates were incubated for 24 hours at 30ºC.

Dilutions corresponding to 20, $2x10^3$ and $2x10^5$ cfu/L were analyzed by the NMKL 136 as explained above and the detection limit of *L. monocytogenes* in sterile water was derived.

2.4 Detection limit for the Fraser method (NMKL136) for detecting *Listeria monocytogenes* in seawater

Seawater was autoclaved for 15 min at 121 ºC. The *L. monocytogenes* S12 strain was used, because there was not much difference between S12 and MB5 strains in the previous experiment. S12 strain was grown as described above. A 10x dilution series to 10^{-9} was performed with autoclaved seawater in 3 parallels. The initial concentration of bacterial culture was calculated as mentioned above. One ml of 10^{-9} dilution was added to 500 ml of autoclaved seawater. Then 50 ml from this dilution was added to 450 ml of autoclaved seawater. Out of this 500 ml, 50 ml was further diluted in 450 ml of autoclaved seawater. This was conducted in 3 parallels. Each diluted solution was filtered through a 0.45µm pore size filter.

Dilutions corresponding to 0.2, 2 and 20 cfu/L were analyzed by the NMKL 136 and the detection limit of *L. monocytogenes* in autoclaved seawater was derived.

2.5 Preparation of the samples

Water samples were filtered using a vacuum pump, filter cup with a membrane filter (diameter 47 mm and pore size 0.45 µm) and Erlenmeyer flask. The membrane was carefully transferred to a 45 ml Falcon tube using a sterile tweezers and 40 ml of Half Fraser was added. Falcon tubes were incubated for 24 hours at 37 ºC. Water samples with blood were first coarse filtered with the Steriflip vacuum-driven filtration system (Millipore, Bedford, MA, USA) with pore size 20 µm in order to remove larger particles enabling subsequent filtration of a larger volume through the 0.45 µm filter.

For the swab samples from the gills, Tween 80 with the emulsifiers effect was added (Nielsen et al. 2016) and all tubes were placed in the vortex machine and shaken for two hours at room temperature. Then they were centrifuged (Hettich Zentrifugen D-78532 Tuttlingen, Germany) at 15 ºC for 30 minutes. The supernatant was carefully removed, and 5 ml of Half Fraser was added to the pellet and incubated for 24 hours at 37 ºC. The swabs were still kept inside the tube.

For cloth samples, 100 ml of Half Fraser was added and homogenized for 180 seconds in a stomacher (Smasher, Biomerieux SA, France). Then 40 ml of homogenate was transferred to 45 ml Falcon tubes and incubated for 24 hours at 37 ºC.

For muscle samples (about 10 g), Half Fraser (about 90 g) was added and homogenized for 120 seconds in a stomacher (Smasher, Biomerieux SA, France). Then 40 ml of homogenate was transferred to 45 ml Falcon tubes and incubated for 24 hours at 37 ºC.

After 24 hours the colour of all Falcon tubes was checked. In the case of colour change, 100 µl of the primary culture was transferred to 15 ml Falcon tubes and 9.9 ml of Full Fraser was added. Then they were incubated for 24 hours at 30 ºC. If the colour changed again, 100 µl of the secondary culture was plated onto the Listeria Brilliance agar using an automatic pipette and single-use spreader. The plates were incubated for 24 hours at 30 ºC. If, after 24 hours there was no growth, the plates were incubated for another 24 hours. Green or turquoise colonies were transferred with a sterile loop needle to new Listeria Brilliance plates several times in order to obtain pure colonies. Plates were incubated again for 24 hours at 30 ºC.

2.6 Detection and identification of *Listeria* spp. and *L. monocytogenes* with traditional microbiological methods

In order to eliminate non-*Listeria*, a catalase test was performed. The catalase test was conducted by dropping a drop of 3% hydrogen peroxide on a colony placed on a clean glass slide. Upon positive reaction, oxygen gas in the form of gas bubbles was developed as shown in Figure 2.6. Colonies were also examined microscopically (Leica Microsystems, DM 1000) to see if these were rod-shaped and not spore forming. Immersion oil was used to achieve 100x magnification.

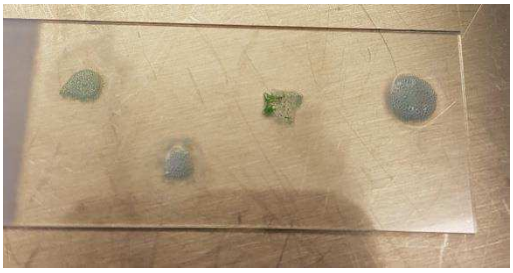

Figure 2.6 Four positive reactions of catalase test. Forming of gas bubbles.

2.7 Biochemical determination of Listeria with API kit

After using the traditional identification methods, catalase-positive and rod-shaped isolates were tested with API Listeria. Current colonies were transferred from the Listeria Brilliance plates to blood plates and incubated for 24 hours at 30 ºC. For the control, *L. monocytogenes* ATCC 51742 and *L. innocua* ATCC 7644 were used, taken from the American Type Culture Collection. Two cryorings were transferred by pipette tip to 50.0 ml TSBYE media using a sterile technique. The bacterial cultures were grown overnight in a rotary incubation cabinet (30 ºC, RPM 150) and transferred to Listeria Brilliance agar plates for incubation for 24 hours at 30 ºC. The bacterial colony was transferred to blood agar plates for incubation for 24 hours at 30 ºC. Bacterial culture was suspended in solution from an API set, and a spectrophotometer (UV mini 120 UV-Vis Spectrophotometer, Shimadzu, Japan)

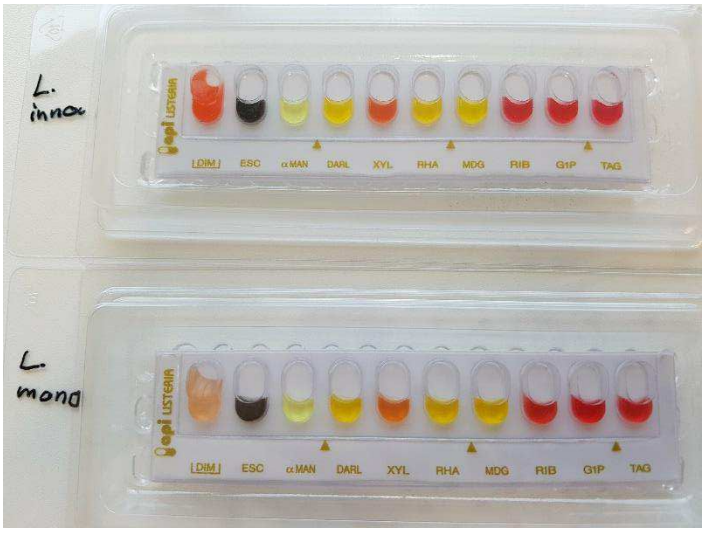

Figure 2.7 The API Listeria tests of L. innocua and L. monocytogenes. The only difference is the DIM-test: positive for L. innocua and negative for L. monocytogenes.

was used to find the density of 1 McFarland (equivalent to OD600 of 0.257). A bacterial suspension with an absorbance of 0.2-0.3 at 600 nm (OD600) was used. Sterile water (3 ml) was emptied at the bottom of the supplied plastic tray to create a damp atmosphere, and the API Listeria strip was placed there. 100 µl of bacterial suspension was transferred to a DIM test, and 50 µl to other test wells. After incubation for 20 hours at 37°C, the ZYM-B reagent was pipetted into the DIM well. The colour reaction was interpreted within 3 minutes after the colour reaction shown in Figure 2.7 following the reading table in the package leaflet. The results from the API test were plotted into the computer program (apiweb.biomerieux.com). Refer to the package instructions for a method description.

