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LISTERIA MONOCYTOGENES IN FISH FARMING AND PROCESSING

Hanna Miettinen

ACADEMIC DISSERTATION

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ABBREVATIONS

AFLP, amplified fragment length polymorphism
ATP, adenosine 5'-triphosphate
a_w, water activity
CAMP, Christine-Atkins-Munch-Petersen test, enhanced β-haemolysis test
CFU, colony forming unit
DNA, deoxyribonucleic acid
D-value, decimal reduction time (min)
EB, *Listeria* enrichment broth
EDTA, ethylenediaminetetraacetic acid
GMP, good manufacturing practices
HACCP, hazard analysis critical control point
ISO, International Organization for Standardization
LMBA, *Listeria monocytogenes* blood agar
MLST, multilocus sequence typing
MVLST, multi-virulence-locus sequence typing
MEE, multilocus enzyme electrophoresis
NaCl, sodium chloride
PC, plate count
PCR, polymerase chain reaction
PD, potato dextrose
PFGE, pulsed-field gel electrophoresis
p-value, pasteurisation value (min)
RAPD, random amplification of polymorphic DNA
RCM, reinforced clostridial medium
REA, restriction endonuclease analysis
RLU, relative light unit
RTE, ready-to-eat
t_d, heat death time (min)
TDT, thermal death time
TPB, tryptic phosphate broth
TBE, tris-borate-ethylenediaminetetraacetic acid
TE, tris-ethylenediaminetetraacetic acid
T_{ref}, reference temperature
UPGMA, unweighted pair group method using arithmetic averages
VTT, Technical Research Centre of Finland
z-value, degrees required for the thermal destruction curve to traverse one log cycle

ABSTRACT

Contamination of fish products with *Listeria monocytogenes*, a bacterium causing listeriosis, presents a risk to consumer health. To better control and prevent this contamination, the present study investigated the prevalence and sources of *L. monocytogenes* in different stages of fish production chain as well as the effects of a pasteurisation method on safety of rainbow trout roe products.

Farmed rainbow trout from different fish farms were found to contain *L. monocytogenes* and *Listeria* spp. at an average rate of 9% and 14%, respectively. *L. monocytogenes* prevalence varied greatly among different fish farms from 0 to 75%. The location of *L. monocytogenes* and *Listeria* spp. in different parts of the rainbow trout differed significantly ($p < 0.0001$). *L. monocytogenes* contamination in rainbow trout occurred almost exclusively in the gills (96%) and only sporadically in the skin and viscera. Special effort, during the transportation and processing of raw fish, should be focused on the isolation and removal of rainbow trout gills before *L. monocytogenes* contamination spreads further.

Presence of *L. monocytogenes* in different Finnish fish species roe products in retail markets varied between 2 to 8%. Recovery of *L. monocytogenes* was significantly ($p < 0.01$) higher in fresh-bought roe products (18%) than in frozen (0%) and frozen-thawed (2%) roe products. In terms of aerobic and coliform bacteria the microbial quality of roe samples was poor in 57% and 73% of the samples, respectively, and 20% of the samples were unacceptable to taste. Pasteurisation, at 62 °C or at 65 °C for 10 minutes, of rainbow trout roe eliminated all inoculated 8 log units of *L. monocytogenes*. Based on the determined D- and z-values, for four *L. monocytogenes* strain mixtures, these pasteurisations theoretically destroyed 46 and 154 log units of *L. monocytogenes* cells, respectively. The quality of pasteurised vacuum packaged rainbow trout roe was found to be consistently good, in terms of microbial as well as sensory quality, for up to six months stored at 3 °C.

L. monocytogenes and *Listeria* spp. appeared on cleaned surfaces of one-third and two-thirds of the 23 studied fish factories, at least sporadically. The presence of *Listeria* spp. on the factory surfaces was indicative of increased possibility of occurrence in the fish products. In factories where *Listeria* spp. was found on surfaces they were often (10/13) found in some products. The overall *L. monocytogenes* contamination level of different ready-to-eat fish products from the fish factories varied from 0 to 20%.

The main *L. monocytogenes* contamination sources in the studied fish farm were the brook and river waters, as well as other runoff waters from environment. Rainy weather conditions were found to increase the probability of finding *L. monocytogenes* in the fish farm environment and in fish. The *L. monocytogenes* contamination in fish gradually disappeared over several months. Such disappearance, however, was faster in the surrounding sea water than in the fish. Presence of certain *L. monocytogenes* pulsed-field gel electrophoresis (PFGE) -types, after the first discovery months earlier in some other sample type, was typical for sea bottom soil samples. The fish farm studied did not spread *L. monocytogenes* contamination, but suffered from *L. monocytogenes* contamination from environmental sources.

The PFGE-typing of *L. monocytogenes* isolates, from 15 fish factories, showed that the same pulsotypes of *L. monocytogenes* occurred in isolates of final fish products as well as both raw fish and fish production environment isolates. Thus, raw fish materials and production environment and machines are sources of *L. monocytogenes* contamination that both need to be properly controlled.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers referred to in the text by Roman numerals I to V:

I Miettinen, H. and Wirtanen, G. 2005. Prevalence and location of *Listeria monocytogenes* in farmed rainbow trout. Int. J. Food Microbiol. 104, 135-143.

II Miettinen, H., Arvola, A., Luoma, T. and Wirtanen, G. 2003. Prevalence of *Listeria monocytogenes* in, and microbiological and sensory quality of, rainbow trout, whitefish and vendace roes from Finnish retail markets. J. Food Prot. 66, 1832-1839.

III Miettinen, H., Arvola, A. and Wirtanen, G. 2005. Pasteurization of rainbow trout roe: *Listeria monocytogenes* and sensory analyses. J. Food Prot. 68, 1641-1647.

IV Miettinen, H., Aarnisalo, K., Salo, S. and Sjöberg, A.-M. 2001. Evaluation of surface contamination and the presence of *Listeria monocytogenes* in fish processing factories. J. Food Prot. 64, 635-639.

V Miettinen, H. and Wirtanen, G. 2006. Ecology of *Listeria* spp. in a fish farm and molecular typing of *L. monocytogenes* from fish farming and processing companies. Int. J. Food Microbiol. In press.

1 INTRODUCTION

The Gram-positive bacterium *Listeria monocytogenes* was probably first recognised in two rabbits in Sweden 1910 (Hülphers 1911). Over a decade later Murray et al. (1926) in the United Kingdom and Pirie (1927) in South Africa recognised a disease in laboratory rabbits, guinea-pigs and gerbils caused by a Gram-positive bacillus. Pirie named the causative bacterium *Listerella hepatolytica* and Murray, Webb and Swann named the bacterium *Bacterium monocytogenes* as it produced large mononuclear leucocytosis. The genus name *Listeria* was given by Pirie (1940) as the genus name *Listerella* was already used.

Recognition of *L. monocytogenes* as a significant food borne pathogen occurred only in the early 1980's, with demonstration of food borne listeriosis outbreak (Schlecht et al. 1983). *L. monocytogenes* is widely distributed in the environment and occurs in almost all food raw materials from time to time. The disease listeriosis usually occurs in high-risk groups, including pregnant women, neonates and immunocompromised adults, but may occasionally occur in persons who have no predisposing underlying condition. Listeriosis is one of the most severe food borne infections, with low morbidity but high mortality 30% (Rocourt et al. 2001). The yearly medical costs and productivity losses from the acute illness from food borne *Listeria* in the USA are estimated to be one, two and three times the costs caused by *Salmonella* spp., *Campylobacter* spp. and *Escherichia coli* O157:H7, respectively, despite the prevalence of these diseases being over 500, 700 and 25 times the number of listeriosis cases, respectively (Anonymous 2000a).

L. monocytogenes is able to multiply in high salt concentrations even at refrigerated temperatures with or without oxygen. It is resistant to diverse environmental conditions and it can survive in industrial environments for years regardless of cleaning procedures (Rocourt et al. 2001, Hoffman et al. 2003). *L. monocytogenes* is ubiquitous in nature and therefore also aquatic creatures are potential bacterium sources. A part of the seafood products undergo various processing steps that inactivate the bacterium, if present on the raw product. *L. monocytogenes* cross-contamination of products after listericidal processing, however, presents a major problem especially for ready-to-eat products. Furthermore, there are seafood products that are eaten raw, without any listericidal step, like cold-smoked and cold-salted fish. A lot of work has been conducted to study the sources of *L. monocytogenes* as well as means to control its growth and contamination in different food sectors (Chasseignaux et al. 2002, Pak et al. 2002, Gudbjörnsdóttir et al. 2004, Thimothe et al. 2004). More research is still needed on focused risk areas, including seafood processing, to prevent problems caused by *L. monocytogenes*.

2 REVIEW OF THE LITERATURE

2.1 *Listeria monocytogenes*

2.1.1 *Listeria monocytogenes*

L. monocytogenes is a small 0.5 μm in diameter and 1 to 2 μm in length, regular Gram-positive rod with rounded ends. Cells are found either singly, in short chains, arranged in V and Y forms or in palisades. Sometimes cells are coccoid and average about 0.5 μm in diameter, causing them to be confused with streptococci (Rocourt 1999*). L. monocytogenes* is facultatively anaerobic. It is motile because of its few peritrichous flagella when cultured at 20 °C to 25 °C. The bacterium does not form spores or capsules (Seeliger and Jones 1986). Its optimum growth temperature is between 30 °C and 37 °C and temperature limits for growth are -0.4 °C to 1 °C and 45 °C to 50 °C (Seeliger and Jones 1986, Golden et al. 1988, Junttila et al. 1988, Walker et al. 1990). The G + C content of the DNA is 36% to 38%. *L. monocytogenes* is widely distributed in nature and is found in water, mud, sewage, vegetation and in the faeces of animals and humans (Seeliger and Jones 1986). The genus *Listeria* includes six species: *L. gray*, *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri* and *L. welshimeri* (Rocourt 1999).

2.1.2 Listeriosis

Listeriosis is a disease caused by bacteria of the genus *Listeria*. *L. monocytogenes* is the pathogenic species in both animals and humans (McLauchlin and Jones 1999). Few cases of human infections, however, are caused by *L. ivanovii* (Cummins et al. 1994, Lessing et al. 1994) and *L. seeligeri* (Rocourt et al. 1986).

Principally listeriosis causes intra-uterine infection, meningitis and septicaemia. Listeriosis during pregnancy manifests as a severe systemic infection in the unborn or newly delivered infant as well as a mild influenza-like bacteraemic illness in the pregnant woman. Pregnancy and neonatal cases comprise 10% to 20% of the listeriosis cases (McLauchlin et al. 2004). In adults and juveniles, the main presentations are as central nervous system infection and/or septicaemia. Most adult and juvenile cases occur amongst the immunosuppressed (Table 1), e.g. patients receiving steroid or cytotoxic therapy or with malignant neoplasms. Other groups include patients with AIDS, diabetics, individuals with prosthetic heart valves or replacement joints and individuals with alcoholism or alcoholic liver disease. The incidence of infection increases with age, with the mean age of adult infection being over 55 years (McLauchlin et

al. 2004). Mild non-invasive listeriosis, with gastroenteritis and fever, has also been reported in otherwise healthy individuals (Riedo et al. 1994, Miettinen et al. 1999, Aureli et al. 2000).

Table 1. People at risk for listeriosis (Hof 2003).

Population	Incidence of listeriosis in some at risk populations (per 100000 individuals per year)
Normal population	0.7
Aged persons (>70 years)	2
Alcoholics	5
Diabetic people	5
Iron overload	5
Pregnant women	12
Cancer patients	15
Steroid therapy	20
Lupus erythematodes	50
Kidney transplant recipients	100
Chronic lymphatic leukaemia	200
AIDS	600
Leukaemia (acute monocytic and acute lymphoblastic)	1000

Although Murray et al. (1926) first suspected an oral route for the bacterial infection observed in animals in 1924, it was not until 1981 that an outbreak of listeriosis in Canada was linked to a contaminated food source (Schlecht et al. 1983). Listeriosis can occur sporadically or epidemically; in both, contaminated foods are the primary vehicles of transmission. Unlike infection by other common food borne pathogens, such as *Salmonella* which rarely results in fatalities, listeriosis is associated with a mortality rate of approximately 20% to 40% (Farber and Peterkin 1991). Listeriosis is, however, a rare disease (Gerner-Smidt et al. 2005), despite of the relatively frequent exposure to the causative bacterium. An average of five to nine exposures to *L. monocytogenes* occur per person per year (Grif et al. 2003). The asymptomatic point prevalence of faecal carriage of *L. monocytogenes* is from 0 to 21% and a cumulative prevalence as high as 77% in high-risk groups, e.g. household contacts of people with listeriosis (Slutsker and Schuchat 1999). The incubation period and infective dose have not been firmly established. Reported incubation times vary from one day to three months (Linnan et al. 1988). The infective dose has so far been reported to be relatively high ($>10^3$ CFU/g) (McLauchlin 1996) or the infection has been suggested to be caused by a prolonged daily consumption of food contaminated with *L. monocytogenes* (10^1–10^5 CFU/g) (Maijala et al. 2001). The attack rate of various strains, including outbreak and sporadic listeriosis strains, is also likely to be low (McLauchlin et al. 2004) explaining the rare incidence <1/100000 inhabitants (Anonymous 2004). In Finland, the yearly incidence rates have varied between 0.35 and 1.0/100000 inhabitants since 1995 (Anonymous 2006). A number of food items

including coleslaw (Schlech et al. 1983), pasteurized milk (Fleming et al. 1985), different cheeses (Linnan et al. 1988, Goulet et al. 1995, Anonymous. 2001, Makino et al. 2005), pâté (McLauchlin et al. 1991), rillettes (Goulet et al. 1998, de Valk et al. 2001), rice salad (Salamina et al. 1996), chocolate milk (Dalton et al. 1997), corn and tuna salad (Aureli et al. 2000), hot dogs and delicatessen meats (Anonymous 1998b, Lin et al. 2006), butter (Lyytikäinen et al. 2000) and turkey (Anonymous 2000b, Frye et al. 2002) have caused listeriosis outbreaks. Some outbreaks have also been connected to the consumption of seafood (Table 2). In addition cold-smoked fish in Finland (Lukinmaa et al. 2003) and seafood in Norway and Sweden (Loncarevic et al. 1998, Rørvik et al. 2000) have been suggested causing listeriosis.

Table 2. Listeriosis outbreaks connected with seafood consumption.

