Pseudomonas anguilliseptica from lumpfish (*Cyclopterus lumpus*) in Norwegian aquaculture – phylogenetic analysis and infection challenge

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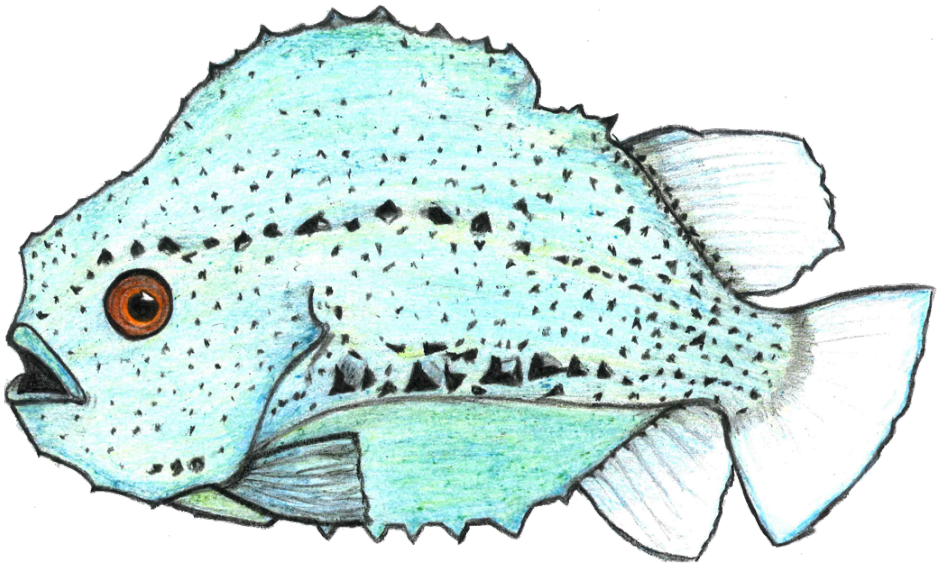

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Front cover motive: *Cyclopterus lumpus*, designed by Kristine Rokke

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"Give someone a truth, they will think for a day. Teach someone to reason, and they will think for a lifetime" ~ Dr. Phil Plait

Bergen, June 2019 Even Bysveen Mjølnerød

Abbreviations

ALO	arginine, lysine and ornithine
aroE	shikhimate dehydrogenase
atpD	ATP synthase F1 beta subunit
BA	blood agar
BA w/2% NaCl	blood agar with 2% added sodium chloride
carA	carbamoyl-phosphate synthase small chain
cfu	colony forming units
DDH	DNA-DNA hybridization
DNA	deoxyribonucleic acid
DPC	days post challenge
ERIC-PCR	Enterobacterial Repetitive Intergenetic Consensus-PCR
FBS	fetal bovine serum
g	grams
glnS	glutaminyl-tRNA synthetase
gyrB	DNA gyrase beta subunit
HK	house keeping
ileS	isoleucyl-tRNA synthetase
i.m.	intramuscular
in vivo	"within the living"
i.p.	intraperitoneal
L	liters
LD	lethal dose
LPS	lipopolysaccharides
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
µl	microliters
MLSA	Multi-Locus Sequence Analysis
MLST	Multi-Locus Sequence Typing
NaCl	sodium chloride
NCBI	The National Center for Biotechnology
NCIMB	National Collections of Industrial, Marine and Food Bacteria
N/A	not applicable
NVI	Norwegian Veterinary Institute
NVIB	Norwegian Veterinary Institute in Bergen
NVIO	Norwegian Veterinary Institute in Oslo
OD	optical density
O/F	oxidative/fermentative
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RAPD	Random Amplification of Polymorphic DNA
recA	recombinase A
RPM	rounds per minute

RNA	ribonucleic acis
tRNA	transfer RNA
rpoB	RNA polymerase beta subunit
rpoD	RNA polymerase, sigma factor
TSA	tryptic soy agar
TSB	tryptic soy broth
ST	sequence type
TN93	Tamura-Nei
UiB	University of Bergen
VIE	visible implant elastomer
VNTR	Variable-Number of Tandem Repeats
WGS	Whole-Genome Sequencing