2.8 Listeria detection with a Polymerase Chain Reaction (PCR)

Five 30 ml samples from the fish cage and three 30 ml samples from the well boat were centrifuged at 23,000 x g for 30 min at room temperature in a centrifuge (Hettich Zentrifugen D-78532 Tuttlingen, Germany). The supernatants were removed, and DNA was isolated from the pellets by use of the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. q-PCR were assayed in an ABI StepOnePlus thermocycler (Applied Biosystems, Foster City, California, USA) using Specific TaqMan primers and a probe for *L. monocytogenes* targeting the invasion-associated protein P60 (iap) gene (Genesig, Primerdesign Ltd., Southampton, UK). qPCR was performed according to the manufacturers instructions Real-time PCR results were recorded as the mean of 2 analytical replicates analysed for each sample against the respective copy of a standard curve with a dynamic range from Log 1 to Log 6 supplied with the kit. A negative control was included in the run.

2.9 Total aerobic bacteria count

The number of CFU (Colony Forming Units) in water and muscle samples was calculated. In total, 34 water samples and 20 muscle samples were examined. 1 ml of homogenate was transferred to 1.5 ml Eppendorf tubes (Fischer Scientific, USA). A dilution series from 10^0 to 10^{-2} were performed with sterile autoclaved MilliQ-water (Millipore, Brussel, Belgium). It was plated out into three parallels from every dilution of 10^0 and 10^{-2} on TSAYE agar plates by using an automated plating machine (Eddy Jet V 1.23, IUL instruments, Barcelona, Spain). Refer to the accompanying manual for the methods used to count colonies and for calculating CFU by plating with Eddy Jet. The plates were incubated for 24-48 hours at 30 °C. The

seawater samples from plant E were plated out both on TSAYE and Marine agar plates to compare the achieved number of colonies on both agars. For colony calculation it was finally decided to use Marine agar, due to the more reliable amounts of bacteria. Samples were plated out by using Eddy Jet and incubated for 48 hours at 25 ^{o}C. After incubation, the number of colonies were calculated.

3. Results

3.1 Determination of the detection limit of *L. monocytogenes* for the NMKL 136 method in sterile milliQ water without filtration and in autoclaved seawater with filtration

Two strains of *L. monocytogenes*, S12 and MB5, were examined to determine the detection limit in sterile milliQ water without filtration. The bacterial stock solutions were calculated to be ~10^9 cfu/ml. For the S12 strain, all three parallels of dilutions 10^{-10} and one of the three parallels of dilutions 10^{-12} were tested positive for the presence of *L. monocytogenes*. This is equivalent to a detection limit of between 10 – 1000 cfu/L.

One strain of L. monocytogenes, S12, was used for the determination of the detection limit in autoclaved seawater with filtration. No significant difference between S12 and MB5 strains in the previous experiment was observed. The bacterial stock solution was calculated to ~10^9 cfu/ml. The three parallels for 10^{-12} dilutions and one of three parallels of 10^{-13} dilutions were tested positive for the presence of L. monocytogenes. This is equivalent to a detection limit of between 2 – 20 cfu/L.

3.2 Detection and determination of *L. monocytogenes* and *Listeria* species with traditional microbiological methods

Listeria species became visible as turquoise bacteria colonies on selective Listeria Brilliance agar plates. Colonies of *L. monocytogenes* had a halo around the colonies. On blood agar plates, the colonies of *L. monocytogenes* were gray-white with small beta hemolysis around colonies after 24 hours. This was easier to see when the plates were held up against a light source. Colonies of *L. monocytogenes* were only observed at the control sample when the API-test was performed. None of the environmental isolates in the present study displayed hemolysis. Figure 3.2.1 shows Listeria Brilliance plates, where only on the s8 sample are there visible green-turquoise colonies, presumptive *L. monocytogenes*.

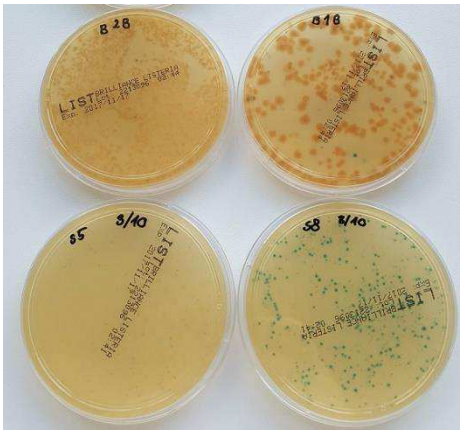

Figure 3.2.1. The Listeria Brilliance plates with presumptive L. monocytogenes on the s8 sample.

The turquoise colonies from the Listeria Brilliance agar plates were tested with a catalase test and were examined microscopically. All tested colonies were catalase positive. The next step was to check the shape of the tested colonies by using a microscope (Leica Microsystems DM 1000). All rod-shaped colonies were further analysed with an API Listeria kit.

No *L. monocytogenes* was detected. It was found *L. welshimeri* in one muscle + skin sample from plant A. *L. welshimeri* is morphologically similar to *L. monocytogenes*, but no beta hemolysis on the blood agar plate, and no halo around the colonies on the Brilliance plates were visible. Many bacterial colonies were similar to colonies of *L. monocytogenes*, but no halo was recognized on the selective agar plates. Figure 3.2.2 and Figure 3.2.3 show two strains of *L. monocytogenes*, S12 and MB5, on the Listeria Brilliance plates, used to determine the detection limit. It is easy to see the halo around the turquoise bacterial colonies.

Figure 3.2.2 L. monocytogenes MB5 on the Listeria Brilliance plate with visible halo around the colonies.

Figure 3.2.3 L. monocytogenes S12 on the Listeria Brilliance plate with visible halo around the colonies.

3.3 Biochemical determination of *Listeria*-species with API Listeria kit

The suspected *Listeria* colonies were isolated and first tested with traditional microbiological methods. Catalase positive and rod-shaped isolates were thereafter identified with an API Listeria kit. From plant A, four muscle + skin samples were examined. One of them (s9) was recognized to be *L. welshimeri*. Figure 3.3 shows the results from API Listeria of the s9 and s1 sample. From the plant B2, one water sample

Figure 3.3 API Listeria test for two muscle + skin samples. The positive (s9) and negative reaction (s1) of the MDG well.

from the well boat before the unloading of fish, and one skin-cloth sample from plant D were checked with an API Listeria kit, but it was not the *Listeria* species. As a control, *L. innocua* and *L. monocytogenes*, taken from the *Listeria* strains collection were used.

3.4 Identification of *L. monocytogenes* with the PCR method

qPCR for *L. monocytogenes* were negative for all samples meaning that the concentration was below the detection limit of 10 bacteria per 30 mL sample.

3.5 Results from an external laboratory

During a visit to plant B2, a double set of samples of the same fish and seawater were taken and sent to an external laboratory (Kystlab prebio). The laboratory used a NordVal no.022 method to detect *L. monocytogenes*. According to the principle of RAPID'L, mono medium relies on the chromogenic detection of the *L. monocytogenes* phosphatidylinositol-specific phospholipase C and on the inability of these species to metabolize xylose (Anonym 2018b). The results from this laboratory coincided with the internal laboratory results. No *L. monocytogenes* were detected. See Appendix for documentation from the foreign laboratory.

Table 3.5 The results of water samples.