Suspected seafood	No. of cases (deaths)	Symptoms	Country, area	References
Shellfish and raw fish	22(6)	Premature labour, fetal distress, respiratory symptoms, meningitis, flu-like illness, urinary tract symptoms, diarrhoea, vomiting	New Zealand	Lennon et al. 1984
Fish	1(0)	Meningitis	Italy	Facinelli et al. 1989
Smoked mussels	3(0)	Vomiting, diarrhoea	Tasmania	Mitchell 1991, Misrachi et al. 1991
Shrimps	11(1)	Fever, nausea, vomiting, musculoskeletal symptoms, diarrhoea, fetal demise	USA	Riedo et al. 1994
Cold-salted 'gravad' rainbow trout	8(2)	Amniotitis, meningitis, premature birth, fever, septic arthritis, septicaemia	Sweden	Ericsson et al. 1997
Smoked mussels	3(0)	Perinatal lethargy, malaise	New Zealand	Brett et al. 1998
Cold-smoked rainbow trout	5(0)	Fever, vomiting, fatigue, arthralgia, headache	Finland	Miettinen et al. 1999
Imitation crabmeat	2(0)	Diarrhoea, cramps, fever, projectile vomiting, nausea	Canada	Farber et al. 2000

2.1.3 Isolation and identification

The first isolation methods were generally based on the direct culture of samples on simple agar media. Isolation was difficult and inoculation into test animals was recommended in case of low numbers of viable *Listeria* cells. At the end of 1930 the first discoveries of the

refrigeration temperatures resulted in more positive samples compared to incubation at elevated temperatures (Beumer and Hazeleger 2003). Ten years later Gray et al. (1948) described a new technique based on cold enrichment at 4 °C for several weeks. The main disadvantage of the method was the long incubation period, up to several months. Modern isolation methods are based on one or two-step enrichment with a variety of selective agents, followed by plating on selective plating media (Anonymous 1996, 1999, 2005, Hitchins 2003). Quantitative and semiquantitative methods are used (Anonymous 1998a, 1999) in addition to detection of *L. monocytogenes*. The selective agents for background flora inhibition include acriflavine, cyclohezimide, cefotetan, ceftazidime, colistin, fosfomycin, lithium chloride, nalidixic acid, potassium thiocyanate, and polymyxin B-sulphate. Enrichments are performed at 30-37 °C for one or two days. Solid media used for isolation of *Listeria* spp. contain selective agents and indicator substrates e.g. blood or chromogens to distinguish *Listeria* spp. from background flora or from different *Listeria* spp. The confirmation of presumptive *L. monocytogenes* colonies on the selective media is performed with Gram-staining, catalase reaction, motility at 25 °C, β-haemolysis test, fermentation of rhamnose and xylose and CAMP-test. Several commercial tests developed for identification of *L. monocytogenes* exist as alternatives to conventional testing. The results of various isolation processes of *L. monocytogenes* differ somewhat with different isolation methods depending on selective compounds, presence of *Listeria* spp. and background flora in the sample, and possible presence of injured *Listeria* cells in the sample (Johansson 1998, Duarte et al. 1999, Vaz-Velho et al. 2001, Pinto et al. 2001, Cornu et al. 2002, Gnanou Besse 2002). Methods can also bias the results and proportions of different *Listeria* spp. (Bruhn et al. 2005, Gnanou Besse et al. 2005).

With or without isolation and further possibility of subtyping, rapid detection of *Listeria* spp. and *L. monocytogenes* is sometimes needed. Clinical and even food specimens can be analysed for the presence of *Listeria* spp. or *L. monocytogenes* from selective enrichment broths with immunoassays using commercial antibodies. Also available are DNA hybridization probes and PCR assays for food and environmental specimens, both for research and commercial use (Allerberger 2003, Gasanov et al. 2005). Using PCR *L. monocytogenes* has been detected e.g. in smoked salmon (Simon et al. 1996, Becker et al. 2005), in cold salted (gravad) rainbow trout (Ericsson and Stålhandske 1997), in salmon and salmon products (Norton et al. 2001, Rodríguez-Lázaro et al. 2005), channel catfish (Wang and Hong 1999), fish seafood products (Bansal et al. 1996, Gouws and Liedemann 2005) and environmental samples (Norton et al. 2001).

2.1.4 Subtyping

Subtyping of closely related *L. monocytogenes* strains is needed to be able to confirm outbreak sources, establish transmission patterns and determine and monitor epidemic strain reservoirs. A wide range of various pheno- and genotyping methods are available for typing of microbes. Genotyping is based on the assumption that strains of the causal organism isolated from different sources are clonally related, and are similar or identical in their phenotypic and molecular characteristics. Therefore, these methods are based on specie-specific proteins or genes that are relatively stable over time and are passed on from generation to generation (Gasanov et al. 2005). A summary of typing methods often used and those that are likely to be used more in the future for subtyping of *L. monocytogenes* are presented in Table 3.

2.2 Growth characteristics of *Listeria monocytogenes*

The viable populations of *L. monocytogenes* start to decrease at the temperatures above 50 °C (Golden et al. 1988). Walker et al. (1990) studied the minimum growth temperature and found that there was slow growth at a temperature range of -0.1 °C to -0.4 °C for three *L. monocytogenes* strains. Also a notable variation in the growth among different strains of *L. monocytogenes* is apparent, especially at refrigeration temperatures (Barbosa et al. 1994, Begot et al. 1997). The generation time of 39 *L. monocytogenes* strains at 4 °C and 10 °C varied between 24.6 to 69 h and 3.5 to 8.6 h, respectively (Barbosa et al, 1994).

L. monocytogenes survives freezing well and the frozen storage causes a limited reduction in the viable population of *L. monocytogenes* (Lou and Yousef 1999). The low pH of the frozen media, like tomato soup (pH 4.7), compared to other foods (ground beef, turkey, frankfurters, canned corn and ice-cream mix) increased the death and injury of *L. monocytogenes* cells during frozen storage (Palumbo and Williams 1991). Harrison et al. (1991) found a less than three log unit decrease in inoculated *L. monocytogenes* counts in fish and shrimps after freezing at -20 °C for three months. Slow freezing at -18 °C is more lethal and injurious than rapid freezing at -198 °C (El-Kest et al. 1991).

L. monocytogenes grows at pH 4.3 to 9.2 (Farber et al. 1989, Parish and Higgins 1989, Petran and Zottola 1989). It has, however, the ability to adapt and survive at even lower pH (3 to 3.5) (O´Driscoll et al. 1996, Shabala et al. 2002, Liu et al. 2005) and higher pH (12) (Liu et al. 2005). Stationary phase cells survive better than exponential phase cells and glucose added to the media helps to protect the bacteria by providing energy and metabolic precursors (Shabala

Table 3. Subtyping methods for *L. monocytogenes*.

Method	Principle	Comments	References
Serological typing	Based on antibodies that specifically react with somatic (O) antigens and flagellar (H) antigens of *Listeria* species.	First-level subtyping. Thirteen serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7. Most of the clinical and food related isolates belong to three serotypes: 1/2a, 1/2b and 4b.	Seeliger and Höhne 1979 McLauchlin 1990 Schönberg et al. 1996
Phage typing	Based on the specific interaction of a particular bacteriophage with its host strain, resulting in host cell lysis.	Able to process relatively large numbers of cultures with good discrimination power. Not all strains are typable, not always reproducible.	Rocourt et al. 1985 McLauchlin et al. 1996
Multilocus enzyme electrophoresis (MEE)	Differentiates isolates according to the electrophoretic mobility of a large number of strains metabolic enzymes. Electromorph profiles (electrophoretic types, ETs) index the whole chromosomal genome.	Discriminatory power of the method is relatively low. ETs distinguished by MEE are more stable than typed strains of many genotyping methods. Used in several seafood studies.	Rørvik et al. 1995, 2000 Caugant et al. 1996 Flint and Kells 1996 Boerlin et al. 1997
Ribotyping	Strains are characterized for restriction fragment length polymorphisms associated with ribosomal operon(s). Chromosomal DNA is digested with restriction enzyme followed with hybridisation using labelled 16S+23S rRNA or rDNA probe.	Less discriminating than bacteriophage typing, MEE or REA. The reproducibility and typeability are good. Suited for long-term epidemiological or phylogenetic studies. Widely used for tracking and subtyping in seafood factories with mostly *Eco*RI as the restriction endonuclease.	Grimont and Grimont 1986 Stull et al. 1988 Nørrung and Gerner-Smidt 1993 Graves et al. 1999 Norton et al. 2001 Thimothe et al. 2004
Restriction enzyme analysis (REA)	Restriction enzymes recognize and cut particular sequences within DNA molecules producing a banding pattern of fragments with varying sizes separated and visualised with gel electrophoresis.	Universally applicable, sensitive, cost effective and easy to do analysis. Limitation is in the difficulty of comparing the complex profiles which consist of hundreds of bands. Exploited e.g. in seafood outbreak study and in epidemiological survey in seafood-processing plants.	Ericsson et al. 1997 Graves et al. 1999 Rørvik et al. 2000 Gasanov et al. 2005
Pulsed-field gel electrophoresis (PFGE)	The intact bacteria are digested using one or more restriction endonucleases that cut infrequently. Large chromosomal DNA fragments are separated by pulsed-field gel electrophoresis.	Discriminating and reproducible. Time (2 to 3 days) needed to complete the analysis is long. Used in several *L. monocytogenes* surveys in seafood industry and in listeriosis outbreak studies caused by seafood.	Destro et al. 1996 Brett et al. 1998 Graves et al. 1999 Olive and Bean 1999 Johansson et al. 1999

Table 3. continued

Method	Principle	Comments	References
Random amplified polymorphic DNA (RAPD)	Genomic DNA is characterized based on the number and size of amplified DNA fragments generated by a single random or universal primer in a PCR. Small changes in the genomic DNA will result in different sizes and numbers of amplified fragments.	Rapid and relatively simple technique. High discriminatory power, screen large number of samples. Suitable for epidemiological typing. Patterns have inconsistent reproducibility. Well-standardised RAPD protocol needed to obtain reliable results. Used in surveys in seafood industry and in listeriosis outbreak studies caused by seafood.	Destro et al. 1996 Wernars et al. 1996 Graves et al. 1999 Farber et al. 2000 Fonnesbech Vogel et al. 2001 Mędrala et al. 2003 Gasanov et al. 2005
Amplified fragment length polymorphism (AFLP)	DNA is digested using restriction enzyme(s), followed by the ligation of the resulting fragments to oligonucleotide adapter complementary to the base sequence of the restriction site. The adapters are designed so that the original restriction site is not restored after ligation, thus preventing further restriction digestion. Selective amplification by PCR of sets of these fragments is achieved using primers corresponding to the contiguous base sequences in the adapter, restriction site plus one or more nucleotides in the original target DNA.	High discriminatory power, excellent typeability and high reproducibility. Can be successfully applied to analyse *L. monocytogenes* routes and ecology in food processing industry.	Vos et al. 1995 Guerra et al. 2002 Autio et al. 2003 Fonnesbech Vogel et al. 2004
Multilocus sequence typing (MLST)	Uses automated DNA sequencing to characterize the alleles present at different housekeeping genes. Also targeted to hypervariable genes.	Highly discriminatory and provides unambiguous result. Differentiate most of the strains better than or equally to PFGE. Hypervariable genes showed low degree of discrimination.	Enright and Spratt 1998 Salcedo et al. 2003 Revazishvili et al. 2004 Meinersmann et al. 2004
Multi-virulence-locus sequence typing (MVLST)	Targets virulence and virulence associated genes.	Virulence associated genes showed higher discriminatory power than ribotyping, PFGE and MLST. MLST and MVLST are valuable typing methods for the future after identification of the applicable discriminatory genes and the number of gene loci that provide optimal resolution. Results can be compared via e.g. worldwide web that assists the observation of global listeriosis epidemics and sources.	Zhang et al. 2004 Chen et al. 2005

et al. 2002). The induction of an acid tolerance response can provide cross-protection against thermal stress, ethanol, osmotic stress, or stress due to a surface-active agent. It has also been shown that acid tolerant strains display increased virulence relative to that of the wild type (O'Driscoll et al. 1996).

L. monocytogenes grows optimally at a_w above 0.97 (Petran and Zottola 1989), however, it has a rather unique ability to multiply at a_w values as low as 0.90 (Lou and Yousef 1999). It can survive for extended periods at even lower a_w values (Shahamat et al. 1980). The bacterium also endures high salt concentrations 25.5 % NaCl (Shahamat et al. 1980) and has been isolated e.g. from brine used in fish industry (Jemmi and Keusch 1994, Autio et al. 1999, Norton et al. 2001, Gudmundsdóttir et al. 2005).

In addition to the above mentioned general growth parameters plenty of additional factors exist effecting the growth, survival and adaptation of different *L. monocytogenes* strains like inoculum size, growth medium with inhibitory as well as protective compounds and structure, atmosphere, and pre-incubation conditions. All these factors also have a combined effect that can not always be predicted based on results tested with an individual variable.

2.3 Thermal resistance of *Listeria monocytogenes*

The heat resistance of *L. monocytogenes* is influenced by many factors such as strain variation, previous growth conditions, exposure to heat shock, acid, as well as other stresses, and composition of the heating menstruum (Doyle et al. 2001). D-value is used to describe the heat resistance of a certain strain at a certain temperature as it is the time needed to destroy 90% of cells at that temperature. z-value is the temperature difference required to destroy 90% of the bacteria with a 10-fold change in heating time. Table 4 presents D- and z-values for some *L. monocytogenes* strains in different seafood. The strain variation of 21 *L. monocytogenes* strains at 55°C in BHI broth at pH 6 was 4.7 fold (23.8 to 111 min) shown by the $D_{55°C}$-values (de Jesús and Whiting 2003). Golden et al. (1988) showed that a *L. monocytogenes* isolate from brie cheese had 1.5 to 2.8 fold higher D_{56}-value than the three listeriosis outbreak strains tested.

Cells in stationary phase of growth appear to be the most resistant to thermal stress (Pagán et al. 1998, Doyle et al. 2001). Stationary phase cells of *L. monocytogenes* had 2.8 to 5.6 times higher $D_{60°C}$-values (0.45 to 12.5 min) than the logarithmic phase cells, each tested in three different media

Table 4. *L. monocytogenes* D- and z-values for seafood products and sea water.

Seafood	Strain	D-values (min) at temperature (°C) of									z-value	References
	(No.)	55	58	60	62	63	65	66	68	70	(°C)	
Salmon	062 (1)		10.73	4.48	2.07		0.87		0.2	0.07	5.6	Ben Embarek and Huss 1993
Salmon	057 (1)		8.48	4.23	3.02		1.18		0.22	0.17	6.7	Ben Embarek and Huss 1993
Cod	062 (1)		7.28	1.98	0.87		0.28		0.15	0.03	5.7	Ben Embarek and Huss 1993
Cod	057 (1)		6.18	1.95			0.27		0.13	0.05	6.1	Ben Embarek and Huss 1993
Imitation crabmeat[1]	Mixture (4)		9.7		2.1			0.4			5.8	Mazzotta 2001
Imitation crabmeat[2]	Mixture (4)		10.2		2.3			0.4			5.7	Mazzotta 2001
Crabmeat	ScottA (1)	12.0		2.61							8.4	Harrison and Huang 1990
Lobster	Mixture (5)		8.3[3]	2.39		1.06[4]					5.0	Budu-Amoako et al. 1992
Crawfish	Mixture (3)	10.23		1.98			0.19				5.5	Dorsa et al. 1993
Mussels	Mixture (7)		16.25	5.49	1.85						4.25	Bremer and Osborne 1995
Salmon caviar[5]	*L. innocua* (1)			2.97		0.77	0.40				5.7	Al-Holy et al. 2004
Salmon caviar[6]	*L. innocua* (1)			3.55		0.85	0.41				5.3	Al-Holy et al. 2004
Sea water[7]	ScottA, KM (2)		1.36	0.69	0.58						10.8	Bremer et al. 1998
Sea water[7,8]	ScottA, KM (2)		2.78	1.15	0.64						6.27	Bremer et al. 1998

[1]Stationary phase cells, [2]Salt adapted cells (5 h, 15% NaCl), [3]57.2 °C, [4]62.7 °C, [5]Aluminum TDT tubes, [6]Glass TDT tubes, [7]Filter sterilized (0.22 μm), [8]Sea water adapted cells (7d)

(minced beef, tryptic phosphate broth TPB and TPB supplemented with 8 g/l lactic acid) at four different pH from 5.4 to 7.0 (Jørgensen et al. 1999). *L. monocytogenes* Scott A strain had 7.6 times higher $D_{56°C}$-value at the early stationary phase than at the exponential phase (Lou and Yousef 1996).