Abstract

The large-scale production and use of lumpfish (*Cyclopterus lumpus*) as a biological delousing agent in Norwegian aquaculture presents novel pathogenic issues. The bacterium *Pseudomonas anguilliseptica* has in recent years emerged as a serious threat to production of lumpfish in Norway. Little is known about the population structure of this bacterium despite its association with disease in a wide range of different fish species throughout the world. The phylogenetic relationships between 53 isolates, primarily derived from lumpfish, were reconstructed by Multi-Locus Sequence Analysis (MLSA) using nine housekeeping genes (*rpoB, atpD, gyrB, rpoD, ileS, aroE, carA, glnS* and *recA*). An infection challenge was also conducted to investigate the pathogenic nature of the bacterium associated with disease in lumpfish. Intraperitoneal (i.p.), intramuscular (i.m.) and cohabitant (exposure of naïve fish to horizontal infection from i.p. and i.m. challenged fish) challenge models were established utilizing an isolate of epidemiological relevance for Norwegian lumpfish production.

MLSA analysis revealed a high degree of relatedness between the studied isolates, yet ultimately described three major phylogenetic clusters consisting of seven different genotypes. While four genotypes were identified amongst Norwegian lumpfish isolates, the majority of isolates, irrespective of geographic origins belonged to a single genotype. This suggests the existence of a dominant genotype associated with disease in production of lumpfish in Norwegian aquaculture. The observed mortality amongst both i.p/i.m and näive cohabitant fish suggests that the bacterium constitutes a 'primary' pathogen in lumpfish. Elucidation of the population structure, and the pathogenic nature of the bacterium associated with production of lumpfish in Norway, has provided valuable information of relevance for future vaccine development.

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1. Introduction:

1.1 Cleaner fish production

Sea lice infestation by *Lepeoptherius salmonis* and *Caligus elongatus* currently represents the biggest economic and fish health related issue in Norwegian production of Atlantic Salmon (*Salmo salar*) (Brooker et al., 2018; Powell et al., 2018). Treatments against sea lice infestations cost the Norwegian aquaculture industry approximately 5 billion Norwegian kroner (NOK) and accounted for 4 NOK/kg of the harvested fish in 2016 (Iversen et al., 2017). Recent increase in production costs are mainly a consequence of the sea lice's increased resistance to chemotherapeutants (Jansen et al., 2016) and the implementation of alternative treatment methods.

In recent years, non-chemical treatments have been developed and increasingly applied in an effort to combat multi-resistant sea lice. Mechanical (i.e. brushing and water jetting), thermic (temperate sea water) and freshwater treatments are today utilized to a greater extent than chemotherapeutants (Hjeltnes et al. 2018). Although most of these treatments have proven effective in de-licing large numbers of fish within a relative short time frame, there are issues regarding fish welfare as well as purely economic issues. Currently, the least stressing and cost-efficient preventive measure against sea lice infestation is the use of cohabiting cleaner fish species that graze sea lice from the salmon.

Commercial fishing for labrid fish species such as ballan wrasse (*Labrus bergylta*), goldsinny wrasse (*Ctenolabrus rupestris*) and corkwing wrasse (*Symphodus melops*) became widespread due to the fish's ability to reduce sea-lice prevalence within net pens. The ever increasing demand for cleaner fish and the requirement for harvesting wild populations in a sustainable manner (Skiftesvik et al., 2014) facilitated the need for commercial production of cleaner fish. However, due to the fact that wrasse species enter a state of winter dormancy in which they do not feed below 6°C (Sayer and Reader, 1996), alternative cleaner fish species that remain active at low temperatures have become the main focus for commercial production of cleaner fish (Powell et al., 2018).

Lumpfish (*Cyclopterus lumpus*) was introduced as a promising alternative to wrasse species as a biological delousing agent. Imsland et al., (2014) demonstrated its efficiency at grazing pre-mature and mature stages of sea lice on cohabiting Atlantic salmon. Unlike the winter dormancy

observed among wrasse species at cold temperatures, Nytrø et al., (2014) described the lumpfish's ability to feed and maintain its metabolic rate at temperatures down to 4°C. At optimal rearing temperatures (approximately 15°C) the growth rate of lumpfish exceeds that of wrasse such that they can be deployed in salmon farms 4 months post hatch, whereas ballan wrasse usually require 1.5 years before deployment (Helland et al., 2013). Due to these properties, lumpfish is now the most commonly used cleaner fish and the second most produced fish in Norwegian aquaculture, after Atlantic salmon (Hjeltnes et. al. 2018).