PLANT	PLACE OF SAMPLING	DEPTH	NUMBER OF SAMPLES IN LITER	PARALLEL	NUMBER OF MIL FILTERED PER FILTER	*LISTERIA MONOCYTOGENES*
A	Fish cage	Surface water	2x1 L	1A	200	Not detected
				1B	200	Not detected
				2A	200	Not detected
				2B	200	Not detected
	Well boat	Surface water	2x1 L	1A	100	Not detected
				1B	100	Not detected
				2A	200	Not detected
				2B	200	Not detected
	Well boat, delousing water, 36°C	Surface water	2x1 L	1A	200	Not detected
				1B	150	Not detected
				2A	150	Not detected
				2B	150	Not detected

B1	Fish cage	Surface water	2x1 L	1A	200	Not detected
				1B	200	Not detected
				2A	200	Not detected
				2B	200	Not detected
	Well boat before fish loading	Surface water	2x1 L	1A	200	Not detected
				1B	200	Not detected
				2A	200	Not detected
				2B	100	Not detected
	Well boat, before fish unloading	Surface water	2x1 L	1A	200	Not detected
				1B	200	Not detected
				2A	200	Not detected
				2B	200	Not detected
C	Fish cage	Surface water	2x1 L	1A	500	Not detected
				1B	500	Not detected
				2A	500	Not detected
				2B	500	Not detected
	Blood water	Surface water	2x1 L	1A	40	Not detected
				1B	40	Not detected
				1C	40	Not detected
				2A	40	Not detected
				2B	40	Not detected
				2C	40	Not detected
B2	Fish cage	Surface water	1x1 L	1A	500	Not detected
				1B	500	Not detected
	Well boat before fish loading	Surface water	1x1 L	1A	500	Not detected
				1B	500	Not detected
	Well boat before fish unloading	Surface water	1x1 L	1A	500	Not detected
				1B	500	Not detected
D	Fish cage 1	Surface water	2x1 L	1A	500	Not detected
				1B	500	Not detected
				2A	500	Not detected
				2B	500	Not detected
	Fish cage 2	Surface water	2x1 L	1A	500	Not detected
				1B	500	Not detected
				2A	500	Not detected
				2B	500	Not detected
	Well boat, before fish unloading	Surface water	2x1L	1A	500	Not detected
				1B	500	Not detected
				2A	500	Not detected
				2B	500	Not detected

	Well boat, before fish unloading	-	1x1 L	1A	500	Not detected
				1B	500	Not detected
E	Inside the fish cage	0.5 m	600 mL		500	Not detected
		5 m	600 mL		500	Not detected
		15 m	600 mL		500	Not detected
	Outside the fish cage	0.5 m	600 mL		500	Not detected
		5 m	600 mL		500	Not detected
		15 m	600 mL		500	Not detected
	Between land and fish cage	0.5 m	600 mL		500	Not detected
		5 m	600 mL		500	Not detected
		15 m	600 mL		500	Not detected

3.6 The general number of bacteria in water samples

Figures 3.6.1 and 3.6.2 illustrates the quantification of the colony count of water samples from the sea and well boat. On the surface of the water in the well was a layer of foam, due to the presence of the protein from fish. Water samples from the bleeding tank were red due to fish blood and the content of organic material. Samples used for the colony count were not filtered. The same volume was used in all samples. The detection limit was set to one colony per plate. Water samples from plant E were shown separately due to a variance in depth.

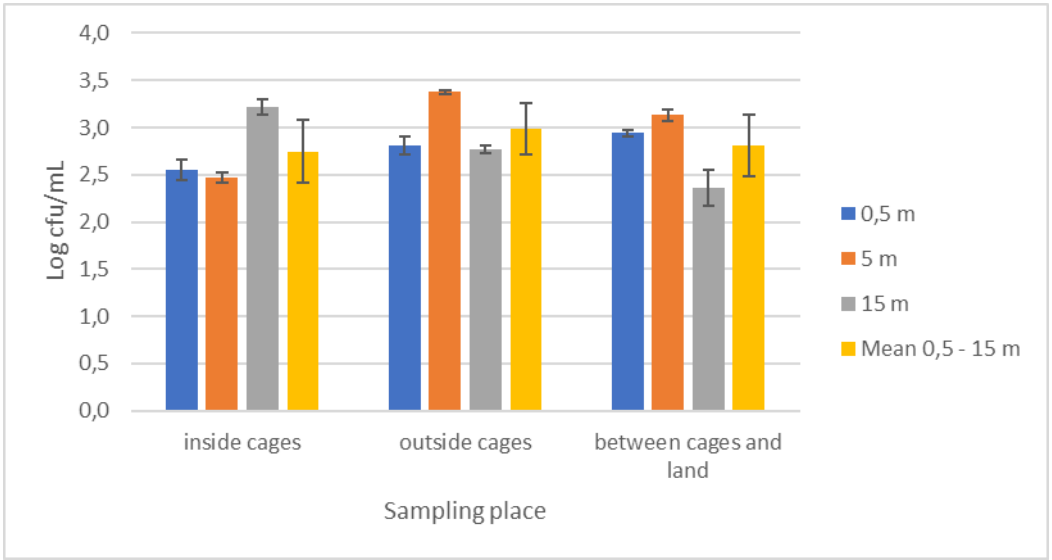

Figure 3.6.1 Total aerobic colony count from water samples incubated at 25 °C taken from plant E in three different places and depths. Every value is an average of 3 parallels. Error bars are standard deviations.

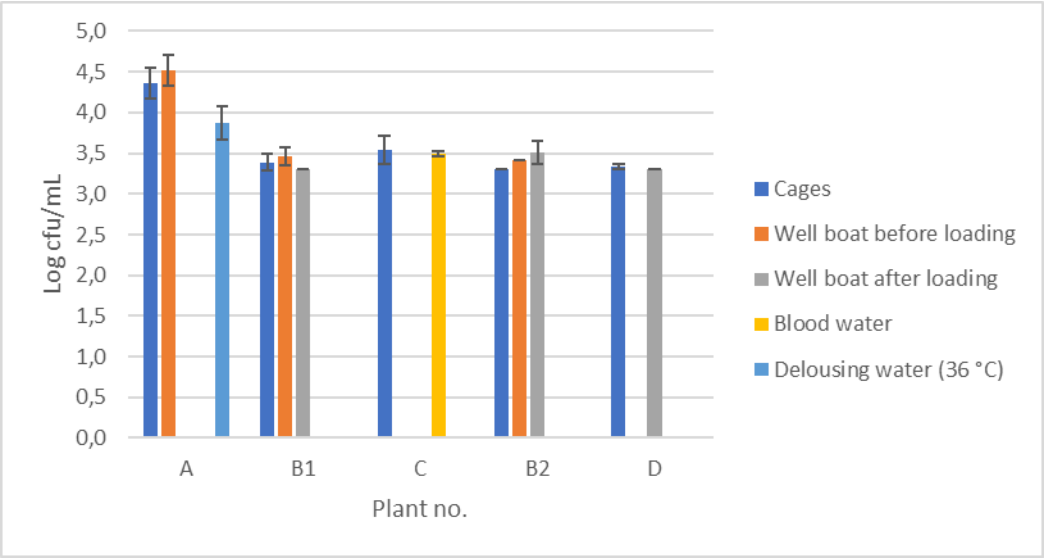

Figure 1.6.2 Total aerobic colony count from water samples incubated at 30 °C. Every value is an average of 3 parallels. Error bars are standard deviations.

3.7 The general number of bacteria in muscle and skin samples

Sampling from plant A was conducted with a service boat. Sampling from plant D was conducted on a slaughter boat. The fish were taken from the fish cage. The average of all the muscle and skin samples from plant A is 4.07 log cfu/mL, and the average of all the muscle and skin samples from plant D is 2.83 log cfu/mL.