The composition of the growth medium, whether a food or laboratory culture broth, affect rates of growth and the synthesis of cellular constituents that determine the thermal tolerance of bacterial cells (Doyle et al. 2001). The $D_{60°C}$-values for *L. monocytogenes* 13-249 were 2 to 6 fold higher in minced beef than in TPB (Jørgensen et al. 1999). In half cream, double cream and butter the D-values of two *L. monocytogenes* strains were 1.1 to 8 fold higher than in TSB also indicative of notable differences in D-values between food products (Casadei et al. 1998). The environment in which cells are grown can be a major determinant of their heat resistance. *L. monocytogenes* –strain Scott A growing in tryptic phosphate broth containing 0.09, 0.5, 1.0 or 1.5 M NaCl was heated in media with the same salt concentrations resulting in 4 log reduction at 60 °C in 1.6, 2.5, 7.4 and 38.1 min, respectively (Jørgensen et al. 1995). Increased heat resistance was also induced by starvation, low pH, and addition of antimicrobial compounds like ethanol, or hydrogen peroxide to the growth media (Lou and Yousef 1996). The growth temperature affects the heat resistance and in general the cells grown at higher temperatures are more heat resistant than those grown at lower temperatures (Smith et al. 1991, Pagán et al. 1998). The rate at which cells are heated during testing also influences their survival. When cells are heated slowly, they exhibit a greater heat resistance than when heated rapidly (Kim et al. 1994, Hassani et al. 2005).

Heat-shock, a short-term exposure of cells to temperatures above the optimum growth, results in increased heat resistance. The degree of enhanced thermal resistance is strain dependent and also varies with the length of the heat shock, the pH of the medium, and the growth phase of the cells. The maximum increase in thermotolerance was 4 and 7 fold for a *L. monocytogenes* strain previously grown at 37 °C and 4 °C and heat-shocked at 45 °C and at 47.5 °C, respectively (Pagán et al. 1997). On the other hand cold-shock decreases the heat resistance of *L. monocytogenes* (Miller et al. 2000). Increased heat tolerance can also be induced by short-term exposure to high salt (Jørgensen et al. 1995) or solute levels (Smith and Hunter 1988). Decreasing a_w values and increasing solute concentrations result in greater heat resistance in *L. monocytogenes* (Doyle et al. 2001). A systemic approach to determine global thermal inactivation parameters for various food pathogens resulted with only a limited number of factors having a significant effect ($p < 0.05$) on the D-value. The collected D-value

data (n=967) for *L. monocytogenes* showed that the presence of 10% salt or a_w<0.92 resulted in a high heat resistance and it became the most heat resistant vegetative pathogen (van Asselt and Zwietering 2005). This did not mean that other effects do not occur, but that the low a_w was statistically significant based on the data studied.

2.4 *Listeria monocytogenes* in nature, seafood industry and seafood products

L. monocytogenes is ubiquitous in nature and therefore aquatic creatures are also potential sources of the bacterium. Part of the seafood products undergoes various processing steps that can inactivate the bacterium if present on the raw product. *L. monocytogenes*, however, can also enter the product both during and after processing due to poor sanitation conditions or inadequate manufacturing practices (Jinneman et al. 1999).

2.4.1 Occurrence of *Listeria monocytogenes* in environment

Forest soil, cultivated and uncultivated fields, mud, feed, feeding grounds, wildlife faeces and birds (Weis and Seeliger 1975) have been found to be substantially (8.4 to 44%) contaminated with *Listeria* spp. Cultivated land is more often contaminated with *L. monocytogenes* than uncultivated land (Weis and Seeliger 1975, Dowe et al. 1997). The soil type influences the survival of *L. monocytogenes*, sandy soil yields a lower level and clay loam and sandy loam soils a higher count (Dowe et al. 1997). Soil has been suggested to be the natural reservoir of *Listeria* spp. since it is able to multiply there (Botzler et al. 1974, Dowe et al. 1997). *L. monocytogenes* has also been found in sludge from a fish farm (Jemmi and Keusch 1994).

Water environments such as coastal sea waters and rivers containing a high organic load have been found to carry *Listeria* spp. In California, river and costal sea waters contained *Listeria* spp. in 81% and *L. monocytogenes* in 62% of the samples (Colburn et al. 1990). In Scotland, *L. monocytogenes* appeared throughout the course of a river passing through areas ranging from sparsely populated mountains to highly populated urban areas (Fenlon et al. 1996). In a northern province of the Netherlands, *L. monocytogenes* contamination occurred in 37% of the surface waters of canals and lakes and 67% of water containing effluent from a sewage treatment plant (Dijkstra 1982). In Italy, *L. monocytogenes* was found in 40%, 58% and 67% of river, treated and untreated sewage water, respectively (Bernagozzi et al. 1994). Most of the treated water (84.4%) and raw sludge (89.2%) was contaminated with *Listeria* spp. in six French urban wastewater treatment plants (Paillard et al. 2005). In Sweden, 12% of raw sludge samples were contaminated with *L. monocytogenes*, however, contamination occurred in only 2% of the treated sludge samples (Sahlström et al. 2004). The presence of

L. monocytogenes in ground or spring water is rare, however, some cases have been reported (Korhonen et al. 1996, Schaffer and Parriaux 2002). Although *L. monocytogenes* has been found in sea water several times, there are sea areas where it has not been detected (Dijkstra 1982, Rørvik et al. 1995, Ben Embarek et al. 1997). The survival of *L. monocytogenes* in water depends on many factors, but it has been found to survive several weeks in suitable water environments (Botzler et al. 1974, Bremer et al. 1998). Hsu et al. (2005) showed that the two *L. monocytogenes* strains studied did not readily survive and compete with the marine flora in sea water or in salmon blood-water in elevated (>7 °C) temperatures.

A large proportion of faecal samples collected from healthy animals with no clinical symptoms of listeriosis may contain *L. monocytogenes* (Wesley 1999). It is also of no surprise that different kinds of fish, squid and crustaceans have been found to contain *L. monocytogenes* (Table 5). The *L. monocytogenes* contamination in salmon, the most studied fish species, also varied widely in the literature (0 to 88%, Table 5). Contamination of other species and shellfish with *L. monocytogenes* was also significant from 0 to 51%. Differences, however, occur in sample collection and transportation, sampling methods, and actual analyses all which may influence the reported results. The mean *L. monocytogenes* prevalence of all studies was 14% (Table 5).

2.4.2 Sources and routes of *Listeria monocytogenes* contamination and its occurrence in seafood industry

Contamination of final products by *L. monocytogenes* in seafood processing plants may occur from various sources. In addition to frequent contamination of raw materials (Table 5), other sources are also significant in terms of final product safety. Table 6 summarises *L. monocytogenes* contamination of environment, raw materials, products during processing and final products in different fish processing factories.

Effective cleaning was found to be an essential preventive measure in reducing the amount of *L. monocytogenes* contamination in fish processing (Jemmi and Keusch 1994, Rørvik et al. 1997, Fonnesbech Vogel et al. 2001, Hoffman et al. 2003). Many times the procedures used for cleaning and disinfection were, however, insufficient in removing persistent *L. monocytogenes* contamination in fish processing factories (Fonnesbech Vogel et al. 2001, Norton et al. 2001, Dauphin et al. 2001, Gudbjörnsdóttir et al. 2004, Thimothe et al. 2004, Gudmundsdóttir et al. 2005). In most of the studied fish processing factories, one or a few *L. monocytogenes* clones were found to persist for several months or years in the processing environment despite the normal washing regime (Johansson et al. 1999, Fonnesbech Vogel et al. 2001, Dauphin et al.

Table 5. Prevalence of *Listeria* spp. and *L. monocytogenes* in live seafood, fresh seafood in retail markets and in fresh raw seafood material from processing factories.

Seafood type (country, area)	Sampling location	Specification	No. of samples	% Positive for *Listeria*		References
				spp.	*monocytogenes*	
Salmon (Norway)	Live, farmed	Gills, skin, guts separately	10	0	0	Ben Embarek et al. 1997
Salmon (Norway)	Processing factory	Skin and belly cavity swabbed	40	12	0	Vaz-Velho et al. 1998
Salmon[1] (Norway)	Processing factories	Collar, tail and belly, 25 g	81		21	Hoffman et al. 2003
Salmon[2] (Norway, Faroe Islands)	Two processing factories	25 g or skin scraping	215		7	Fonnesbech Vogel et al. 2001
Salmon (Norway)	Processing factory	Skin swabbed	7		86	Dauphin et al. 2001
Salmon[1] (Norway)	Producer	25 g	46		4	Mędrala et al. 2003
Salmon (Norway)	Processing factory	25 g	50	2	0	Rørvik et al. 1995
Salmon (UK)	Commersial outlets	Flesh and skin, 25 g	5		0	Davies et al. 2001
Salmon (UK)	Processing factory	Skin swabbed	8		88	Dauphin et al. 2001
Salmon[1] (USA)	Two processing factories	Collar, tail and belly, 25 g	61		30	Hoffman et al. 2003
Salmon[1] (USA)	Freezer warehouse	Slime layer, 2 g	19	26	21	Eklund et al. 1995
Salmon[1] (USA)	Freezer warehouse	Skin, 25 g	46		65	Eklund et al. 1995
Salmon[1] (USA)	Freezer warehouse	Flesh under skin, 25 g	22	0	0	Eklund et al. 1995
Salmon[1] (USA)	Freezer warehouse	Belly-cavity lining, 25 g	7		0	Eklund et al. 1995
Salmon[1] (USA)	Freezer warehouse	Head, 25 g	17	65	47	Eklund et al. 1995
Salmon[1] (USA)	Freezer warehouse	Tail, 25 g	9		67	Eklund et al. 1995
Salmon[1] (USA)	Freezer warehouse	Trimmings, 25 g	15	80	7	Eklund et al. 1995
Salmon (Chile)	Processing factory	Flesh, 25 g	50		8	Hoffman et al. 2003
Salmon trout (Portugal)	Processing factory	Skin and surface swabbed	48	6	2	Vaz-Velho et al. 1998
Trout (Portugal)	Commercial outlets	Flesh and skin, 25 g	10		0	Davies et al. 2001
Seatrout (Norway)	Producer	25 g	26		15	Mędrala et al. 2003
Trout (UK)	Commercial outlets	Flesh and skin, 25 g	22		10	Davies et al. 2001
Rainbow trout (Finland)	Processing factory	Head, 25 g	60		2	Autio et al. 1999
Rainbow trout (Finland)	Processing factory	Heads, 25 g	140		4	Markkula et al. 2005
Rainbow trout[1] (Finland)	Processing factory	Heads, 25 g	117		4	Markkula et al. 2005
Rainbow trout (Spain)	Two fish farms	Gills, gut, skin 10 g, separately	30		0	González et al. 1999
Rainbow trout (Switzerland)	Three fish farms	Flesh, 10 g	27	0	15	Jemmi and Keusch 1994
Rainbow trout (Switzerland)	Three fish farms	Faecal content swabbed	45	13	22	Jemmi and Keusch 1994
Rainbow trout (Switzerland)	Three fish farms	Skin swabbed	45	33	11	Jemmi and Keusch, 1994
Rainbow trout (USA)	31 retail markets	Flesh, 25 g	74	54	51	Draughon et al. 1999
Brown trout (Spain)	Live	Gills, gut, skin, 10 g, separately	30		0	González et al. 1999
Pike (Spain)	Live	Gills, gut, skin, 10 g, separately	12		0	González et al. 1999
Whiting (France)	Commercial outlets	Flesh and skin, 25 g	26		0	Davies et al. 2001

Table 5 Continued

Seafood type (country, area)	Sampling location	Specification	No. of samples	% Positive for *Listeria*		References
				spp.	*monocytogenes*	
Plaice (UK)	Commercial outlets	Flesh and skin, 25 g	5		0	Davies et al. 2001
Sardine (Portugal)	Commercial outlets	Flesh and skin, 25 g	10		0	Davies et al. 2001
Whitefish (USA)	Two processing factories	Flesh, 25 g	67		7	Hoffman et al. 2003
Sablefish[1] (USA)	Processing factory	Collar, tail, belly, 25g	56		4	Hoffman et al. 2003
Different species[2] (USA)	Three processing factories	Collar, belly flap area, 25 g	102		9	Norton et al. 2001
Different species (USA)	Two retail markets	Fillets, 40 g	320		23	Cao et al. 2005
Different species (Denmark)	Retail markets	25 g	232		14	Nørrung et al. 1999
Different finfish species (India)	Fish market, processing factories	25 g	29	72	17	Jeyasekaran et al. 1996
Different species (Middle East)	Live	25 g	40	37	17	El-Shenawy and El-Shenawy 2006
Hake (Argentina)	Retail stores	25 g	42	2	0	Laciar and de Centorbi 2002
Mackerel (Argentina)	Retail stores	25 g	26	8	0	Laciar and de Centorbi 2002
Blackback (Iran)	Live	25 g	28		0	Basti et al. 2006
Silver carp (Iran)	Live	25 g	39		10	Basti et al. 2006
Different fish species (Japan)	Retail stores	25 g	125		2	Handa et al. 2005
Different fish species (Portugal)	Producers, retail stores	25 g	25		12	Mena et al. 2004
Fish, shellfish, shrimp, etc. (Japan)	Municipal fish market	10 g	781		1	Iida et al. 1998
Squid (Argentina)	Retail stores	25 g	17	29	18	Laciar and de Centorbi 2002
Oysters (USA)	Live, collected	25 g	35	3	0	Colburn et al. 1990
Oysters (USA)	Live, collected	25 g	75	0	0	Motes 1991
Oysters, mussels, cockles (France)	Live, collected on shores	25 g	120	55	9	Monfort et al. 1998
Shrimps (USA)	Live, collected	25 g	74	11	11	Motes 1991
Shrimp (All over the world)	Imported to USA, fresh and frozen	25 g	205	7	4	Gecan et al. 1994
Mussel (Argentina)	Retail stores	25 g	15	27	0	Laciar and de Centorbi 2002
Crawfish (USA)	Two processing factories	25 g	78	30	4	Thimothe et al. 2002
Crawfish (USA)	Two processing factories	25 g	179	45	8	Lappi et al. 2004b
Different shellfish (India)	Fish market, processing factories	25 g	36	44	12	Jeyasekaran et al. 1996
Shelfish (Middle East)	Live	25 g	15	53	33	El-Shenawy and El-Shenawy 2006

[1]frozen, [2]some frozen

Table 6. *L. monocytogenes* contamination in fish processing factories and comments on the contamination.