1.2 Bacterial diseases associated with production of lumpfish

The widespread use of cleaner fish for control of sea lice infestations in Norwegian aquaculture presents novel virologic, parasitic and bacteriological issues. Since the start of production of farmed lumpfish, the most prevalent bacterial species associated with disease outbreaks have been *Vibrio anguillarum* (Marcos-López et al., 2013), atypical *Aeromonas salmonicida* (Rønneseth et al., 2017)*, Moritella viscosa, Tenacibaculum* spp., and *Pasteurella* sp. (Alarcón et al., 2016; Ellul et al., 2018). In recent years, *Pseudomonas anguilliseptica* has emerged as an additional and serious threat to lumpfish production in Norway (Alarcón et al., 2016) and the number of outbreaks in this fish species is currently increasing annually (figure 1) (Walde et al., 2019). In fact, these figures are most certainly underestimates as the disease is not notifiable and these are limited to cases investigated by the Norwegian Veterinary Institute (NVI) only. *P. anguilliseptica* associated mortalities have also been reported in lumpfish farmed in Scotland (Treasurer and Birkbeck, 2018), Ireland, Iceland and the Faroe Islands (D. Colquhoun pers. comm).

Table 1: Total numbers of outbreaks of selected diseases/agents associated with production of cleaner fish in Norway from 2012-2018. Data registered by the Norwegian Veterinary Institute (Walde et al., 2019).

Fish species	**Disease/agent**	**Number of positive sites per year**						
		2012	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**
Lumpfish	Atypical *Aeromonas salmonicida*	1	8	5	51	27	24	20
	Classical *Aeromonas salmonicida*	0	0	0	1	4	0	0
	Pasteurella sp.	1	16	8	14	28	23	14
	Pseudomonas anguilliseptica	0	0	1	4	8	15	17
	Vibrio anguillarum	7	6	8	12	12	7	7
	AGD	0	0	2	2	8	2	4
Wrasse	Atypic *Aeromonas salmonicida*	12	13	16	32	18	14	13
	Vibrio anguillarum	6	6	6	2	2	2	3
	Pseudomonas anguilliseptica	0	0	0	0	0	1	0
	AGD	0	5	2	2	1	1	0

1.3 *Pseudomonas anguilliseptica*

Pseudomonas anguilliseptica is an opportunistic pathogen primarily affecting fish in marine and brackish environments (Wiklund, 2016). It was originally described in 1971 as the causative agent of "red spot disease" (sekiten-byo) from pond cultured Japanese eel (*Anguilla japonica*) (Wakabayashi and Egusa, 1972). The bacterium was in 1981 isolated from European eel (*Anguilla anguilla*) in Scotland (Nakai and Muroga, 1982), and thereafter became a frequently occurring pathogen associated with eel farming throughout Europe. Initially the bacterium was considered to be exclusively associated with disease in eel culture (Wiklund, 2016). However, the bacterium displays little or no host specificity as it has shown to be pathogenic in a range of cultured and wild fish species from different geographic origins (Table 2).

Table 2: Species of wild caught and cultured fish shown to develop disease due to infections of *P. anguilliseptica.*

Species		Geographic origin	Reference
Japanese eel	*Anguilla japonica*	Japan	(Wakabayashi and Egusa, 1972)
European eel	*Anguilla anguilla*	Scotland	(Nakai and Muroga, 1982)
Ayu	*Plecolossus altivelis*	Japan	(Nakai, Hanada & Muroga 1985)
Atlantic salmon	*Salmo salar*	Finland	(Wiklund & Bylund 1990)
Sea trout	*Salmo trutta*	Finland	(Wiklund & Bylund 1990)
Whitefish	*Coregonus* sp.	Finland	(Wiklund & Bylund 1990)
Rainbow trout	*Onchorhynchus mykiss*	Finland	(Wiklund & Bylund 1990)
Baltic herring	*Clupea harengus*	Baltic sea	(Lonnstrom et al., 1994)
Sea bass	*Dicentrarchus labrax*	France	(Berthe, Michel & Bernardet 1995)
Turbot	*Scophthalmus maximus*	Spain	(López-Romalde et al., 2003a)
Gilthead seabream	*Sparus aurata*	France, Spain	(Doménech et al. 1999; Berthe, Michel & Bernardet 1995)
Cod	*Gadus morhua*	UK/Canada, Scotland	(Ferguson et al. 2004; Balboa et al., 2007)
Striped beakperch	*Oplegnathus fasciatus*	Korea	(Kim et al. 2011)
Lumpfish	*Cyclopterus lumpus*	Norway	(Alarcón et al. 2016)