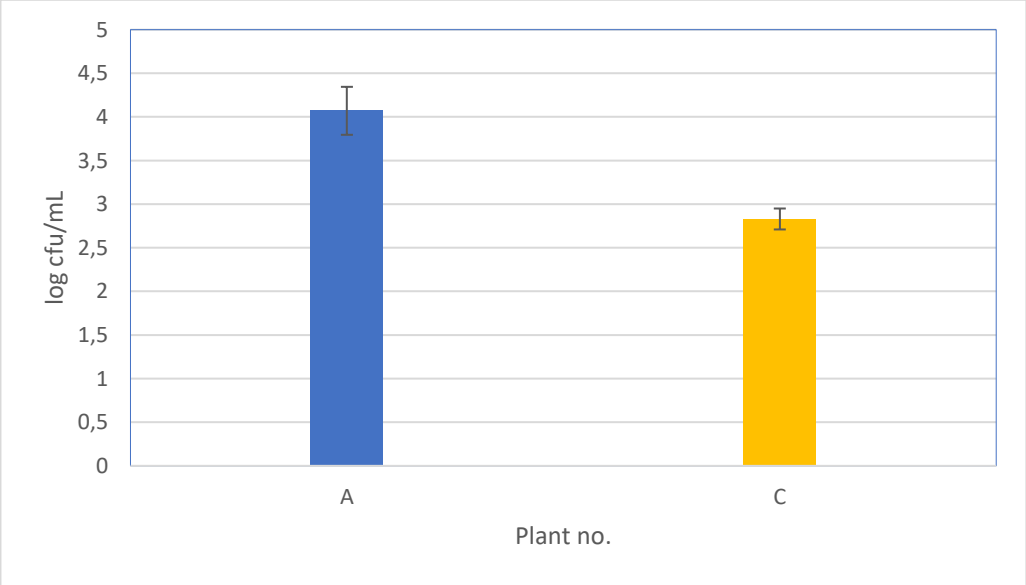

Figure 3.7 Total aerobic colony count from muscle + skin samples. The average of 10 fish samples from each plant was calculated.

4. Discussion

In this study, seawater and fish from Salmon-farms were tested for the presence of the bacterium *L. monocytogenes*. *L. monocytogenes* was not detected in any of the 126 samples. Many similar studies obtained the same conclusions (Johansson et al. 1999; Heir and Langsrud 2013). Non-pathogenic *L. welshimeri* was found in 1 of 16 samples (6.25%) collected from plant A. Duplicates of the samples collected at plant B2 were sent to an external laboratory. The results from this laboratory were in accordance with the internal laboratory results; both confirmed the absence of *L. monocytogenes* in all samples from plant B2. The absence of both living and dead *L. monocytogenes* was also proved by a PCR analysis of three water samples from plant D.

4.1 Methods used to detect *L. monocytogenes* and *Listeria* spp.

Sodibox cloths were used to collect the mucus from fish skin. Sampling from plants B1, C and B2 were conducted on dead fish, while sampling from plant D were conducted on live fish. The cloths were used to take samples from larger areas, while the swabs were used for smaller surfaces like gill sampling. The swab had the ability to reach into the smaller, tighter spaces. All samples were kept cold until analysis, therefore it is assumed that bacteria did not multiply significantly after sampling.

The Millipore membrane filter with pore size 0.45 µm was examined by (Besse et al. 2004) relative to recovering *L. monocytogenes* on the filter during filtering. A known concentration of *L. monocytogenes* in a solution was filtered through the filter and the results were compared with the concentration of *L. monocytogenes* on the filter. This method proved to be precise in the enumeration of *L. monocytogenes* (Besse et al. 2004). A filter with a pore size of 20 µm was used to prefilter blood water which had many larger particles of organic matter. This was done in order to enable the filtration of sufficient volumes of blood water through the 0.45 µm filter. The 20 µm filter is not expected to affect the detection of *Listeria* due to the large pore size.

The samples were analyzed for the presence of *L. monocytogenes* with NMKL136, a method with good detection ability (2-20 cfu/L) in autoclaved seawater, as demonstrated by this study. The NMKL 136 method included the use of Fraser Broth Supplements and is a good way to prove the presence of *L. monocytogenes*. This method showed excellent detection

results and satisfactory results in the enumeration of *L. monocytongenes* in foods (Loncarevic et al. 2008). The sensitivities of ALOA, LCA, OCLA and LMBA in the detection of *L. monocytogenes* in food samples after one-step enrichment (Half-Fraser) were 94.4–96.4% and after two-step enrichment (Half-Fraser followed by Fraser) 97.7–100% (Loncarevic et al. 2008). The Listeria Brilliance agar works on a similar principle and is equally sensitive to the agar mentioned above. The Listeria Brilliance agar is better at distinguishing between *L. monocytogenes* and another *Listeria* species, forming a halo around the *L. monocytogenes* colony, due to the enzyme lecithinase (Park et al. 2014). In this study, the samples in the first-step enrichment culture were observed after 24 hours, but not so many of these went black within 24 hours. The study of (Truong 2008) has shown that the samples, which did not change colour within 24 hours, contain *L. monocytogenes*. Therefore, the samples were left for further observation for the next 24 hours. Most of the samples went black between the first and second day. A great minority of samples in the second-step enrichment culture needed more than 24 hours to turn black. After the second-step enrichment culture went black, it was plated out on the Listeria Brilliance plates. After 24-48 hours of incubation, 6 of 126 samples (4.76%) were considered to be Listeria spp. The black enrichment cultures were not black after 24 hours for a reason, because the samples did not contain *L. monocytogenes*. The conclusion is to follow the protocol of the method, where the test manufacturers describe the colour change after 24 hours of incubation. More than 24 hours of incubation may produce unreliable results. After a longer incubation time, the enrichment may react with bacteria other than *Listeria*.

Park et al. (2014) showed that Listeria Brilliance agar had a selectivity of 95.5% for 200 food samples not infected with *L. monocytogenes*. In the study of (Eri 2016), as in this study, it was discovered that not only *Listeria* were grown on the selective Listeria Brilliance agar plates. Therefore, it was necessary to use other tests, like API Listeria which confirmed the presence of the *Listeria* species. It was observed that green-turquoise colonies appeared on the selective agar. These green-turquoise bacteria colonies may be *Bacillus*-species. *Listeria* is closely related to *Bacillus*, *Lactobacillus*, and *Streptococcus* (Jay 2005). *Bacillus subtilis* have many similarities with *L. innocua* and *L. monocytogenes* in sequence analysis (Glaser et al. 2001). *B. subtilis* also has a similar morphology. It is gram-positive, rod-shaped and catalase positive, like *L. monocytogenes*. Under a microscope, the spores of *B. subtilis* may not always be visible. It is possible that *B. subtilis* will not produce spores and will appear to be very

similar to *L. monocytogenes*. *Bacillus*-species may have the same nutritional requirements as *Listeria*-species and grow on the selective Listeria Brilliance agar.

The API Listeria kit was used to distinguish between different *Listeria* species or to eliminate *Listeria* from further consideration. One of the six samples analysed was identified to be *L. welshimeri*. The rest of the samples did not show a positive result for *Listeria* spp. The interpretation of the colour reaction of the wells is subjective. Therefore, it was performed two controls: *L. monocytogenes* and *L. innocua*, to check the colour difference produced by the DIM-test. *L. monocytogenes* has a negative reaction to the DIM-test, and *L. innocua* has a positive one. That is the only detectable difference between these species. It is an advantage that the DIM-test is not based on hemolysis. This makes it possible to distinguish between atypical, nonhemolytic *L. monocytogenes* from *L. innocua* (Bille et al. 1992).

4.2 *L. monocytogenes* in the environment

Soil is often considered to be the source of *Listeria* contamination, particularly for silage (Fenlon 1988). The fertile agricultural soil is rich in decaying plant material, animal waste, and sewage sludge. Many studies confirm the presence of *Listeria* in agricultural soil. The surface soil has a higher level of the pathogen than the soil at a depth of 10 cm. This indicates that vegetation is a principle component in the *Listeria* contamination of the soil (Ryser and Marth 1999). In the present study, the samples were collected in the autumn and winter. These results overlap with the research of (Welshimer and Donker-Voet 1971) who obtained negative results from soil and dead vegetation samples collected in early autumn. However, analogue samples collected the following spring were almost all positive for *L. monocytogenes* (Welshimer and Donker-Voet 1971). This may explain the negative results observed in the present study. In the spring, there is far more runoff from agriculture found in seawater, due to the spreading of sewage sludge as fertilizer and the thawing of the earth after the winter time. The occurrence of *L. monocytogenes* may be higher during the spring and summer.