Product type, No. of factories	Sample types	No. of samples	During/before processing[1]	% positive for *L. monocytogenes*	Typing if used	Comments on *L. monocytogenes* contamination	References
Hot-smoked fish, 1	Raw fish	9		0			Jemmi and Keusch 1994
	Fish during processing	36		3			
	Final product	15		0			
	Environment	57		0			
Cold-smoked fish, 1	Raw fish	9		44		No regular cleaning and disinfection.	Jemmi and Keusch 1994
	Fish during processing	36		19			
	Final product	16		6			
	Environment	113		32			
Hot-smoked fish, 1	Raw fish	9		0			Jemmi and Keusch 1994
	Fish during processing	36		0			
	Final product	18		0			
	Environment	76		0			
Cold-smoked fish, 1	Raw fish skin	46		65		Primary source of *L. monocytogenes* external surface of fish.	Eklund et al. 1995
	Environment, raw area	85	d	33			
	Environment, process area	23	d	30			
	Fish raw area	37	d	59			
	Fish process area	89	d	71			
Cold-smoked salmon, 1	Environment, slaughterhouse	83		7	MEE	Final product contamination from process environment.	Rørvik et al. 1995
	Environment, smokehouse	147		29			
	Fish during processing	71		23			
	Final product	65		11			
Cold-smoked salmon, 40	Drains		d	63		Risk factors: rotation of duties, finding of *L. monocytogenes* in drains.	Rørvik et al. 1997
	Fish during process		d	33			
Cold-smoked and cold-salted fish, 1	Environment	163		15	PFGE	Critical contamination sites: salting and slicing. One persistent clone for 14 months.	Johansson et al. 1999
	Raw material	55		0			
	Products	37		22			

Table 6 continued

Product type, No. of factories	Sample types	No. of samples	During/before processing[1]	% positive for *L. monocytogenes*	Typing if used	Comments on *L. monocytogenes* contamination	References
Cold-smoked fish, 1	Raw fish	60		2	PFGE	Two major contamination sites of final products: brining and slicing. Raw material not important source of *L. monocytogenes* of final products. Some *L. monocytogenes* clones predominated. Eradication program successful.	Autio et al. 1999
	Fish during processing	75		29			
	Final product	22		100			
	Environment	122	b	13			
	Environment	65	d	30			
	Brine	6	d	67			
	Personnel	19	d	32			
	Air	125	d	0			
	Environment, brine, products	94		0			
Cold-smoked fish, 3	Raw fish	102		8	Ribotyping	*L. monocytogenes* clones persisted in two factories. Two contamination sources: raw materials and environment. All factories had own *L. monocytogenes* strains.	Norton et al. 2001
	Fish during process	127		18			
	Environment	206		30			
	Final products	96		7			
Cold-smoked fish, 1	Raw fish	18		0	RAPD	One *L. monocytogenes* clone persisted over four years. Slicing area associated contamination of final products.	Fonnesbech Vogel et al. 2001
	Fish during processing	4		0			
	Final product	128		47			
	Contact surfaces	50		40			
	Environment	96		17			
Smoked, cold-smoked, and gravad fish, 1	Raw fish	136		11	RAPD	Brining area associated contamination of final products. Prevalence may vary significantly over time at the same factory.	Fonnesbech Vogel et al. 2001
	Surfaces	281	d	15			
	Surfaces	375	b	6			
	Final products	900		6			
Cold-smoked salmon, 1	Raw salmon	18		11	PFGE	One dominant and persisted *L. monocytogenes* clone that was not found in raw materials. Process environment potential source of final product contamination. Raw materials not major source of final product contamination.	Dauphin et al. 2001
	Environment, contact	7	d	71			
	Environment, no contact	8	d	88			
	Environment, contact	4	b	50			
	Environment, no contact	5	b	80			
	Salmon during processing	14		64			
	Final product	21		10			

Table 6 continued

Product type, No. of factories	Sample types	No. of samples	During/before[1] processing	% positive for *L. monocytogenes*	Typing if used	Comments on *L. monocytogenes* contamination	References
Cold-smoked fish, 2	Drains	128	d[2]	63	Ribotyping	Raw fish and process environment had different *L. monocytogenes* populations. *L. monocytogenes* clone persisted over two years. Factories with similar *L. monocytogenes* prevalence values for raw materials, had disparate values for process contamination.	Hoffman et al. 2003
	Other environmental sites	96	d[2]	32			
	Food contact sites	32	d[2]	3			
	Raw fish	187	d[2]	4 to 30[3]			
	Environment	256	d[2]	1			
	Raw fish	128	d[2]	4 to 30[3]			
Smoked fish, 4	Raw fish	234		4	Ribotyping	Environment and cross-contamination the sources of final product contamination. Raw materials not a major source of *L. monocytogenes* of process environment and final products. Other risk factors poor employee hygiene and GMP.	Thimothe et al. 2004
	Final product	233		1			
	Environment	553	d	13			
	Contact surfaces	125	d	5			
	Employee contact surfaces	135	d	10			
	Non food surfaces	162	d	12			
Shrimp, salmon, cod, 5	Environment	309	b	10		Cleaning procedures insufficient.	Gudbjörnsdóttir et al. 2004
	Environment	214	d	20			
	Floors and drains	91	b	19			
	Floors and drains	75	d	27			
	Personnel	48		6			
	Brine	23		9			
	Raw material	74		14			
	Fish during processing	102		4			
	Final product	104		18			
Cold-smoked salmon, 4	Raw material	86		16	PFGE	Cleaning did not eliminate *L. monocytogenes*. Raw materials and processing environment contamination sources of *L. monocytogenes*. All factories had own *L. monocytogenes* flora.	Gudmundsdóttir et al. 2005
	Brine	14		21			
	Final and unfinished products	125		4			
	Environment	134	b	3			
	Environment	99	d	11			
	Floors and drains	68	b	18			
	Floors and drains	69	d	25			
	Personnel	48		6			

[1]d = during and b = before processing, [2] At the beginning of the daily production process, [3] According to different fish species.

2001, Hoffman et al. 2003, Lappi et al. 2004a, Thimothe et al. 2004, Gudmundsdóttir et al. 2005). In addition to persistent *L. monocytogenes* clones, sporadic *L. monocytogenes* clones were also found in the factories (Johansson et al. 1999, Fonnesbech Vogel et al. 2001, Dauphin et al. 2001, Lappi et al. 2004a, Thimothe et al. 2004). The prevalence of *L. monocytogenes* in a certain fish factory varied significantly over time. This was suggested to be dependent on how busy the period was and the time available for performing the cleaning and disinfection between the shifts (Fonnesbech Vogel et al. 2001). The processing environment was found to be the major contamination source for the final products (Rørvik et al. 1995, Autio et al. 1999, Johansson et al. 1999, Dauphin et al. 2001, Fonnesbech Vogel et al. 2001, Norton et al. 2001, Lappi et al. 2004a, Thimothe et al. 2004, Gudmundsdóttir et al. 2005). Especially contamination associated with slicing and brining was established (Autio et al. 1999, Johansson et al. 1999, Dauphin et al. 2001, Fonnesbech Vogel et al. 2001). In addition potential sources of final product contamination were cross-contamination, job rotation, employee hygiene and food handling practices (Rørvik et al. 1997, Thimothe et al. 2004). The raw materials have not always been reported as an important final product contaminant source (Johansson et al. 1999, Dauphin et al. 2001, Thimothe et al. 2004). The raw materials, however, have clearly been found to be a source of *L. monocytogenes* contamination of the final products in certain factories (Eklund et al. 1995, Fonnesbech Vogel et al. 2001, Norton et al. 2001, Gudmundsdóttir et al. 2005). Another observation was that all the factories had their own *L. monocytogenes* contamination patterns and contamination degree at the process environment and on final products (Dauphin et al. 2001, Hoffman et al. 2003, Lappi et al. 2004a) despite the use of similar raw materials (Thimothe et al. 2004). This was particularly influenced by the factory design, structure and conditions as well as operational and sanitation procedures (Hoffman et al. 2003, Thimothe et al. 2004).

2.4.3 Occurrence of *Listeria monocytogenes* in seafood products

The contamination of seafood products with *L. monocytogenes* has been widely studied. Differences occur in the prevalence of *L. monocytogenes* in different product types as well as by producers (Table 7). In the case of cold-smoked fish the *L. monocytogenes* contamination ranged from 0 to 100%, the mean contamination rate of the sampled products being 30%. The highest number of *L. monocytogenes* cells encountered in cold-smoked fish was 2.5×10^4 CFU/g (Loncarevic et al. 1996). The contamination of hot-smoked fish products, including those reported only to be smoked, ranged from 0 to 33%. The largest amount of *L. monocytogenes* cells found in hot-smoked fish product was 1.3×10^5 CFU/g (Loncarevic et

Table 7. Prevalence of *L. monocytogenes* and *Listeria* spp. in seafood products and confirmed highest numbers of *L. monocytogenes*.

Product type	Country	Sampling location and No.	No. of samples	% positive for *Listeria* spp.	% positive for *L. monocytogenes*	Highest number of *L. monocytogenes* (CFU/g)	References
Cold-smoked salmon	Denmark	Producers	380		37	> 1000	Jørgensen and Huss 1998
Cold-smoked salmon	Denmark	Producer	1000		6		Fonnesbech Vogel et al. 2001
Cold-smoked salmon	Denmark	Producer	128		47		Fonnesbech Vogel et al. 2001
Cold-smoked salmon	Poland	Producer	44		61		Mędrala et al. 2003
Cold-smoked salmon	Italy	Producers, 3	165		19	> 1100	Cortesi et al. 1997
Cold-smoked salmon	France	Producer	21		10		Dauphin et al. 2001
Cold-smoked salmon	Norway	Producer	65	11	11		Rørvik et al. 1995
Cold-smoked salmon	USA	Producers, 6	61		79	34	Eklund et al. 1995
Cold-smoked salmon and trout	Japan	Retail stores	50		24	460	Nakamura et al. 2004
Cold-smoked salmon and trout	Spain	Retail outlets	54	6	0		González-Rodríguez et al. 2002
Cold-smoked rainbow trout	Finland	Producer	22		100		Autio et al. 1999
Cold-smoked rainbow trout	Finland	Retail outlets	62		15		Lyhs et al. 1998
Cold-smoked halibut	Denmark	Producers	40		53	> 1000	Jørgensen and Huss 1998
Cold-smoked silver carp	Iran	Retail fish market	20		35	100 to 1000	Basti et al. 2006
Cold-smoked fish	Sweden	Retail markets	26		12	25400	Loncarevic et al. 1996
Cold-smoked fish	Canada	Retail outlets	116	6			Dillon et al. 1994
Cold-smoked fish	UK, Chile	Retail outlets, producers	49	6	4		Fuchs and Nicolaides 1994
Cold-smoked fish	Switzerland	Import- and export-control	814		14		Jemmi et al. 2002
Cold-smoked fish	Switzerland	Producer	16	6	6		Jemmi and Keusch 1994
Cold-smoked fish	Finland	Retail outlets	30		17		Johansson et al. 1999
Cold-smoked fish	Finland	Producer	37		22		Johansson et al. 1999
Cold-smoked fish	USA	Producers, 3	96		11		Norton et al. 2001
Cold-smoked and smoked fish	USA	Producers, 4	233	9	1		Thimothe et al. 2004
Cold-smoked fish and shellfish	Different countries	Processors, importers	291		18		Heinitz and Johnson 1998
Hot-smoked rainbow trout	Finland	Retail outlets	42		2		Lyhs et al. 1998
Hot-smoked salmon and whitefish	Finland	Retail outlets	6		0		Lyhs et al. 1998
Hot-smoked whitefish	Finland	Retail outlets	47		0		Lyhs et al. 1998
Hot-smoked fish	Switzerland	Import- and export-control	471		12		Jemmi et al. 2002
Hot-smoked fish	Switzerland	Producers, 2	33	3	0		Jemmi and Keusch 1994
Hot-smoked fish	Sweden	Retail markets	66		2	132000	Loncarevic et al. 1996
Hot-smoked fish	Finland	Retail outlets	48		2		Johansson et al. 1999
Hot-smoked fish and shellfish	Different countries	Processors, importers	234		8		Heinitz and Johnson 1998
Hot-smoked fish	UK, Ecuador	Retail outlets, producers	32	13	0		Fuchs and Nicolaides 1994

Table 7 continued

Product type	Country	Sampling location and No.	No. of samples	% positive for *Listeria* spp.	% positive for *Listeria monocytogenes*	Highest number of *L. monocytogenes* (CFU/g)	References
Smoked salmon	Belgium	Super markets, 4	42	19	19		van Coillie et al. 2004
Smoked salmon	Spain	Retail outlets	52	38	27		Aguado et al. 2001
Smoked salmon	Spain	Producers, 8	100	41	28		Vitas et al. 2004
Smoked salmon	Norway	Producers	33		9		Rørvik and Yndestad 1991
Smoked salmon	Japan	Retail stores	92		5	< 10	Inoue et al. 2000
Smoked trout	Belgium	Super markets, 4	15	0	0		van Coillie et al. 2004
Smoked halibut	Belgium	Super markets, 4	18	33	33		van Coillie et al. 2004
Smoked fish	Iceland	Retails stores, producers	31	35	3		Hartemink and Georgsson 1991
Smoked fish	Spain	Retail outlets	170		22	>100	Dominguez et al. 2001
Smoked fish	Canada	Retail outlets	142	25			Dillon et al. 1994
Smoked RTE fish	USA	Producers, 4	519	8			Lappi et al. 2004a
Smoked seafoods	USA	Retail markets	2644		4	> 100000	Gombas et al. 2003
Heat-treated seafood	Denmark	Producers	79		13	> 1000	Jørgensen and Huss 1998
Salmon sauce	Italy	Producer	32		6		Pourshaban et al. 2000
Salmon sauce	Italy	Producer	42		0		Pourshaban et al. 2000
Processed seafood	Japan	Fish markets	247		5		Iida et al. 1998
Seafood salads	Belgium	Super markets, 4	45	49	27		van Coillie et al. 2004
Seafood salads	Belgium	Supermarket chain	362		27		Uyttendaele et al. 1999
Seafood salads	Iceland	Retails stores, producers	37	32	16		Hartemink and Georgsson 1991
Seafood salads	USA	Retail markets	2446		5	100 to 1000	Gombas et al. 2003
Preserved fish products	Denmark		335		11	> 100	Nørrung et al. 1999
Fish fingers	Ireland	Retail outlets	20	95	15	1000 to 10000	Sheridan et al. 1994
Fish fingers/fish cake	Singapore	Retail outlets, producers	16		19		Ng and Seah 1995
Fish and fish products	Italy	Retail outlets, producers	3160		6		Busani et al. 2005
Fish butter	Belgium	Super markets, 4	3	0	0		van Coillie et al. 2004
Marinated fish	Switzerland	Import- and export-control	125		38		Jemmi et al. 2002
Cold-salted (gravad) fish	Sweden	Retail markets	58		21	3400	Loncarevic et al. 1996
Cold-salted fish	Iceland	Retails stores, producers	23	61	22		Hartemink and Georgsson 1991
Cold-salted fish	Denmark	Producers	176		29	> 1000	Jørgensen and Huss 1998
Cold-salted fish	Finland	Retail outlets	32		50		Johansson et al. 1999
Cold-salted rainbow trout	Finland	Retail outlets	43		33		Lyhs et al. 1998

Table 7 continued

Product type	Country	Sampling location and No.	No. of samples	% positive for *Listeria* spp.	% positive for *Listeria monocytogenes*	Highest number of *L. monocytogenes* (CFU/g)	References
Cured seafood	Denmark	Producers	91		4	< 10	Jørgensen and Huss 1998
Shrimp	Iceland	Producers, 26	3331	8	2		Valdimarsson et al. 1998
Shrimp	Norway	Producers	16		18		Rørvik and Yndestad 1991
Shrimp	Different countries	Wholesales	49		8		Farber 1991
Shrimp	Different countries	Retail outlets	20		20		Farber 1991
Shellfish and shrimp	Iceland	Retails stores, producers	22	5	5		Hartemink and Georgsson 1991
Shellfish	Portugal	Producers, retail stores	8		0		Mena et al. 2004
Shellfish	Italy	Retail outlets, producers	1494		0		Busani et al. 2005
Shellfish	Chile	Producers, markets, restaurants	268		12		Cordano and Rocourt 2001
Shellfish	Japan	Retail stores	16		0		Handa et al. 2005
Crawfish	USA	Producers	78	0	0		Thimothe et al. 2002
Crawfish	USA	Retail stores	31		3		McCarthy 1997
Crabmeat	USA	Producers	126	10	8		Rawles et al. 1995
Crab	USA, China	Wholesales	7		14		Farber 1991
Crabmeat/scallops	Singapore	Retail outlets, producers	16		0		Ng and Seah 1995
Crustaceans	Italy	Retail outlets, producers	347		0		Busani et al. 2005
Surimi	Different countries	Wholesales	25		0		Farber 1991
Minced fish	Norway	Producers	8		12		Rørvik and Yndestad 1991
Roe	Japan	Retail stores	67		10		Handa et al. 2005
RTE products	Switzerland	Import- and export-control	151		7		Jemmi et al. 2002
RTE-salmon	Different countries	Wholesales	32		31		Farber 1991
RTE-seafood	Nordic Countries	Producers	63	5	5		Gudbjörnsdóttir et al. 2004
Raw RTE-seafood	Japan	Retail stores	213		3	> 100	Inoue et al. 2000

al. 1996). Seafood salad *L. monocytogenes* contamination occurred at a rate between 4.7 to 27% and the highest number of cells found was 100-1000 CFU/g (Gombas et al. 2003). The contamination of cold-salted fish varied between 21 to 50% and the largest number of *L. monocytogenes* cells found was $3.4x10^3$ CFU/g (Loncarevic et al. 1996). In Japan, contamination occurred in 10% (7/67) of the studied roe samples (Handa et al. 2005). The overall *L. monocytogenes* contamination rate of seafood products was 7.8% according to Table 7.

The amounts of *L. monocytogenes* found in different seafood products that have been suspected of causing listeriosis cases vary. In Finland, a listeriosis outbreak was connected with the consumption of cold-smoked rainbow trout. The cold-smoked rainbow trout from the same lot and the same retail store was found to contain $1.9x10^5$ CFU/g of *L. monocytogenes* (Miettinen et al. 1999). In a case with smoked mussels, *L. monocytogenes* amounts of $1.6x10^7$ CFU/g and $3.2x10^6$ CFU/g occurred in the mussels from the patients' refrigerators (Mitchell 1991). High numbers of *L. monocytogenes* ($2.1x10^9$ CFU/g) were also found in imitation crab meat that caused a listeriosis outbreak (Farber et al. 2000). On the other hand in a listeriosis outbreak associated with the consumption of cold-salted rainbow trout the detected amounts of *L. monocytogenes* in the fish from two patients' refrigerators were low (<100 CFU/g, 6200 CFU/g) (Ericsson et al. 1997).

2.5 Control of *Listeria monocytogenes* in fish processing

The policy regarding the presence and acceptable amounts of *L. monocytogenes* in different seafood products varies in different countries, from zero tolerance (e.g. in the USA) to an acceptable amount of less than 100 CFU/g that has been estimated as a level when the risk of listeriosis is very low even for high-risk groups (Farber et al. 1996, Buchanan et al. 1997). In Finland, the Finnish food safety authority also has a guideline for the control of *L. monocytogenes*. The level of *L. monocytogenes* in seafood products at the time of consumption should be less than 100 CFU/g. When the product leaves the production plant *L. monocytogenes* should not be detectable. In addition there are instructions like additional samplings or product recalls, for situations when *L. monocytogenes* or *Listeria* spp. are found in products or from plant environment.

To efficiently control *L. monocytogenes*, every potential route of entry and cross contamination must be monitored (Gravani 1999) plus good manufacturing practices need to be followed (Blanchfield 2005). The production facilities are important, as plants in which production facilities were in a good state of repair had a lower risk of *L. monocytogenes*

contamination than those with moderate or heavy wear and tear (Rørvik et al. 1997). In addition, the facilities should be designed or arranged to restrict or eliminate the transmission of people, equipment and conveyance between raw, processing, packaging and shipping areas as the cross contamination between raw and finished product areas is a major source of contamination (Lappi et al. 2004a). Well-designed lines and facilities enable good working routines and effective departmentalisation of facilities help in controlling both employees and product traffic (Autio et al. 2004). The employee movement between departments, due to rotation of the assigned duties, was found to be a risk factor for *L. monocytogenes* contamination of final products, especially if limited or no precautions were taken to avoid spread of bacteria (Rørvik et al. 1997). Cross contamination by employees and the environment of the processing plants may represent important causes of contamination of the finished product (Thimothe et al. 2004). Separate equipment, including tools employed by maintenance persons, should be available for use in raw and finished product areas (Gravani 1999). In addition all equipment within the factory should be designed to minimise cross contamination to products. Certain points of the processing chain, such as the machines, are particularly susceptible to contamination, because of the difficulties in efficient cleaning (Dauphin et al. 2001). The slicing and brining processes were the most critical steps of the fish production line mainly due to difficulties with cleaning the equipment thoroughly (Autio et al. 1999, Johansson et al. 1999). In addition, *L. monocytogenes* was extremely difficult to eliminate using routine disinfection procedures in a meat-bone separator which is complex equipment (Lappi et al. 2004a). The prevention of *L. monocytogenes* contamination in products is based on avoiding the colonisation of processing environment and equipment with *L. monocytogenes*. The hygienic aspects should be stressed when selecting new processing equipment as the complex construction of equipment often hampers the cleaning and disinfection practices that cannot be applied due to the effects on equipment materials (Autio et al. 2004).

Listeria spp., including *L. monocytogenes*, have been most frequently isolated from floor drains and floors, thus suggesting that these areas may function as reservoirs for *Listeria* in food processing facilities (Rørvik et al. 1997, Norton et al. 2001, Thimothe et al. 2004). These should be thoroughly cleansed and disinfected daily but high-pressure hoses should never be used, since such practices readily promote the spread of *Listeria* to nearby equipment and other areas of the factory through splashing and the generation of aerosols (Gravani 1999). Clean and especially dry floors are important for the control of *Listeria* in processing plants (Thimothe et al. 2004).

Disinfection is the final step in eliminating *L. monocytogenes* and other food borne pathogens as well as the myriad of spoilage organisms present in the production environment. Since the presence of organic debris readily decreases the effectiveness of disinfecting agents against *L. monocytogenes* (Best et al. 1990), it is important to remember that every item must first be thoroughly cleaned before it is disinfected. *L. monocytogenes* is sensitive to disinfectants commonly employed in the food industry. Disinfection with hot water is not advised, since sufficiently high water temperature can not be easily maintained (Gravani 1999). In some cases, however, the use of hot water (80 °C), heating in oven (80 °C) and treating with gas flame or a hot steam treatment has proven efficient in eradication of *L. monocytogenes* in a fish factory (Autio et al. 1999, Lappi et al. 2004a). In addition to inadequate separation between raw and finished product resulting from faulty factory design, indifferent attitudes of employees toward proper cleaning and disinfecting has been most frequently cited as factors that promote post processing contamination. Effective cleaning and disinfection programs for standard operating procedures for every factory job along with master schedules listing the frequency of cleaning and disinfection procedures are needed (Gravani 1999). The effectiveness of the cleaning and disinfection programs should be verified through daily microbiological analysis of both product and environmental samples gathered from all areas of facility. During environmental sampling, the efficacy of cleaning and disinfection procedures can be easily determined through the use of ATP bioluminescence monitoring systems. Routine testing of environmental samples for *Listeria* spp. remains, however, a critical component of any disinfection verification program (Gravani 1999). Development of focused clean-up and disinfection procedures as well as implementation of the HACCP programme are of great importance in the prevention of *L. monocytogenes* colonisation (Autio et al. 2004).

Consistent monitoring of *L. monocytogenes* contamination over time should be a component of every *L. monocytogenes* control strategy (Hoffman et al. 2003). The prevalence data and subtyping of *L. monocytogenes* provide a base for implementing effective cleaning and disinfecting procedures focusing on *L. monocytogenes* niches and transmission pathways (Thimothe et al. 2004). A plant-specific *Listeria* control program should also include strategies to minimise both the raw material and the environmental contaminations, procedures to prevent cross-contamination and employee training (Lappi et al. 2004a). Even when handled under the best possible conditions, raw seafood or processing environment will probably never be completely free from *L. monocytogenes* (Gravani 1999, Fonnesbech Vogel et al. 2001, Autio et al. 2004).

3 AIMS OF THE STUDY

The aims of the present thesis were to investigate the prevalence and sources of *L. monocytogenes* in different stages of the fish production chain and to improve the safety of rainbow trout roe products. The specific aims were as follows:

1. to determine the prevalence and location of *L. monocytogenes* in farmed rainbow trout (I),

2. to determine the prevalence of *L. monocytogenes* and the quality of Finnish roe products in the retail level (II),

3. to ensure the safety of rainbow trout roe in regards to *L. monocytogenes* elimination and quality maintenance of pasteurisated, refrigerator stored roe (III),

4. to study the presence of *L. monocytogenes* and *Listeria* spp. on fish factory surfaces and in final fish products as well as the association of surface hygiene and occurrence of *Listeria* spp. (IV),

5. to investigate the sources and ecology of *L. monocytogenes* and *Listeria* spp. in fish farming (V) and

6. to study the distribution of *L. monocytogenes* isolates from raw fish, fish production factories and the final fish products typed with pulsed-field gel electrophoresis (V).

4 MATERIALS AND METHODS

4.1 Sampling of raw material rainbow trout (I)

A total of 510 rainbow trout in lots of 10 to 50 fish each originating from fish farms in Finnish lakes (four lots) and sea areas (14 lots) were studied. The fish were individually packed into plastic bags straight after slaughter, and kept on ice. In addition to full-grown fish, two juvenile rainbow trout lots of 50 fish each from different farms were investigated. Three separate samples from each full-grown fish were taken: 1) the fish gills, with gill arches, were aseptically removed, 2) the peritoneal cavity was opened and the viscera without heart and kidneys were removed aseptically and 3) the fish skin was peeled off. The gill, viscera and skin samples were each thoroughly chopped using scissors. After chopping, pooled samples were formed separately from gill, viscera, and skin samples of five fish, resulting in three samples altogether. Only part of these original samples were taken into the pooled sample, and the rest of the original samples were incubated for five days at 5 °C, after which the samples were frozen. All original samples in the pool were analysed later if the pooled sample was found to be *Listeria* spp. positive. Two juvenile fish lots were chopped without separation of sample locations and pooled, each pool containing five fish.

4.2 Rainbow trout roe and its pasteurisation (II, III)

4.2.1 Retail level and fresh roe (II, III)

A total of 141 fresh, frozen or frozen-thawed roe samples from Finnish rainbow trout (*Oncorhynchus mykiss*), whitefish (*Coregonus lavaretus*) and vendace (*Coregonus albula*) were bought. The roe samples were bought from 46 different retail stores, shops and markets of various sizes in eight towns, and they originated from 26 different factories and suppliers in Finland. Frozen roe samples were allowed to thaw at 5.0 °C for 18-24 h before analysis. From at least 30 roe samples of each fish species (total 92) the amount of *Listeria* spp., aerobic and coliform bacteria as well as sensory quality were analysed and an additional 49 roe samples were analysed for *Listeria* spp. presence.

Fresh Finnish rainbow trout (*Oncorhynchus mykiss*) roe, used in study III was obtained from a wholesaler where it was cleaned, salted (2.5% wt/wt), and vacuum packed one to two days before further studies and processing in the laboratory.

4.2.2 *Listeria monocytogenes* strains (III)

Study III used four *L. monocytogenes* strains isolated during study II from rainbow trout, whitefish and vendace roes. The strains were adapted to 10 °C by incubating them separately three times at 10 °C for five days in Trypticase Soy Broth (Becton, Dickinson and Company, Sparks, MD, USA).

4.2.3 D- and z-value determination (III)

Determination of D-values for a mixture of equivalent amounts of four *L. monocytogenes* strains in rainbow trout roe was performed twice at both test temperatures (60 °C and 63 °C). Cold adapted *L. monocytogenes* strains were inoculated into fresh rainbow trout roe and mixed well resulting in a *L. monocytogenes* concentration of around $5x10^5$ CFU/g. The roe was incubated for five days at 10 °C so the cells achieved the stationary growth phase and were the most resistant to heat treatment. The amount of *L. monocytogenes* cells in roe was 10^8 CFU/g before heat treatment. The inoculated roe was packaged into heat sealed plastic film pouches (6 cm x 6 cm x 0.4 cm). Each pouch contained 15 g of roe. The heat treatment was performed in a water bath (WB 29, Memmert, Schwabach, Germany) and the temperature was measured inside a control roe pouch not inoculated with *L. monocytogenes* and in a water bath with temperature sensors (K-type thermo element, Fluke 52 II, Fluke Corp., Everett, WA, USA). The stability of the water temperature during the experiment was ± 0.2 °C. The test was initiated when the control pouch reached the desired temperature (60 °C or 63 °C). Triplicate samples were taken out of the water bath each time (30 s to 150 s intervals) and put into an ice-water mixture. The amount of *L. monocytogenes* in the pouches was determined by cultivation on the same day. Linear regression analysis of the thermal destruction of *L. monocytogenes* cells was performed in each heat treatment experiment. The D-value was calculated as a negative reciprocal of the mean of duplicate slopes at both 60 °C and 63 °C.

The z-value is the temperature difference required to yield a 10-fold change in the heating time and still destroy 90% of the *L. monocytogenes* cells. The z-value was calculated using the equation $z = (T_2-T_1) / \log(D_1/D_2)$, where D_1 is the time needed to destroy 90% of *L. monocytogenes* cells at temperature T_1, and D_2 is the time needed to destroy 90% of the *L. monocytogenes* cells at temperature T_2.

4.2.4 Pasteurisation of rainbow trout roe (III)

Roe was packaged into glass jars (100 g) and vacuum sealed (Mini-VacuumTwister, MVT-34/3, Rainer Naroska engineering, Bad Salzuflen, Germany). Pasteurisation of the roe was performed in an autoclave (Stock Pilot Rotor 900, Herman Stock Maschinenfabrik, Neumünster, Germany) as a water spray circulation run. Four temperature sensors (T type thermo element, Cu/CuNi, Ellab A/S, Roedovre, Denmark) installed inside four glass jars situated in different positions in the autoclave monitored the run temperature. One sensor monitored the temperature of the water spray. Temperatures were recorded with the Dasylab (Dasytec, Amherst, NH, USA) measurement program. Pasteurised roe jars were cooled with water spray circulation (7 °C) in the autoclave after pasteurisation.

Two experiments were conducted to evaluate the shelf-life of the pasteurised roe. Fresh vacuum packaged roe was pasteurised for 10 min at 65 °C and 15 min at 62 °C. Control samples were frozen (-18 °C) from the same fresh roe lot, but without pasteurisation. After pasteurisation the jars of roe were stored at 3 °C for 31 weeks and the sensory and microbial quality were analysed five and four times, respectively. Further microbiological analysis was performed on five jars of each roe type after the storage time had elapsed.

Two pasteurisation experiments with *L. monocytogenes* -inoculated roe were performed (10 min at 62 °C and at 65 °C). Before pasteurisations the roe was similarly inoculated and incubated as the roe in the D-value experiments. After pasteurisation the jars were stored for two weeks at 10 °C and *L. monocytogenes* was qualitatively analysed.

4.2.5 Pasteurisation values (III)

Pasteurisation values for pasteurisation experiments were calculated using the equation $\frac{P}{t_d} = 10^{(T-T_{ref})/z}$ (Stumbo, 1973), where P is the pasteurisation value (min), t_d is the heat death time (min), T_{ref} is the reference temperature, and z-value was already determined with the help of D-values. Because the reference temperature should be lower than the actual pasteurisation temperature T_{ref} value of 60 °C was chosen. The pasteurisation values were calculated on a spreadsheet program (Microsoft Excel 2002, Microsoft Corporation, Redmond, WA, USA) at 30 s intervals and added together. Calculation was started when the last temperature sensor inside the roe jar reached 50 °C and ended as it dropped below 50 °C.

4.3 Sampling in a fish farm (I, V)

A fish farm that had severe *L. monocytogenes* contamination in fish according the study I was further sampled 14 times. The fish cleaning house was sampled (17) during the first sampling occasion. The fish farm environment samples included fish (78), farming nets (17), equipment (5), water (78) and sea bottom soil (43) samples. Fish were analysed as pooled samples of gills from one or two fish (25 g). Water samples were directly analysed from 25 ml and bigger volumes (three or four litre) were first prefiltered and then sterile filtered. Surface samples were taken with sterile cotton gauzes, the gauzes being moistened with sterile peptone water if necessary. Surface areas of at least 100 cm² were sampled. The sea bottom soil samples (25 g) were mainly taken inshore, except for seven samples that were taken from five to 20 m deep sea areas. The fish farming nets and facilities were situated near (40 m) the shore. A nearby (10 km) meteorological measurement site measured the mean monthly rainfall (1970 to 2000) and rainfall from 2001 to 2004.