4.2.1 *L. monocytogenes* in seawater

There were 34 water samples collected from different places. Water from the well boat before (3) and after (7) fish were loaded from the cages, seawater in or around fish cages (20), blood water (2), and delousing water (36 ºC), where the fish were held for a few seconds to get rid

of lice (2). No *L. monocytogenes* were detected. Different studies mention a great divergence in the detection of *L. monocytogenes* in water samples from various environments. The prevalence of this bacterium in river waters, seawater, surface water, and spring water ranges from 0 to 62% (Ben Embarek 1994). The spring water and free ocean water tested negative for the organism (Ben Embarek 1994). The *L. monocytogenes*-positive samples were detected only in freshwater from rivers and lakes in the late spring (Arvanitidou et al. 1997). Brackett (1988) reports that surface waters, sewage effluents, and agricultural runoff may potentially contribute *Listeria* spp. to the aquatic environment. The highest number of positive samples was found in waters exposed to runoff from agricultural or urban areas. Plants B and D were not situated near farmlands, but plants A and C were quite close. It was reported that *L. monocytogenes* has been isolated from the fecal material of healthy grazing animals, like sheep, goats, and cattle. Watkins and Sleath (1981) noted >18,000 cfu/L in trade effluents associated with animals and sewage sludges from treatment plants in northeast England. Studies in northeastern Scotland reported about 120 cfu/mL of L. monocytogenes in untreated sewage, and 2-21 cfu/mL in treated effluent (Fenlon et al. 1996). Samples of silage and bird feces were examined by Fenlon (1985). Fecal samples indicated that seagulls feeding at sewage works had a higher rate of carriage than those found elsewhere. Normally, a low level of occurrence of *L. monocytogenes* was observed in bird fecal samples, with an increased presence of *L. monocytogenes* in the nesting season and the peak period for listeriosis in sheep (Fenlon 1985). Because of the *L. monocytogenes* presence in seawater, gulls can be another source of fish and shellfish contamination (Fenlon 1985).

Recent research proves that sunlight (visible 470 nm and UV-A light) adversely affects the viability of L. monocytogenes in seawater. The bacteria are able to survive in seawater over a longer period of time when sunlight is excluded (NicAogain et al. 2018). This finding has been confirmed by the results obtained by this study. Due to the small amount of sunlight in the winter, the number of the pathogenic bacteria is expected to be higher after winter or/and in early spring time due to more bacteria surviving, than after summer and in the autumn time, when seawater was exposed to sunlight over a longer period of time.

4.2.2 *L. monocytogenes* in fish raw material

In this study, *L. monocytogenes* was not detected in the salmon collected directly from the seawater. There were 92 salmon samples collected in total (skin (40), gills (30), muscle (20), and pooled samples (skin + gills of three fish (2)). These results coincide with where *Listeria*

was not detected in salmon before processing (Heir 2014). Other studies also show that no *L. monocytogenes* was detected in samples from salmon farms and harvested salmon (Hsu et al. 2005). Rørvik et al. (1995)'s research, show that *L. monocytogenes* was not detected in 50 slaughtered fish. Eri (2016) did not detect L. monocytogenes on 17 swab samples from fish necks and gills. This indicates a very low incidence of *L. monocytogenes* in live salmon. Ben Embarek (1994) reported that no bacteria were found in the intestinal tract, skin, and gills of 10 live salmon. The pathogen was not found in either the 199 salmon or in environmental samples taken from a fish farm in Bergen (Embarek 1997). The published studies report a very low occurrence of *L. monocytogenes* in raw fish, from 0–1 % to 10% (Autio et al. 1999; Johansson et al. 1999). In Japan, only 1.3% of the 781 fish samples and 0% of the salmon samples tested positive for *L. monocytogenes* (Jin et al. 1994; Iida et al. 1998).

4.2.3 *L. monocytogenes* in the processing environment

As mentioned earlier, *L. monocytogenes* is rarely found in fish and in seawater. Despite this, the fish processing environment is exposed to the establishment of pathogenic bacteria. Omnipresent moisture and the presence of organic material promotes bacterial growth. The key issue is to eliminate bacterial niches which may cause biofilm formation and become the immediate cause of product contamination. Although the bacteria are introduced to the processing factory in very low concentrations, often so low that it is not detectable, they may find perfect conditions for division and grow to significant concentrations. *L. monocytogenes* was often detected inside the gutting/slicer machine, conveyor belt, on the floor, and in the purification/cooling tank (Heir 2014; Eri 2016). The gutting and slicer machines are prone to contamination with *L. monocytogenes*, because of the collection of organic materials, such as blood and mucus, and they are usually hard to clean because of their advanced machinery, tubes, suction, nozzles etc. Slicer machines, locations under the conveyor belt, and inaccessible places between plastic and steel were mentioned as problem areas. The fabric conveyor belts are made of a material which provides a favourable attachment area for bacteria, especially if it is worn (Rørvik 2000). To solve this problem and reduce the risk of contamination, the conveyor belt could be more frequently exchanged and/or could be made from another material, which is not conducive to the attachment of bacteria. The NFR (Norges forskningsråd) funded a project (Fillet-O) which should solve the problem of killing, slaughtering, and processing of salmon, promoting automation (Eri 2016).

There are no formal demands on the internal control systems in the processing environments. The contagious hygiene demands are general, that it must be ensured that personnel, workwear, equipment, or machines do not constitute a hygiene risk and prevent the spread of disease. The food processing standards are supervised, controlled and legislated by the Food Safety Authority, which may include a program for the daily environmental and food product sampling for *Listeria* and coliform bacteria. The Norwegian food industry is further subjected to EU enforced Regulation (EC) 178/2002, laying down the General Principles and requirements of food safety, and later Regulation (EC) 852/2004, for the Hygiene of foodstuffs (Løvdal et al. 2015). In order to achieve proper hygiene in the processing areas, it may be necessary to implement elaborate internal control systems which provide basic rules for hygiene and which are common for all salmon processing factories.

There are studies showing that *L. monocytogenes* arrives with raw salmon to the smoke house (Rørvik 2000). Analysis with PFGE has proven that the same bacteria occur on raw fish, floor, staff, and finished products (Gudmundsdottir et al. 2005). The risk of contamination increases with the expanded degree of processing (Rørvik et al. 1997). Studies have shown that *L. monocytogenes* in fish may occur in very low concentrations. This makes the detection of *L. monocytogenes* difficult. Although *L. monocytogenes* appear in fish in very low numbers, the transport of several tonnes of fish through machines with potential niches suitable for the establishment of bacteria, may lead to bacterial accumulation inside the machines.

L. welshimeri was probably detected on one muscle + skin sample from plant A. The sample was taken on a service boat which handles the dead fish. The *L. welshimeri* found on the salmon could have come initially from the service boat. The presence of *L. welshimeri* on one sample increases the probability that maybe *L. monocytogenes* was also current on the same or other fish, but that the appearance was under the detection limit. All fish samples in this study tested negative for *L. monocytogenes*. Based on these results, it has not been proven that *L. monocytogenes* enters the processing environment via the fish, but because of the presence of *L. welshimeri*, the entrance of *L. monocytogenes* to the factory via fish cannot be excluded.

There are many studies which confirm the presence of *L. monocytogenes* on different processing equipment. *L. monocytogenes* was detected on the hands, gloves, and footwear of the staff (Heir and Langsrud 2013). The wheels of trolleys that are moved between clean and unclean zones tested positive for this pathogen, therefore it poses a serious risk of

contamination (Heir and Langsrud 2013). It appears that hygiene and disinfection are insufficient. There is a lot of speculation. Is it due to the negligence of employees who do not respect the principles of hygiene, are washing procedures badly designed or are disinfectants inadequate? Every food processing plant must struggle with diverse issues.