4.4 Sampling in fish processing factories (IV)

A total of 943 surface samples were taken for *Listeria* spp. analyses from 23 factories. Hygiene samples from surfaces (866) with four other methods (Table 8) were taken from 28 factories. Surface samples were taken in the morning after washing and cleaning just before the start of a normal workday. Most of the samples, on average 30-40 samples from each factory, were taken from surfaces considered important for process hygiene, e.g. knives, chopping boards, conveyor belts, control panels and aprons. Some samples for *Listeria* spp. analyses, five to seven per factory, were also taken from floors, drains and other lower hygiene areas. In addition, two to six samples of raw, marinated, cold-salted, cold-smoked or smoked fish or roe products were taken from each factory to be analysed for *Listeria* spp. presence.

4.5 Microbial analyses

4.5.1 Isolation and identification of *Listeria* spp. and *Listeria monocytogenes* (I, II, III, IV, V)

Listeria spp. were analysed according to the ISO method (Anonymous 1996) for detection with a two-step enrichment with half-Fraser (Half Fraser broth, Oxoid, Basingstoke, UK; 30 °C, 1d) and Fraser (Fraser broth, Oxoid; 37 °C, 2d) enrichment broths (studies I, II, III, V) or according to Nordic Committee on Food Analysis method (Anonymous 1999) using one-step enrichment with EB-broth (Listeria Enrichment broth, Oxoid; 30 °C, 2d; study IV). The selective plates used were Oxford (Oxoid; 37 °C, 2d; studies I, II, III, IV, V), LMBA (Tammer-Tutkan Maljat, Tampere, Finland; 37 °C, 2d; studies I, II, III, V) and PALCAM

(Oxoid; 37 °C, 2d; study IV). Sample size was 25 g or 25 ml. The filter and surface gauze samples were weighed and incubated in enrichment broth in the same ratio as the 25 g samples. *Listeria* from the factory surfaces (study IV) was sampled using contact plates (25.5 cm^2) of Oxford-agar (Oxoid). The contact plates were incubated for two days at 37 °C. Gram-staining, catalase test, haemolysis testing (Orion Diagnostica, Espoo Finland or bioMérieux, Marcy l'Etoile, France, 37 °C, 2 d) and API-Listeria test (bioMérieux) were performed for suspected *Listeria* spp. colonies instead of testing the carbohydrate utilization and the CAMP test.

4.5.2 Enumeration of *Listeria* spp. (II, III)

Performance of *Listeria* spp. enumeration (study II) was according to ISO method for enumeration with 25 g samples on PALCAM (Oxoid; 37 °C, 2d) plates (Anonymous 1996). At least five suspected *Listeria* spp. colonies per plate were identified as mentioned above. Direct plating of roe samples in the D-value determination study (III) was performed from 10 g samples on Oxford agar plates not supplemented with selective components. Plates were incubated for two days at 37 °C.

4.5.3 Analysis of aerobic, anaerobic, coliform and enterobacteria, fungi and ATP (II, III, IV)

The microbial analysis of retail roe samples and surface hygiene samples were performed according to Table 8.

Table 8. Microbial methods used in analysis of aerobic, anaerobic and coliform bacteria and fungi in roe samples and the methods used for surface hygiene samples in fish factories.

Analysis	Sample	Method	Plate/kit	Incubation
Aerobic bacteria	10 g, roe	Spread-plating	Plate count agar (Difco Sparks, MD, USA)	30 °C, 3d
Coliform bacteria	10 g, roe	Pour-plating	Violet red bile agar (Difco)	37 °C, 2d
Fungi	10 g, roe	Spread-plating	Potato dextrose agar[1] (Difco)	25 °C, 5d
Anaerobic bacteria	10g, roe	Spread-plating	Reduced plate count agar (Difco)	10 °C, 10d[2]
Anaerobic bacteria	10g, roe	Spread-plating	Reduced plate count agar (Difco)	30 °C, 3d[2]
Anaerobic bacteria	10g, roe	Spread-plating	Reinforced clostridial medium (Difco)	37 °C, 7d[2]
Aerobic bacteria	Surface	Contact agar	Hygicult® TPC (Orion Diagnostica)	30 °C, 2d
Enterobacteria	Surface	Contact agar	Hygicult® E (Orion Diagnostica)	37 °C, 2d
Fungi	Surface	Contact agar	Hygicult® Y&F (Orion Diagnostica)	25 °C, 5d
ATP	Surface	Swabbing	Surface monitoring kit (Bio-Orbit, Turku, Finland)	

[1]Supplemented with 0.4% chloramphenicol (Sigma Chemical, St. Louis, MO, USA), 0.4% chlortetracycline (Sigma) and 0.02% Triton-X (Fluka Chemie, Buchs, Germany)
[2]Incubation in anaerobic jars (Anoxomat WS8000, Mart® Microbiology, Lichtenvoorde, Netherlands)

4.5.4 Identification of bacteria from pasteurised roe (III)

Identification of isolated bacteria from pasteurised roe samples was performed after Gram-staining, oxidase, catalase, and indole tests with API50 CHB (bioMérieux) or Chrystal ID (Becton, Dickinson and Company) according the manufacturer's instructions.

4.6 Sensory analysis (II, III)

A modified general descriptive profile method (study II, Anonymous 1985) and a descriptive sensory analysis method, conventional profiling (study III, Anonymous 2003) were used. In study II the panel scored four attributes: appearance as dryness (freeness from visible liquid), odour freshness, texture as egg firmness and freshness of taste on a continuous intensity line scale from zero (low intensity) to ten (high intensity). The panellists were also allowed to give verbal descriptions of the samples and add comments. In study III the following eight attributes were evaluated: freshness of odour and taste, taste intensity, brightness of colour, amount of free liquid, intactness and firmness of eggs and total quality. The attribute intensities were rated on a continuous unstructured graphical line scale from zero (low intensity) to five (high intensity).

4.7 Typing of *Listeria monocytogenes* isolates

4.7.1 Ribotyping (I)

Ribotyping was performed for 14 *L. monocytogenes* positive samples including water, surface and fish samples from the first sampling occasion in the studied fish farm. Ribotyping was carried out for two isolates per sample, altogether 28 isolates. Performance of ribotyping followed the manufacturer's standard instructions, as described by Bruce (1996). DNA was digested with *Eco*RI (DuPont Qualicon, Wilmington, Del., USA). The automated system processed the batches and generated a pattern for each sample and a marker lane using proprietary algorithms. Isolates were assigned to a ribogroup from the database, or a new one was created and similarities were calculated (Qualicon software v. 12.2). Definition of a ribogroup was a set of closely related patterns (threshold similarity $\geq$ 0.97) that were mathematically indistinguishable from one another by the system.

4.7.2 PFGE-typing (V)

Fish farm ecology and the relationships of *L. monocytogenes* isolates between farmed fish, fish processing factories and fish products were studied with PFGE-typing. Before subtyping with PFGE 98 *L. monocytogenes* isolates from the fish farm samples (study V), 47

L. monocytogenes isolates from raw rainbow trout (study I) and 36 *L. monocytogenes* isolates from nine fish processing factories collected earlier partly in study III were frozen stored.

DNA was isolated according to the PulseNet 4-h protocol (Graves and Swaminathan 2001) with minor modifications. In brief, the cell density of overnight grown bacteria was adjusted to 0.8 in TE-buffer (0.01 M Tris [Sigma], 0.01 M EDTA [Riedel-de Haën, Seelze, Germany], pH 8.0). Cell suspensions with 10 mg/ml lysozyme (Sigma), molten InCert Agarose (BioWhittaker Molecular Applications, Rockland, ME, USA), 1% sodium dodecyl sulphate (Sigma) and 0.2 mg/ml Proteinase K (Roche Molecular Biochemicals, Mannheim, Germany) were dispensed into plug molds (Bio-Rad Laboratories, Hercules, CA, USA). The agarose plugs were lysed in lysis buffer (0.05 M Tris, 0.05 M EDTA, 1% sodium lauryl sarcosine [Sigma], 0.15 mg/l Proteinase K) for 2 h at 53 °C in a water bath shaker. After proteolysis, the lysis solution was removed and the plugs were washed. Plugs were digested with *Asc*I (New England BioLabs, Beverly, MA, USA) in buffer solutions according to the manufacturer's instructions. DNA fragments were electrophoretically separated through 1% agarose gel (Pulsed Field Certified Agarose, Bio-Rad Laboratories) in 0.5 x TBE buffer (Bio-Rad Laboratories) at 200 V, 14 °C in CHEF Mapper® XA PFGE apparatus (Bio-Rad Laboratories). The pulse times ramped from 1 to 35 s for 18 h. Lambda Ladder PFG marker (New England BioLabs) was used for fragment size determination. After electrophoresis, the gels were stained with ethidium bromide and subsequently photographed (Gel Doc 2000 system, Bio-Rad Laboratories). The resulting pulsed-field electrophoretic macrorestriction patterns were compared using Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium). A dice coefficient (position tolerance 1.0%) expressed different PFGE pattern similarity. The clustering and construction of dendograms were performed by the unweighted pair group method using arithmetic averages (UPGMA).

4.8 Statistical analyses (I, II)

Listeria spp. and *L. monocytogenes* prevalence values for different locations in fish were compared using Pearson's chi-square tests (I). Differences in the microbial and sensory quality of roe between the fish types and storage conditions were analysed with 3x3 multi- and univariate analyses of variances (II). In addition, one-way multivariate analyses of variances were performed separately for each fish type and storage condition. Tukey's post hoc test was used to determine which of the fish types and storage conditions differed from each other. Pearson product-moment correlations were calculated to analyse relationships between the sensory and microbiological measures (II).

5 RESULTS

5.1 Prevalence and location of *Listeria monocytogenes* in farmed rainbow trout (I)

Two-thirds of the fish lots (11/18) contained *Listeria* spp. and more than one-fourth contained *L. monocytogenes* (5/18). Of the pooled fish samples, 15% (15/103) were contaminated with *L. monocytogenes* and 35% (36/103) with *Listeria* spp. Contamination of individual fish, studied after frozen storage, was lesser (Table 9). The freezing destroyed part of the *Listeria* spp. population in fish samples since seven pooled samples positive for *Listeria* spp. were not found to contain *Listeria* spp. in any of the individual fish samples after the frozen storage.

Listeria spp. occurred most often in the fish gill samples (Table 9), only a few times in skin samples and once in viscera samples. Almost all identified *L. monocytogenes* isolates were found in gills, while only one in viscera and one in skin samples. Many gill samples within the lot with the *L. monocytogenes* positive skin sample were also contaminated with *L. monocytogenes*. In the case of the *L. monocytogenes* positive viscera sample, the viscera were the only sample containing *L. monocytogenes*.

Table 9. Prevalence of *Listeria* spp. and *L. monocytogenes* in individual fish according to location. Percentages given in brackets refer to the division of number of positive samples by the total number (510).

Sample	*Listeria* spp. No. (%)	*L. monocytogenes* No. (%)
Gills	82 (16)[a]	43 (8.4)[a]
Skin	14 (2.7)[b]	1 (0.2)[b]
Viscera	1 (0.2)[b]	1 (0.2)[b]
Total fish	91 (18)	44 (8.6)

[ab] Different superscripts within a column refer to statistically significant ($p < 0.0001$) difference in prevalence.

Clear differences occurred in the *Listeria* spp. and *L. monocytogenes* prevalences among the fish farms. All or most of the pooled fish samples at seven farms were contaminated with *L. monocytogenes* or other *Listeria* spp., at four farms contamination of fish lots was only sporadic and at seven farms no *Listeria* spp. appeared in any fish. No association emerged between certain sea or lake areas and *Listeria* spp. contamination. All sampled areas contained fish farms with and without *Listeria* spp. contamination. No *Listeria* spp. was found in the juvenile fish studied.

5.2 Safety and quality of Finnish retail level roe (II)

5.2.1 Prevalence of *Listeria monocytogenes* (II)

Nearly a fifth of roe samples were contaminated with *Listeria* spp. and a minor amount with *L. monocytogenes* (5%). The prevalence of *L. monocytogenes* and *Listeria* spp. ($p < 0.01$) differed significantly between storage conditions (Table 10) fresh-bought roe products containing more often *L. monocytogenes* than fresh and frozen-thawed roes. *Listeria* spp. occurred more often in rainbow trout roe ($p < 0.001$) than in roes of other fish species (Table 10). The interactions between fish species and storage condition were not significant ($p = 0.60$). *Listeria* spp. quantities found with direct plating were normally low. One fresh rainbow trout roe sample contained *L. monocytogene*s at a level of 60 CFU/g. *L. innocua* occurred in three fresh rainbow trout roe samples and one fresh whitefish roe sample with greater than 10 but less than 100 CFU/g. Most of the *Listeria* positive samples resulted from enrichment procedures. *Listeria* spp. or *L. monocytogenes* occurrences did not depend on the producer, since all the *L. monocytogenes* positive roes were processed by different producers and 25 *Listeria* spp. positive roes originated from 18/26 different producers.

Table 10. Prevalence of *Listeria* spp. and *L. monocytogenes* in different roe product types.

Roe storage condition/ fish species of roe	No. of samples	Positive for *Listeria* spp. (%)	Positive for *L. monocytogenes* (%)
Frozen	59	5 (8.5)[b]	0 (0)[a]
Frozen-thawed	48	5 (10)[b]	1 (2.1)[a]
Fresh	34	15 (44)[a]	6 (18)[b]
Rainbow trout	50	18 (36)[a]	4 (8.0)
Whitefish	45	5 (11)[b]	2 (4.4)
Vendace	46	2 (4.3)[b]	1 (2.2)
Total roe samples	141	25 (18)	7 (5.0)

[ab] Different superscripts within storage types ($p < 0.01$), fish species ($p < 0.001$) and columns refer to statistically significant difference in prevalence.

5.2.2 Microbial and sensory quality (II)

The mean count and median of total aerobic bacteria of all roe samples was 6.6 ± 1.5 logCFU/g and 7.1 logCFU/g, respectively. Bacterial counts of vendace roe were significantly ($p < 0.05$) higher than counts in rainbow trout roe, but not significantly different from those of whitefish. This difference between fish species was highest when the roes were bought fresh ($p < 0.05$), although a similar tendency was also observed in frozen roe. With regard to individual fish species and storage conditions, the contamination was significantly higher

($p < 0.05$) in fresh vendace roe samples than in those of frozen rainbow trout and whitefish roes.

The mean count and median of coliform bacteria of all roe samples was 3.2 ± 1.7 logCFU/g and 3.4 logCFU/g, respectively. There were no significant differences among fish species, but significantly ($p < 0.05$) fewer coliform bacteria occurred in frozen roe than in fresh roe samples. In addition, significantly ($p < 0.05$) more coliform bacteria were found in fresh vendace roe than in either frozen vendace or frozen rainbow trout roes.

Multivariate tests showed significant differences in the sensory scores between the storage conditions ($p < 0.001$), fish species ($p < 0.001$) and interactions between the two ($p < 0.01$). The ratings of all sensory characteristics correlated highly with each other. With respect to taste and odour freshness the roe quality was best in rainbow trout, however, appearance and structure evaluation was best in vendace roe samples. Roe samples bought fresh had on average a fresher odour and taste than frozen and frozen-thawed roe samples, seen in all three fish species. Sensory defects in roe samples were described as rancid, musty, fishy, metallic, yeast- and mould-like, slimy, gruel-like and eggs having hard skin. There was no significant correlation between results obtained by sensory and microbial methods.