4.3 Methods for *Listeria* detection

New hygiene rules impose requirements on the manufacturer to assess and manage the risk of contamination individually. The salmon industry is forced to implement documentation of *Listeria* control throughout the production chain. The monitoring process over product quality may increase knowledge about the contamination risk food processing plants and give rise to the opportunity to introduce better measures against *L. monocytogenes*. Samples are often analysed in external laboratories by using microbiological standard methods. These methods are reliable, but they often take too long time. In the time it takes to analyse samples, the products may be further distributed or already eaten. Therefore, simple in use, fast, and reliable *Listeria* detection methods for companies are being researched and tested. The diversity of the so called quick methods is considerable (Heir 2014). The advantage of using these methods is the relatively short waiting time compared to microbiological standard methods. Unfortunately, the quick detection methods are not always reliable. They may show false negative or false positive results. The false negative results pose a risk to human health. And the false positive results may expose the factory to unnecessary cost linked to additional tests, or the withdrawal of the product from sale. In this study, for *L. monocytogenes* detection, a microbiological standard method, NMKL 136 was used, it takes a long time, but it is reliable and has a low detection limit.

In the world of industry, especially industries which work with raw materials, the risk of the establishment of pathogenic bacteria in the processing environment is ever present. In this case, the salmon industrial environment is prone to the establishment of *L. monocytogenes*. There are many research avenues being funded to find an effective method for reducing *L. monocytogenes* on the surface of fish. Ovissipour et al. (2018) investigated the effect of different electrolyzed water solutions, including acidic electrolyzed water (AEW) and neutral electrolyzed water (NEW) on the reduction of *L. monocytogene*s on salmon fillets. Another way to reduce the number of *L. monocytogenes* on the fillet, is irradiation with UV and Pulse UV. It was proposed that UV-light killed 70-80% of bacteria. For the best results, it is wise to use UV right before packing (Heir 2015). The next method used for bacteria reduction was

lauryl arginate. It showed a good antimicrobial effect, and *L. monocytogenes* reduction of 90%, but there were no inhibition effects on the surviving *Listeria* (Heir 2015). The next method to be investigated was Freebac-Mucosol, which removes mucus and bacteria from fish. It inhibits bacterial growth on the fish skin to a small degree but has no impact on the fish gills. For increased effect, the methods for the reduction and inhibition of *L. monocytogenes* may be combined (Heir 2015).

4.4 New concepts for Salmon slaughtering and processing

Due to hygiene considerations, it may be necessary to modernize the salmon industry, to make it similar to the chicken industry. This would involve a fully automated line, from killing, bleeding, gutting, filleting, and secondary processing. Automation may limit the use of buffer tanks for; cooling, rinsing, and grading of the fish, and introduce more hygienic controllable fish processing of individual fish through all the steps.

The salmon slaughterhouses are cleaned after one or two production shifts. Cleaning consists of flushing with clean water to get rid of fish residuals and blood, foaming with acid or alkaline based soap and spraying with disinfection chemicals. Several workers walk around flushing the surfaces with a hose, and after the production is finished, the cleaning shift spray soap-foam on different machines and production surfaces. The foam is left for some time. Then hot water is sprayed to rinse off the soap. After that, disinfectants are applied to deactivate microorganisms and are left to vaporize until the production process starts again (Løvdal et al. 2015).

The use of a fully automated processes would produce other advantages, like reducing staff requirements, increasing profitability, and advancing the fish processing and distribution industry of Norway. This would enable the control of quality at all stages and reduce transport costs (Digre 2014). Fish processing at the individual level is a big step forward in the maintenance of hygiene in the processing environment. It may help to prevent cross contamination inside the processing area. Reducing the number of staff working in the factory may contribute to a reduction in the movement of employees between the clean and unclean zone inside the factory, moving the tools, and thus lowering the risk of bacterial transmission. Fewer staff also decreases the probability of healthy carriers of *L. monocytogenes* being employed at the factory. *L. monocytogenes* can also be excreted by both symptomatic and asymptomatic human carriers. Throughout Europe, 1.8-9.0% of healthy individuals excrete *L.*

monocytogenes in their feces (Ralovich 1984). Therefore, it is very important that proper hand hygiene procedures are followed.

A new slaughter boat called "Norwegian Gannet" will be completed in September 2018. It will be the biggest slaughter boat in Norway (Refvik 2017). The boat will carry salmon directly from fish cages in Norway to Hirtshals in Denmark, where it will be packed and sent to the markets. Bleeding and gutting occurs on the boat on the way to Denmark. This slaughter boat is built according to the design of the already existing slaughter boat "Tauranga", but it will be much bigger than "Tauranga". This slaughter boat will allow fish to arrive in the markets twice as fast as the normal processing method (Refvik 2017). A full boat will replace 50 trailers to Europe, thus significantly reducing CO_2 emissions (Arnesen 2018). Therefore, the "Norwegian Gannet" is considered to be environmentally-friendly and sustainable. The shorter route to the consumer will extend the shelf life of the product. The team behind "Norwegian Gannet" have released reports about the reduced risk of contamination, mortality, and escapement of fish, because of the elimination of the transport of live fish and waiting cages (Arnesen 2018). This boat will be capable of carrying and processing 1,000 metric tons of salmon per trip (Refvik 2017). There will also be huge amounts of organic matter and bacteria generated at the location where the fish will be slaughtered and kept. The accumulation of organic matter and bacterial growth may arise. After every transport of fish, the machines, rooms, and all equipment must be well cleaned and disinfected, before a new fish load is taken on board. It is very important to wash equipment after every fish transport to avoid the formation of a biofilm. To keep the boat free of pathogenic bacteria over a long period on such a large surface could be a colossal challenge. However, the staff employed on the boat must be sure that the establishment and domestication of pathogenic bacteria on the slaughter boat does not arise. Otherwise, an immense amount of fish may be contaminated with the pathogen. This eventuality could lead to a gigantic loss of raw products and money for the fish industry.

4.5 *L. monocytogenes* viability in natural and artificial environments

Hansen et al. (2006) investigated the survival of *L. monocytogenes* and *L. innocua* in different environments. She discovered that *L. monocytogenes* fails to thrive in natural seawater and fresh water. The counts decreased rapidly when *L. monocytogenes* had to compete with natural microflora. When natural seawater and fresh water were autoclaved or filtered (0.2µm pore size), the counts of *Listeria* decreased much more slowly than in non-autoclaved or non-

filtered water (Hansen et al. 2006). *L. monocytogenes* died off in seawater within 36 h at room temperature in the presence of natural microflora (Hsu et al. 2005). If the temperature is 11 ºC or lower, *L. monocytogenes* loses its viability, but is still detectable after more than 6 days of incubation. The conclusion is that the natural microflora outperforms *Listeria* and pathogen reduction in natural water is a natural biological mechanism. When the growth of marine flora and *L. monocytogenes* in salmon blood water at ≤11 ºC and 22 ºC was investigated, the marine flora showed good growth at higher temperatures and a corresponding decrease in the growth rate as the temperature was lowered (Hsu et al. 2005). *L. monocytogenes* demonstrated very low growth at ≤11ºC and just marginally more growth at 22 ºC. Restricted growth of *L. monocytogenes* under these conditions in the presence of organic material shows that the marine microflora inhibit the pathogen, because *L. monocytogenes* grew well in filter-sterilized blood water (Hsu et al. 2005).

4.6 Total bacteria count

The aerobic bacteria count obtained for the present study was relatively low. This was expected due to sampling collection in the winter period.

The highest colony count from water samples was in plant A. The samples from plant A were collected earliest, in September. The temperature of the water was not measured, but the water temperature could be higher in September than during the rest of the winter months. The higher seawater temperature could contribute to the better survival conditions for bacteria. The number of bacteria from plants B1, C and B2 was quite stable. The lowest bacterial content was found in plant D. The samples were collected at the latest, in January, so water temperature was probably at its lowest.

The seawater samples taken at different depths in plant E present quite a stable number of bacteria. Between the samples collected at 0.5 m depth, the sample from between the fish cage and land contained the highest amount of bacteria. The reason for this may be the shortest distance to land and therefore water contamination from the runoff produced by agriculture. The sample taken from inside the cage at 15 m and the sample taken outside the cage at 5 m are almost the same. This may be explained by the large number of fish in the sea cage which is located at that depth. A large number of fish in a small area causes a lot of organic material and bacterial accumulation in and around the fish cage. The means of all

samples taken at the same depth are very similar, and the difference between them is estimated to be due to natural variation.

4.7 Conclusion

In the present study, five different fish farms were examined for the presence of *L. monocytogenes* on well boats, fish, and seawater from fish cages, in the period from September 2017 to January 2018. The pathogenic bacterium *L. monocytogenes* was not detected. The results show that seawater and the fish from sea cages do not represent a major contamination pressure for slaughterhouses in the autumn and winter seasons. However, it may be possible that *L. monocytogenes* enters the processing environments in very low concentrations although *L. monocytogenes* is undetectable in seawater and on fish raw material. The unhygienic design of processing machines, especially slicer machines, does not allow for cleaning and disinfection in hard-to-clean places. The presence of organic matter, omnipresent humidity and poor hygiene in the processing environment allows bacteria to accumulate in niches forming a biofilm. The transmission of bacteria within the slaughterhouse does not help to maintain cleanliness within the factory. The accumulation of undetectable amounts of the pathogenic bacteria poses a high risk for final product contamination during processing. The accumulation of bacteria over a long period of time makes it difficult to trace back to the origin of the bacteria. Because of its ubiquity in nature, it is not possible to prevent the entry of *L. monocytogenes* to the processing factory. Therefore, the crucial challenge for the industry is to find effective methods of eradicating the pathogenic bacteria from the processing environment and ensure safe food production.

4.8 Further work

The research and results of the present study provoked to think about further work which could reduce risk for product contamination with the pathogenic bacteria *L. monocytogenes*. Among these are:

- **Research on seasonal and climate variations.** Research on the existing fish farms should be related to seasonal variations. Sampling collection should take place throughout the whole year at different times and in various weather conditions. Only in this way will it be possible to draw any reliable conclusions concerning seasonal variation.

- **Land-based and off-shore farms.** Further work should relate to more research on land-based farms and off-shore plants. Maybe it would be easier to gain control over contamination sources on land-based farms? It would be well protected from the soil pollution from agriculture and fewer external factors would affect the farms. Or is it better for salmon to mature in more natural conditions and build off-shore based plants far away from land and agriculture contamination?

- **Processing modernization with easy-to-clean design with automated (robotized) cleaning.** Before the fish is killed, it is fasted for a few days to get rid of as much feces from the intestine as possible. However, the possibility that some residues remain in the intestine is high. During the gutting process, the fish product may become contaminated with the bacteria from the intestine. It is worth investigating if it is possible to gut fish more carefully in order to avoid damaging the intestine and exposing the product to contamination. The processing environments like smokehouses, slaughterhouses, well boats and slaughter boats should be equipped with modern machines with easy-to-clean designs. More research should be related to effective hygiene rules and protection against the establishment of pathogenic bacteria in the processing environments. The solution may be automated/robotized cleaning. The advantages of automated cleaning may be shorter cleaning time and using warmer water.

- **Hygiene legislation and internal control routines.** Revision of current legislations and guidelines should be performed. Some food producers do not have *Listeria*-problems inside theirs factories. Which internal control routines do they follow? Can other factories learn from them? *Listeria* detection methods used at the processing factories should be checked for its reliability and be properly selected to meet the company's needs for detecting *Listeria*.

- **Vectors for bacteria.** More studies should be conducted concerning factors that may act like a vector for bacteria. It may be the well boat which collects fish from different locations and cages, creating a risk for bacterial transmission between plants and fish cages. Another suggestion for further research is to investigate the feed tanks. The moisture getting into feed tanks, may pose a risk for the establishment of bacteria and if poorly secured may provide food for seagulls which, in turn, may pose a further risk

of contamination with a pathogen and become a vector for bacterial transmission. There are many areas of the food industry which require modernization in order to provide pathogen free food production and distribution.

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Appendix

Composition and function of media

Composition:

1. Half Fraser broth. REF. 42 627 (BioMérieux, 69280 Marcy 1'Etoile, France)

Meat peptone	5.0 g/L
Casein peptone	5.0
Meat extract	5.0
Yeast extract	5.0
Sodium chloride	20.0
Buffer mixture	13.35
Esculin	1.0
Lithium chloride	3.0
Ammonium ferric citrate	0.5
Acriflavin	0.0125
Nalidixic acid	0.01
Water to 1 liter	

2. Fraser broth. REF. 42 072 (BioMérieux, 69280 Marcy 1'Etoile, France)

Animal peptones	10.0 g/L
Meat extract	5.0
Yeast extract	5.0
Sodium chloride	20.0
Buffer mixture	13.35
Esculin	1.0
Lithium chloride	3.0
Ammonium ferric citrate	0.5
Acriflavin	0.025
Nalidixic acid	0.02
Water to 1 liter	

Characteristics:

The Half-Fraser broth or Fraser broth is used for the enrichment of bacteria in the genus *Listeria*. The broths contain a rich nutrient base consisting of a mixture of peptones and a buffer, which maintain the pH close to neutral to favour growth of the main species of *Listeria*. The selectivity of the broths is provided by lithium chloride, acriflavine, and nalidixic acid which inhibit the background flora. The ammonium ferric citrate present in the broths detects the presence of *Listeria* species by revealing the hydrolysis of esculin. Thus, the colour change of the medium from the initial yellow to black colour may be considered to be a presumptive test for the presence of *Listeria*.

TSAYE (agar)

0.6% yeast
20 g TSA (Oxoid)
3 g yeast (Merck)
500 ml distilled water

TSBYE (broth)

0.6 % yeast
15 g TSB
3 g yeast
500 ml distilled water

Marine agar

18.7 g Marine broth
8 g agar-agar
500 ml distilled water

Brilliance Listeria selective agar

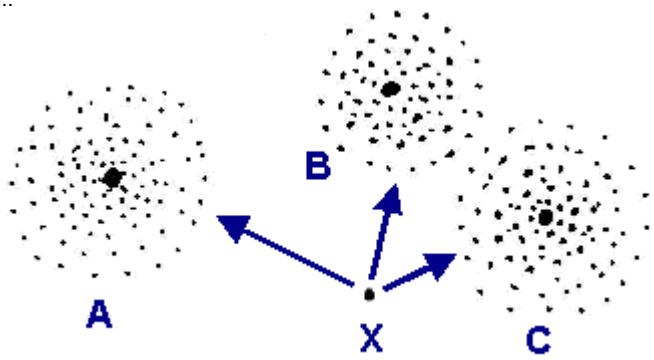
33.6 g Brilliance agar
480 ml distilled water
Autoclave 121 ºC, 15 min
Cool to 46 ºC.
Add one ampoule diff.supplement dissolved in 1 ml autoclaved milliQ water
Add one ampoule selective supplement dissolved in 1 ml autoclaved milliQ water

PRINCIPLE

To identify an organism, the profiles obtained have to be compared to the profiles of the taxa in the database. This comparison is done by calculating for the profile obtained:

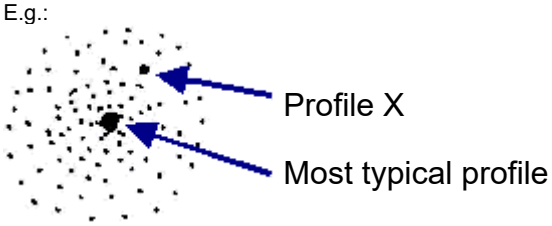
- Its relative proximity to the different taxa of the database (%id). In this way it is possible to determine whether the profile obtained is close to a taxon.