5.3 Pasteurisation of rainbow trout roe (III)

5.3.1 D- and z-values of four *Listeria monocytogenes* strain mixture in rainbow trout roe (III)

Heat treatments of the mixture of the four *L. monocytogenes* strains resulted in D-values of 1.60 min and 0.44 min at 60 °C and at 63 °C, respectively, and the z-value calculation was 5.36 °C. In both temperatures the linear regression lines of the thermal death of *L. monocytogenes* cells were parallel with the duplicate experiment at the same temperature.

5.3.2 Microbial quality (III)

Microbial quality of pasteurised roe was excellent during the entire 31 week storage time as the aerobic, coliform and fungal counts were below detection levels. Frozen control roes were also of good microbial quality and the level of aerobic and coliform bacteria as well as fungi were low, mostly under 10^3 CFU/g. No anaerobic psychrotrophic bacteria were detected in any of the roes nor were there aerobic psychrotrophic bacteria in the pasteurised roes at the end of the storage time. The level of aerobic psychrotrophic bacteria in control roes were below the level of aerobic mesophilic bacteria that was detected in the same roes. One

pasteurised roe (1/10) and one control roe (1/5) showed growth of a few anaerobic mesophilic colonies on plate count agar. *Bacillus circulans* was identified from the pasteurised (62 °C) roe sample. From three pasteurised roe (3/8) and one control roe sample (1/5) a few colonies growing on clostridium medium were found. As these colonies, however, could not be identified with Chrystal ID they were not *C. botulinum* nor *C. perfringens*.

5.3.3 Sensory quality (III)

The quality of both the pasteurised and frozen control roe samples were good until 24 weeks of storage (mean scores of total quality 3.1 to 3.5). After that, the sensory quality of the pasteurised samples began to decrease in comparison to the control sample. The quality decrease was most clearly detectable in the freshness of odour and taste as well as in colour brightness. The differences between the experimental and control samples were not, however, statistically significant. The texture remained good throughout the entire storage period. At the end of the storage period the quality of all samples was still acceptable.

5.3.4 Prevalence of *Listeria monocytogenes* (III)

No *L. monocytogenes* were detected in inoculated (10^8 CFU/g) roes pasteurised for 10 min at 65 °C or at 62 °C with enrichment cultivation. The pasteurisations were stronger than theoretical pasteurisation at the same temperatures and duration due to the exclusion of the heating up and cooling down times in the theoretical calculations. The roe jars were at elevated temperatures (> 50 °C) for 56 min and 51 min at 65 °C and at 62 °C pasteurisations, respectively, which are five times longer than the actual pasteurisation time of 10 minutes.

5.3.5 Pasteurisation values (III)

In order to assess the effectiveness of the different roe pasteurisation treatments, pasteurisation values were calculated for experimental pasteurisations and for theoretical 1D pasteurisation based on the determined D-values at 60 °C and 63 °C. The effect of the heating up and cooling down processes is included in the pasteurisation values of the experimental pasteurisations. Pasteurisation values ranged between 73 to 247 minutes and the corresponding 1D treatment pasteurisation value was 1.6 min. Based on the division of these figures the performed experimental pasteurisations at 62 °C and 65 °C destroyed 46 to 154 log units of *L. monocytogenes* cells. In other words, the mildest pasteurisation temperature (62 °C, 10 min) corresponds to almost four times the theoretical 12D treatment.

5.4 Occurrence of *Listeria monocytogenes* and *Listeria* spp. and surface hygiene in fish processing factories (IV)

L. monocytogenes and *Listeria* spp. were found from 30% and 65% (7/23 and 15/23) of the factories sampled, respectively. In the factories that were *Listeria* spp. positive, 7.2% of all the surfaces sampled tested positive. One-third of the positive samples identified were *L. monocytogenes* and the rest were mainly *L. innocua*. *Listeria* spp. was found from e.g. the brine basin, brine machine, packaging table, cold-smoke skewer, grindstone, skinning machine, vacuum packaging machine, scale, apron, conveyor belt, door handle, light switch, grilling machine, floors and drains.

About half (48%) of the washed and cleansed surfaces of the fish factories were at least moderately contaminated with aerobic bacteria (> 1.8 CFU/cm²) and one-fourth (26%) of all the samples were heavily contaminated (> 5 CFU/cm²). In 43% of the samples ATP activity exceeded 1 RLU that indicates evident microbial activity. The amount of enterobacteria was over 0.1 CFU/cm² in 20% of the samples and in 9% of the samples the amount of enterobacteria exceeded 1.0 CFU/cm². One-third of the surfaces were at least moderately (> 0.6 CFU/cm²) contaminated with moulds and over 10% of the surface samples exceeded 1.6 CFU/cm². Yeast contamination was only slightly lesser than the mould contamination. Of the *Listeria* spp. positive surfaces, 71% (22/31) were at least moderately (> 1.8 CFU/cm²) contaminated, and 68% were heavily contaminated (≥ 5 CFU/cm²) in terms of total bacteria counts, and 59% had an ATP result higher than 1 RLU. Enterobacteria (≥ 0.1 CFU/cm²) were found in 56% of the *Listeria* spp. positive samples. Yeast and mould contaminations of the *Listeria* spp. positive surfaces were 44% and 43%, respectively. On the other hand, 8.8% and 7.5% of the all enterobacteria and aerobic bacteria contaminated surfaces were also *Listeria* spp. contaminated.

Almost half (45%) of the ready-to-eat fish products had *Listeria* spp. contamination. *L. monocytogenes* was found in 13% of the ready-to-eat fish products, including cold-smoked (17%) and hot-smoked fish (8%), cold-salted fish (20%) and roe (0%). The contamination of raw fish was slightly lesser (33%) for *Listeria* spp. and (11%) for *L. monocytogenes*. *Listeria* spp. was detected from the raw fish or fish products in 70% (16/23) and *L. monocytogenes* from 30% (7/23) of the factories. In five factories all product samples examined had *Listeria* spp. contamination and one out of three brines examined had *L. monocytogenes* contamination. In this factory *L. monocytogenes* contamination occurred in all the products studied.

5.5 Contamination sources of *Listeria monocytogenes* at the fish farm (I, V)

During the three year survey, 17% (41/240) of the samples taken from the farm and its surroundings were contaminated with *L. monocytogenes.* At the beginning of the follow-up the surfaces, water and fish in the farming area were heavily contaminated with both *L. monocytogenes* and *L. innocua*. All wet surfaces had *Listeria* spp. contamination. The washed fish cleaning house, however, was free from *Listeria* spp. except for one *L. innocua* contaminated drain (1/17). There was no *Listeria* spp. found in the ten fish collected simultaneously from further out to sea. The contamination of the fish farm environment and fish with *L. monocytogenes* varied from heavy contamination at the beginning of the follow-up through sporadic incidence to a situation with no *Listeria* spp. found until a new extensive contamination at end of the follow-up. Fig. 1 demonstrates the association of the mean monthly rainfall and the *L. monocytogenes* occurrence in the fish farm. *L. monocytogenes* was found in some of the samples in all sampling occasions when the mean monthly rainfall exceeded 50 mm. No *L. monocytogenes* but other *Listeria* spp. were found in sampling occasions when the mean monthly rainfall was less than 50 mm. In two occasion, however, when the mean rainfall was less than 10 mm, no *Listeria* spp. were found in any of the samples.

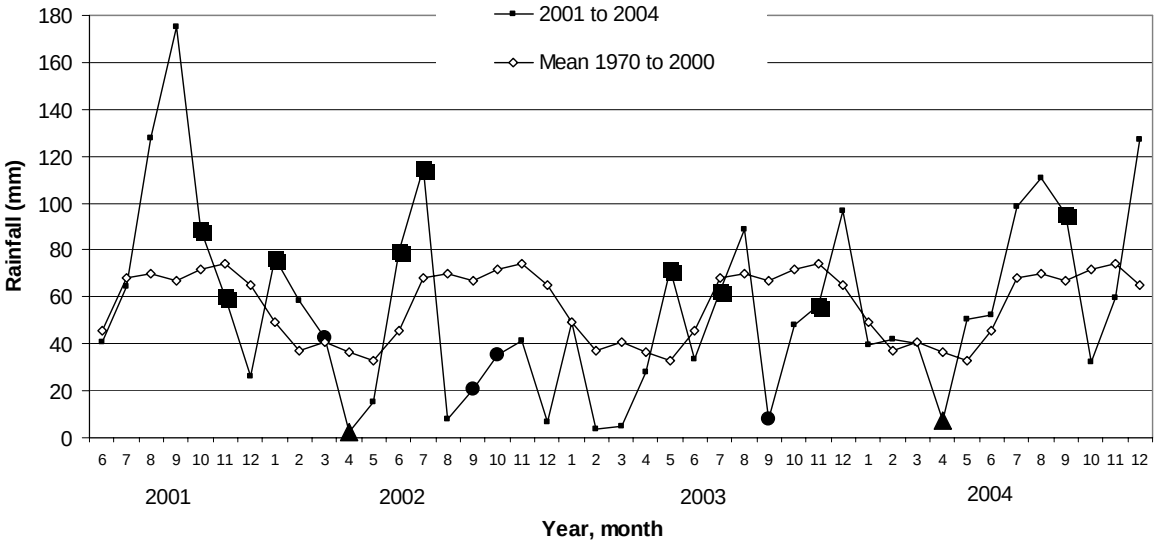
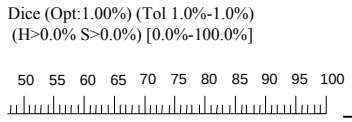


Figure 1. The rainfall per month measured near the fish farm location from June 2001 until the end of 2004 compared with the mean rainfall during 1970 to 2000. The performed samplings are marked with bigger symbols (■ *L. monocytogenes* found in sampling, ● other *Listeria* spp. but no *L. monocytogenes* found, ▲ no *Listeria* spp. found). *L. monocytogenes* findings are clarified in Table 11.

L. monocytogenes isolates (98) from the fish farm studied fell into 12 PFGE pulsotypes (Table 11). During the follow-up time, the occurrence of different pulsotypes changed. Six different pulsotypes were present in the fish farm at the beginning of the study, in the following years the old pulsotypes disappeared and new ones emerged. The fish were contaminated only two times and both times only one *L. monocytogenes* pulsotype was found. These pulsotypes were not found in any other sample types. The fish contaminants belonged to different clusters. Ribotyping of the 28 *L. monocytogenes* isolates from the first contamination wave formed seven ribotypes. The same isolates formed ribotypes according to their pulsotypes except ribotyping separated one pulsotype into two groups (Table 11).

Table 11. Dendogram, sample places, pulsotypes, ribotypes, serotypes, sampling dates and the number of PFGE isolates (98) of *L. monocytogenes* isolates from one fish farm typed with pulsed field gel electrophoresis using *Asc*I enzyme and ribotyped with *Eco*RI enzyme.



Sample place	Pulso-type	Ribo-type	Sampling date (month/year)	No. of PFGE isolates
Ground	a1		9/04	3
River water	a2		11/03	4
Brook water	b		9/04	2
Fish	c1	1	10/01	19
Fish	c1		1/02	9
Surface	c2	2	11/01	1
Sea water	c2	2	11/01	1
Fish	d		9/04	6
Sea water	e	3	11/01	1
Ground	e		5/03	5
Ground	f1		5/03	5
Sea water	f2	4	11/01	2
Ground	f2		6/02	5
Brook water	g1		9/04	1
Sea water	g1		9/04	3
Sea water and surface	g2	5, 6	11/01	5, 2
Ground	g2		7/02	8
Sea and brook water	g2		7/03	5, 5
Sea water	h	7	11/01	6

5.6 Distribution of *Listeria monocytogenes* PFGE-types isolated from different areas of fish production chain (V)

From 15 different fish companies 181 *L. monocytogenes* isolates formed a total of 30 PFGE-types. Nine of the pulsotypes comprised *L. monocytogenes* isolates from more than one company (two to four companies), but most (21/30) of the pulsotypes were only found in one company. *L. monocytogenes* contaminated raw fish from six fish farms harboured one or two *L. monocytogenes* PFGE-pulsotypes in each fish lot. The fish processing factories and the fish farm environment mostly harboured several *L. monocytogenes* pulsotypes at one sampling time. There were nine *L. monocytogenes* pulsotypes found from ready-to-eat fish products. Seven of these final product pulsotypes included isolates from other sources. Five included isolates from a fish processing environment and four from raw fish samples, consequently two pulsotypes included isolates from all three sources. *L. monocytogenes* isolates, originating from a fish processing environment, formed 11 pulsotypes. In addition to the five pulsotypes belonging to the final product pulsotypes, a raw fish material isolate shared one pulsotype and five did not occur in other sample types. Raw fish isolates formed ten pulsotypes, four shared with the final products, one with the processing environment and five not shared with any other sample types. Ten pulsotypes found from the environment of the studied fish farm were not shared with other sample types.

6 DISCUSSION

6.1 The role of raw fish materials as a source of *Listeria monocytogenes* (I, V)

The incoming raw fish material is occasionally an important *L. monocytogenes* source in fish production. The *L. monocytogenes* prevalence in different fish lots ranged from 0 to 75% for individual thawed fish and from 0 to 100% according to pooled unprocessed fish samples. The mean prevalence of *L. monocytogenes* in pooled rainbow trout was 15% and 9% was found for *L. monocytogenes*, in the individual fish samples. The actual prevalence is something between these figures as they either overestimate or underestimate the real situation because all fish in pooled samples were not necessarily contaminated and freezing destroyed *L. monocytogenes* bacteria in some of the individual samples. The *L. monocytogenes* prevalence results found in the literature varied greatly from 0 to 88% (Table 5) for raw fresh fish. When considering all data, however, the mean prevalence of *L. monocytogenes* was 14%, which is near the results in this study.

An essential factor in the transmission of *L. monocytogenes* contamination between raw fish and fish processing is the location of *L. monocytogenes* in fish. The location of

L. monocytogenes in different parts of raw fish material differed significantly ($p < 0.0001$) in rainbow trout. Up to 96% of the *L. monocytogenes* positive samples were gill samples. Only 4% (2/45) of the *L. monocytogenes* positive samples were skin or viscera samples. Rainbow trout is therefore contaminated by *L. monocytogenes* almost exclusively in the gills and only sporadically in the skin and viscera. On the basis of these results special effort should be focused on the isolation and removal of rainbow trout gills before the *L. monocytogenes* contamination can spread further.

The prevalence of *L. monocytogenes* varied greatly between different fish farms as mentioned above from 0 to 100%. The results from other fish farm studies support the finding that *Listeria* spp. contamination varies greatly between different farms. In a small Norwegian salmon study, no *Listeria* spp. was found in gill, skin and guts of ten salmon (Ben Embarek et al. 1997). In Switzerland (Jemmi and Keusch 1994), three studied freshwater rainbow trout fish farms showed no *Listeria* spp. in fish at one first farm, but the other two farms had *Listeria* spp. in fish skin (6/15 and 9/15) and in faecal content (6/15 and 4/15) sampled with swabs. Gills were not studied in that investigation (Jemmi and Keusch 1994). In USA, *L. monocytogenes* was isolated from combined skin and gut samples of aquacultured hybrid striped bass in two out of three fresh water ponds (Nedoluha and Westhoff 1993). If fish grow in areas where the presence of *L. monocytogenes* is possible, like polluted waters and waters with a high content of organic material (Ben Embarek 1994), it is probable that occasionally the fish will also harbour *L. monocytogenes*.

Typing of 181 *L. monocytogenes* isolates from 15 fish farming and fish processing factories, including raw fish and final fish products, showed a wide range of different *L. monocytogenes* pulsotypes (30). Both the raw fish and the processing environment *L. monocytogenes* isolates were often found together with product isolates within the same PFGE-type. The importance of factory environment as the main source of *L. monocytogenes* contamination of final products has previously been reported (Destro et al. 1996, Autio et al. 1999, Fonnesbech Vogel et al. 2001). This study, however, also emphasises the importance of the raw materials as a source of *L. monocytogenes* at least occasionally when they are heavily contaminated. Few other studies exist that also suggest that the raw materials have a relevant role in contamination of products and the process environment (Eklund et al. 1995, Fonnesbech Vogel et al. 2001, Norton et al. 2001, Markkula et al. 2005).

6.2 Safety and quality of Finnish roe at retail level (II)

Roe products are a potential source of *L. monocytogenes* in Finland. The presence of *L. monocytogenes* in different fish species roe varied from 2 to 8%. Fresh-bought roes, however, contained 20 times more often *L. monocytogenes* (18%) than frozen or frozen-thawed roe products. This may be caused by the freezing process destroying and injuring *L. monocytogenes* cells despite the fact that they are quite resistant to the freezing process and normally only a small amount is inhibited during the frozen storage (Golden et al. 1988, Harrison et al. 1991). In Japan, the prevalence of *L. monocytogenes* in roe products in retail markets was 10% (Handa et al. 2005) which is quite near the values found here. The detected amounts of *L. monocytogenes* were low (< 100 CFU/g), which supports the inactivation effect of freezing. The storage time of fresh or frozen-thawed roe, before consumption, is only a few days and therefore the risk of the development of high counts of *L. monocytogenes* is relatively low if the roe is kept appropriately refrigerated (< 3 °C).

Wide variations in the microbial as well as sensory quality of the roe products exist. The microbial quality was poor in 57% of the roe samples, having aerobic bacteria over 10^7 CFU/g, and 73% of the samples had coliforms over 150 CFU/g. The overall microbial quality was worst in vendace roe. This is probably because the small size of the ovaries necessitates more handling during production. Jokinen et al. (1992) also found high bacterial counts ($10^{7\text{-}8}$ CFU/g) in 62% of salmon, rainbow trout, whitefish and vendace roes bought from wholesale and retail markets in Sweden. They found, however, only small amounts of coliform bacteria. Himelbloom and Crapo (1998) found that aerobic counts increased in pink salmon ikura as the 30-day Alaska production season progressed, ending with 4.5×10^7 CFU/g in fresh eggs. The total coliform counts during the season varied from below a detectable level to 2.4×10^3 CFU/g. These results are similar to the findings in the present study.

The correlations between microbial and sensory measures were low. Unacceptable quality in the taste of freshness, by sensory analyses, was found in 20% of the roe samples. Roe samples bought fresh had a better sensory quality than products bought frozen or frozen-thawed. The deterioration in quality was often reflected in all evaluated sensory characteristics, partly because olfactory and taste perceptions as well as chemical feeling factors are all perceived in the mouth and the flavour terms easily intermingle (Meilgaard et al. 1999). Defects were most consistently found in vendace roe samples and the rainbow trout roe was rated as the freshest. Musty and rancid off-odours and off-tastes were detected in roes from every fish species. Yeast-like or mould-like off-odours and off-tastes were also perceived in many samples.

These defects suggest the presence of temperature abuse along the cold chain or poor production plant hygiene. Rancidity is a common indicator of time temperature-dependent spoilage of fish material (Huss 1995). Too long of a storage time can also explain deterioration in quality, especially in frozen-thawed roe, since there was often no best before expiration date on the products sold at service counters.

6.3 Safety and quality of pasteurised rainbow trout roe (III)

Pasteurisations of rainbow trout roe for 10 min at 62 °C and 65 °C were effective in eliminating inoculated *L. monocytogenes* cells (10^8 CFU/g), which is about the highest possible *L. monocytogenes* cell count to grow in rainbow trout roe. Experimentally determined D- and z-values for the mixture of four *L. monocytogenes* strains were a little lower than the values found in the literature on fish or crab meat (Ben Embarek and Huss 1993, Mazzotta 2001, Table 4). Based on the determined D- and z-values, however, the experimental pasteurisations were calculated to eliminate at least 45 log units of *L. monocytogenes* that is sufficiently even in case of a more heat resistant *L. monocytogenes* strain.

The quality of cold stored pasteurised vacuum packaged rainbow trout roe, stored for six months, was consistently good in terms of microbial as well as sensory quality. Pasteurisations inactivated most of the bacteria in rainbow trout roe. The initial bacterial load in roe, however, was relatively low. In addition, *B. circulans* was identified from roe pasteurised for 10 min at 62 °C, which demonstrates that bacterial spores can survive pasteurisation. The storage temperature of the pasteurised roe has to be below 3 °C to prevent spore-forming bacteria from multiplying and forming toxins, especially since there is a risk of group II *C. botulinum* spores being found in the fish roe (Hyytiä et al. 1998, Lindström et al. 2004). In addition, the low storage temperature is important to ensure the stability of the sensory quality of roe. Inhibition of lipid hydrolysis is the main way to ensure sensory quality of roe products stored for an extended time period. Enzymatic lipid hydrolysis is effectively retarded by either freezing or pasteurisation (Kaitaranta 1982). In addition, vacuum packaging is essential in minimizing the oxidative rancidity of lipids. The lipid hydrolysis was successfully avoided as the sensory quality of all the roe samples remained good after a six month storage period. Pasteurisation offers a feasible choice for the production of safe, good quality rainbow trout roe products for consumers.

6.4 *Listeria monocytogenes, Listeria* spp. and surface hygiene in fish processing factories (IV)

L. monocytogenes and *Listeria* spp. were found on surfaces of one-third and two-thirds of the factories, at least sporadically. Many important process surfaces were contaminated with *Listeria* spp. The washing and cleaning practices in most (26/28) of the studied fish factories were inadequate and the cleaned process surfaces were contaminated with aerobic bacteria, enterobacteria and fungi. The *Listeria* spp. positive surfaces contained increased (> 1.8 CFU/cm²) numbers of aerobic bacteria in 71% of the samples. In comparison to another fish industry study in which 94% of the *Listeria* spp. positive surface samples were contaminated with aerobic bacteria (Aarnisalo et al. 1998). Surface samples having increased amount of enterobacteria or aerobic bacteria, were two and one and a half times more likely contaminated with *Listeria* spp. than samples with increased ATP levels or fungi amount. None of the studied hygiene monitoring methods or microbes was an effective indicator of the presence of *Listeria* spp. on the surfaces. The presence of *Listeria* spp. on the factory surfaces was, however, an indicator of its increased possibility to be found in the fish products. In factories where *Listeria* spp. was found on surfaces it was often found (10/13) in some products too. The contamination of different ready-to-eat fish products with *L. monocytogenes* was the same level (0 to 20%) as previously found in Finland (Johansson et al. 1999).

The contact plate surface detection method for *Listeria* spp. was easy to perform and preliminary results were obtained rapidly. On the other hand, the effectiveness and reliability of the contact plate method was low as the sampled area was small (25.5 cm²) and the stressed *Listeria* spp. cells from the washed surfaces may have suffered from the selective compounds of the agar. Disruptive microbial populations, due to the lack of enrichment stages, also complicated the identification of *Listeria* spp. colonies.

6.5 Sources of *Listeria monocytogenes* in fish farming (I, V)

During the three year follow-up period, the primary routes of *L. monocytogenes* contamination of the fish farm were clarified. The fish were contaminated with *L. monocytogenes* in the costal farm area, as the fish farmed further out to sea were *Listeria*-free, but became heavily contaminated in the costal farm area. The thorough contamination of the fish lot occurred within a four-week period when the fish lot was kept in the coastal area before sampling. Interestingly, the typing results revealed that the two *L. monocytogenes* PFGE-types found in the fish samples during the follow-up were different from the *L. monocytogenes* PFGE-types isolated from the water environment at the fish farm. The

brook and river waters, as well as other runoff waters, seemed to be the main contamination source at the fish farm, regardless of the fact that the typing did not reveal any place to be the main *L. monocytogenes* source. Twice the same *L. monocytogenes* PFGE-type was found in the brook and sea waters (Table 11) at the same time, and once *L. monocytogenes* was found in the river water, but not in the nearby sea water. *Listeria* spp. was also found in the river and brook waters, but not in sea water samples taken at the same time. The results by Colburn et al. (1990) support this conclusion, as they found that the freshwater tributaries draining into Humboldt-Arcata Bay were consistent sources of *Listeria* spp. The primary *L. monocytogenes* source contaminating the river and brook waters was not extensively studied. There was no specific source, like agriculture or cattle, that could be suspected of direct contamination of water. On the other hand, it is possible that *L. monocytogenes* may naturally be present, at least sometimes, in the soil of a river or brook, as it is frequently found in soil and water environments (Weis and Seeliger 1975, Ben Embarek 1994, Schaffter and Parriaux 2002). The widespread presence of *L. monocytogenes* in soil has often been attributed to contamination from decaying plant and faecal material, with damp surface soil providing a cool, moist protective environment and the decaying material the substrate, which together enable the survival of *L. monocytogenes* from season to season (Fenlon 1999). This may be the case, to some degree, with *L. monocytogenes* in wet soil environments like river, brook and sea bottom soils.

Weather conditions had a strong influence on the probability of finding *L. monocytogenes* and other *Listeria* spp. in the fish farm environment (Fig. 1). The number of samples contaminated with *Listeria* spp. was typically larger after rainy periods. The occurrence of *L. monocytogenes* and *Listeria* spp. in samples decreased, however, during dry periods. In addition, it was also noted that when sampling was performed immediately after a few days of rain showers during a relatively dry period the same *L. monocytogenes* PFGE-type was found in the brook and sea water. This suggests that the *L. monocytogenes* contamination in a water environment is a phenomena occurring rapidly if a suitable source is present in the environment and the weather conditions are favourable, but disappearing soon if no further contamination arrives.

The original *L. monocytogenes* contamination in fish gradually disappeared. The time needed for this was several months. Such disappearance was faster in sea water than in fish. Hsu et al. (2005) showed that *L. monocytogenes* does not readily survive in sea water and does not compete well with microbial marine population at elevated temperatures (22 °C) in the

presence of organic material. At temperatures below 11 °C, *L. monocytogenes* lost viability throughout storage but was detectable after six days of incubation (Hsu et al. 2005). The soil samples often contained *L. monocytogenes* PFGE-types found earlier in other sample types, even 18 months after the first discovery. It is possible that the *L. monocytogenes* strains did not survive in the soil for the entire period, but reappeared via e.g. brook water. It has, however, been shown earlier that many bacteria including *L. monocytogenes* survive longer in sediments than in water (Botzler et al. 1974, Burton et al 1987). The difference in the occurrence of *Listeria* spp. in different sea bottom soil samples was clear. No *Listeria* spp. was found from the deeper sea areas whereas all inshore samples taken at the same time contained *L. monocytogenes* or other *Listeria* spp.

It has been established that cattle contribute to the amplification and dispersal of *L. monocytogenes* in a farm environment and that ruminant farm ecosystems maintain a high prevalence of *L. monocytogenes* (Nightingale et al. 2004). This fish farm did not seem to be contributing to the maintenance of the amplification and dispersal of *L. monocytogenes* in the water environment. On the contrary, the fish did not become contaminated easily and only two out of twelve *L. monocytogenes* strains found from the farm were also found in fish. In addition, the viscera of rainbow trout did not contain *L. monocytogenes* at this fish farm and overall the viscera were contaminated only once with *L. monocytogenes* in all the studied rainbow trout samples (Table 10). After gavage ingestion of live *L. monocytogenes* cells by salmon no *L. monocytogenes* were detected after three and seven days in the fish stomachs (Hsu et al. 2005). Thus, fish do not spread and increase the environmental contamination with *L. monocytogenes* contaminated faeces, since the bacterium is not persistent in fish. The fish farm studied did not spread *Listeria* contamination, but on the contrary suffered from *L. monocytogenes* contamination from outside sources like the brook water.

7 CONCLUSIONS

1. The prevalence of *L. monocytogenes* in pooled unprocessed fresh rainbow trout was on average 15%. In the individual thawed fish samples were found a prevalence of 9% for *L. monocytogenes*. The prevalence of *L. monocytogenes* varied greatly between different fish farms: from 0 to 100% in pooled samples and from 0 to 75% in individual fish samples. The location of *L. monocytogenes* in different parts of the raw fish material differed significantly ($p < 0.0001$) in rainbow trout. Rainbow trout contaminates with *L. monocytogenes* almost exclusively in the gills and only sporadically in the skin and viscera. Up to 96% of the *L. monocytogenes* positive samples were gill samples and only 4% of the *L. monocytogenes* positive samples were skin or viscera samples. Special effort should be focused on the isolation and removal of the gills of rainbow trouts before the *L. monocytogenes* contamination spreads further.

2. The presence of *L. monocytogenes* in different Finnish fish species roe products in the retail market varied from 2 to 8%. Especially fresh-bought roe products contained significantly ($p < 0.01$) more often *L. monocytogenes* (18%) than frozen and frozen-thawed roe products (0.9%). The microbial quality of the roe samples was poor in relation to aerobic and coliform bacteria in 57% and 73% of the samples, respectively, plus 20% of the roe samples were unacceptable to taste.

3. Based on the determined D- and z-values for four *L. monocytogenes* strain mixture the performed pasteurisations at 62 °C and at 65 °C for 10 min theoretically destroyed 46 to 154 log units of *L. monocytogenes* cells in rainbow trout roe. The experimental pasteurisations of *L. monocytogenes* inoculated rainbow trout roe samples, carried out at 62 °C and at 65 °C for 10 min, destroyed 8 log units of *L. monocytogenes,* which has shown to be the highest possible *L. monocytogenes* cell count to grow in roe. The experiments performed showed that the quality of pasteurised vacuum packaged rainbow trout roe was consistently good regards to of both microbial and sensory quality for up to 6 months when stored at 3 °C. Pasteurisation offers a feasible choice for the production of safe, good quality rainbow trout roe products for consumers. The storage temperature of the pasteurised roe, however, has to be below 3 °C to prevent the formation of toxins, especially as there is a risk for the growth of group II *C. botulinum* spores.

4. *L. monocytogenes* and *Listeria* spp. were found on surfaces of one-third and two-thirds of the fish factories at least sporadically. The surface contact agar and ATP-based techniques were not effective indicators of the presence of *Listeria* spp. on the surfaces. The presence of *Listeria* spp. on the factory surfaces was, however, an indicator of its increased possibility to be found in the fish products. In factories where *Listeria* spp. was found on surfaces it was often (10/13) found in some products. *L. monocytogenes* contamination level of different ready-to-eat fish products varied from 0 to 20%.

5. The brook and river waters, as well as other runoff waters from environment, were the main contamination sources of *L. monocytogenes* in the studied fish farm. Weather conditions had a strong influence on the probability of finding *L. monocytogenes* and *Listeria* spp. in the fish farm environment and in fish. The number of samples contaminated with *L. monocytogenes* and *Listeria* spp. was often greater after rainy periods. The occurrence of *L. monocytogenes* and *Listeria* spp. in samples decreased during dry periods. The *L. monocytogenes* contamination in fish gradually disappeared over several months. Such disappearance, however, was faster in the surrounding sea water than in the fish. Presence of certain *L. monocytogenes* PFGE-types, after the first discovery months earlier in some other sample type, was typical for sea bottom soil samples. The two *L. monocytogenes* PFGE-types found in fish were different than the *L. monocytogenes* PFGE-types found elsewhere in the fish farm environment. The fish farm studied did not spread *L. monocytogenes* contamination, but on the contrary suffered from *L. monocytogenes* contamination from environmental sources.

6. *L. monocytogenes* PFGE-types, isolated from raw fish and from fish production environments, were found also among final fish products. Raw fish materials and production environment and machines are sources of *L. monocytogenes* contamination that both need to be monitored.

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