  E.g.:



- Its proximity to the most typical profile in each of the taxa (T index). The most typical taxon is the one that has no tests against the identification in relation to the percentages shown in the database for the taxon in question.

  E.g.:



The classification of the taxa allows an identification result to be proposed.

The example overleaf enables the different identification operations to be followed.

1- The **database** is made up of the percentage (P) of positive reactions for each taxon/test combination.

2- The **frequency of occurrence** of the observed reactions (F_+ and F_-) :

- is derived from the percentage of positive or negative reactions $P+ = P/100$ and $P_- = 1 - (P/100)$
- takes into account the risk of reading errors: α_+ for α positive reading and α_- for a negative reading.
- The calculation formulae are as follows:
  - reaction read positive $F_+ = P+ (1 - \alpha_+) + (\alpha_+ \times P_-)$
  - reaction read negative $F_- = P_- (1 - \alpha_-) + (\alpha_- \times P+)$

$P = 60$	$P_+ = 0.60$	$P_- = 0.40$	$\alpha_+ = 10^{-3}$	$\alpha_- = 10^{-2}$
$F_+ = 0.60 (1 - 0.001) + (0.001 \times 0.40) = 0.5998$				
$F_- = 0.40 (1 - 0.01) + (0.01 \times 0.60) = 0.402$				

At the same time are: the frequency of occurrence of the reactions of the most typical profile are calculated, with $P+$ when $P >= 50$ and P_- when $P < 50$.

$P = 70$	$F_+ = 0.699$	$P = 50$	$F_+ = 0.50$	$P = 20$	$F_- = 0.79$

3- **The frequency of occurrence of the profile observed** (Po) in each taxon is the product of all the frequencies of occurrence, within the taxon, for observed reactions (Fo).
At the same time the frequency of occurrence of the most typical profile (Pt) is calculated.
This is the product of the frequency of occurrence of the most typical reactions.
These frequencies are then normalized according to the LAPAGE method, by dividing each by their sum, and multiplying by 100 to give the **percentage of identification** (% id).

At the same time, the **modal frequency (Fm)** for each taxon is calculated from the most typical reactions (DYBOWSKI).

4- **Taxa** are then sorted by decreasing values of their % id.

- For the first 4 taxa, the ratio (R) of their % id to that of the next taxon is calculated. One of these ratios has a maximum value. The taxon with the maximum ratio is selected for identification, along with any taxa situated before it in the classification.
- A T index is calculated from each of the modal frequencies (*).

  (*) T index calculation formula: = (log Fm - log S)/ -log S S is a value based on the number of tests. In the example, S = 10^{-2}.

If only one taxon is selected, and its % id is >= 80.0, it is proposed for the identification, with a comment selected on the basis of its % id and T index values.
If several taxa are selected and the sum of the % id is >= 80.0, they are proposed for identification, with a comment based on the value of the sum of the % id and the average of the T indexes.

RESULT EXPRESSION

The %id (or the sum of the %id) >= 80 is taken into account for the selection of the taxon (or taxa) proposed for identification.
A comment, selected on the basis of the values of the %id (or the sum of the %id) and the T index (or the sum of the T indexes), shows whether the identification is reliable.

The threshold values selected for the comments on the accuracy of the identifcation are:

EXCELLENT IDENTIFICATION	%id >= 99.9 et T >= 0.75
VERY GOOD IDENTIFICATION	%id >= 99.0 et T >= 0.50
GOOD IDENTIFICATION	%id >= 90.0 et T >= 0.25
ACCEPTABLE IDENTIFICATION	%id >= 80.0 et T >= 0

- Identification to the taxon : one single taxon has been selected.

- Identification to the genus level : 2, 3 or 4 taxa belonging to the same genus have been selected.

- Low discrimination : 2, 3 or 4 taxa belonging to different genera have been selected.

- The identification is "not reliable" if the sum of the %id proposed if less than 80.0.

- The profile is "doubtful" if a taxon having several tests against the identification is present among the those proposed (i.e. with a frequency of 0 or 100%). This can be due to a very atypical profile or an error in reading or coding.

- The profile is "unacceptable" if the number of choices proposed is 0, all the gross frequencies being less than the threshold value. The profile is very far from the taxa of the data base.

- The profile is listed along with the following information :

Tests against identification
The tests against, if any, followed by their percentage of positive reactions (i.e. tests with a frequency of occurrence of the observed reaction < 0.25).

Complementary tests
In case of identification to the species level, to the genus level or of low discrimination, COMPLEMENTARY TESTS are proposed to complete the identification. These tests are extracted from the literature.

Notes
Comment associated with a species. This note appears if the species is identified as a significant choice.

API Listeria

Close

NEGATIVE TESTS

POSITIVE TESTS

KYSTLAB preBIO

Avdeling Sunnmøre

NORSK AKKREDITERING TEST070

Marine Harvest Norway AS
Org.nr.959352887
Att: Erik Lind Dragesund (Slakteri)
Evangervegen 25
6092 EGGESBØNES

Date: 03.12.2017
Sample ID: 2017-7226

ver 1

TEST REPORT

Sample arrival: 01.12.17 at 13:30 | Test period: 01.12.17 - 03.12.17 | Sampler: Customer

2017-7226-1 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 1.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

2017-7226-2 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 2.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

2017-7226-3 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 3.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

2017-7226-4 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 4.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

2017-7226-5 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 5.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

2017-7226-6 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 6.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

The laboratory is not accredited for sampling or evaluation and interpretation of test results.
Results applies only to received sample. Measurment uncertainty regarding analysis can be obtained from the laboratory.
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2017-7226-7 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 7.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

2017-7226-8 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 8.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

2017-7226-9 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 9.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

2017-7226-10 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 10.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

2017-7226-11 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 1+2+5.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

2017-7226-12 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 8+9+10.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

Yours sincerely Kystlab-PreBIO AS

Ingebjørg Worren

Ingebjørg Worren
Technician

Copy to
Rima Marcinkeviciene (E-mail)
Mindaugas Dauksas (E-mail)
Darius Dauksas (E-mail)
Kvalitet (E-mail)
Linn Bigset (E-mail)
Justinas Pisarevucius (E-mail)

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Avdeling Sunnmøre

NORSK AKKREDITERING TEST070

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Att: Erik Lind Dragesund (Slakteri)
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Date: 03.12.2017
Sample ID: 2017-7227

ver 1

TEST REPORT

Sample arrival: 01.12.17 at 13:30 | Test period: 01.12.17 - 03.12.17 | Sampler: Customer

2017-7227-1 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 1.Sjøvann.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

2017-7227-2 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 2.Sjøvann.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

2017-7227-3 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 3.Sjøvann.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

2017-7227-4 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 4.Sjøvann.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

2017-7227-5 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 5.Sjøvann.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

2017-7227-6 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 6.Sjøvann.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

The laboratory is not accredited for sampling or evaluation and interpretation of test results.
Results applies only to received sample. Measurment uncertainty regarding analysis can be obtained from the laboratory.
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2017-7227-7 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 7.Sjøvann.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

2017-7227-8 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 8.Sjøvann.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

2017-7227-9 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 9.Sjøvann.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

2017-7227-10 **Swab sample** Sampled: 30.11.17 at 11:00

Labeled: 10.Sjøvann.

Parameter	Method	Result	Unit
Listeria monocytogenes	NordVal no.022	**Not detected**	/swab

Yours sincerely Kystlab-PreBIO AS

Ingebjørg Worren

Ingebjørg Worren
Technician

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