P. anguilliseptica is an obligate aerobe, gram negative, motile and slim rod-shaped bacterium. It produces small pin-head sixed colonies on a range of general-purpose media (i.e. blood agar (BA), with and without added salt (2% NaCl), marine agar, and tryptic soy agar (TSA), as well as on Ordals medium. Incubation is usually performed at 15 or 22°C (Balboa et al., 2007; Michel et al., 1992) with colonies starting to appear after 3-4 days, depending on temperature. In mixed cultures, colonies of *P. anguilliseptica* are easily overgrown by more rapidly multiplying bacteria. The bacterium is cytochrome oxidase and catalase positive, as well as

having a low metabolic reactivity for most carbohydrates (Wiklund, 2016).

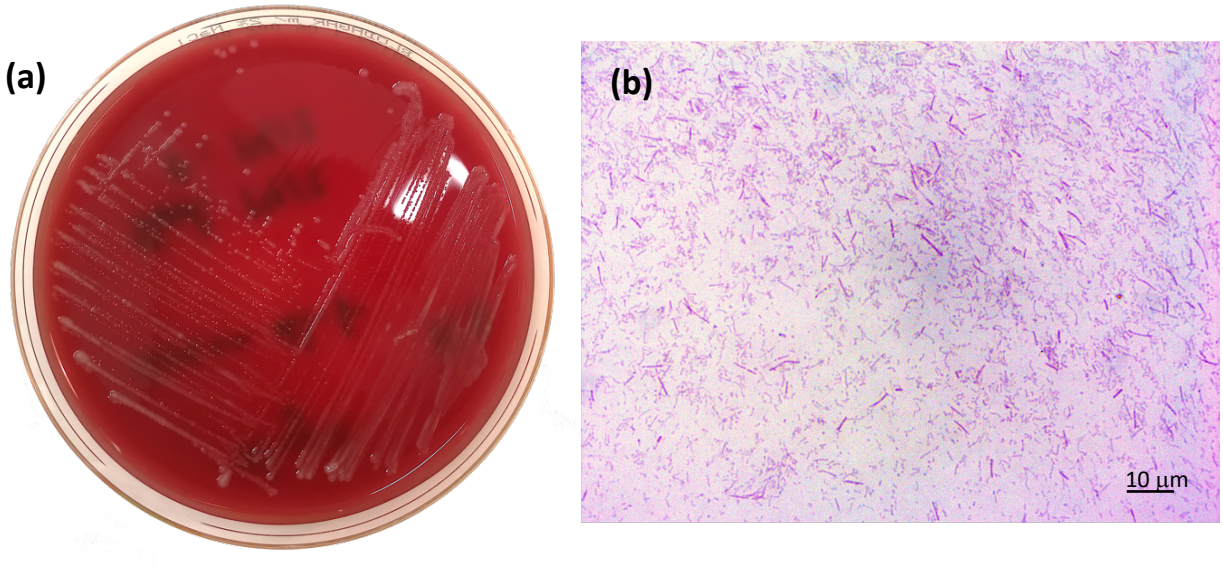


Figure 1: Pure culture displaying colony morphology of *P. anguilliseptiga* on blood agar (2% NaCl) (a) and gram staining of the bacterium (100X) (b).

López-Romalde et al., (2003a) conducted a phenotypic and genetic characterization of 32 isolates of *P. anguilliseptica* from seabream and turbot in Spain. By comparing those with a further 18 isolates originating from different geographic localities, fish species and time of isolation, they described intraspecific heterogeneity. Phenotypic analyses revealed a great degree of homogeneity with few discrepancies compared with previously published characteristics (Doménech et al., 1997; Lonnstrom et al., 1994; Wiklund and Bylund, 1990). DNA fingerprinting by Random Amplification of Polymorphic DNA (RAPD) did however describe two major genetic groups. The groups were almost exclusively divided between eel isolates and every other isolate included in the study (López-Romalde et al., 2003a).

A complementary study was later published by the same authors regarding the serological characteristics of the same collection of isolates (López-Romalde et al., 2003b). Through techniques such as slide agglutination, microagglutination and dot blot utilizing antisera raised against an isolate from Japanese eel (CECT899) and turbot (TW-P19), they managed to differentiate the isolates into two distinct groups consistent with the previous genetic analysis. Analyzing the lipopolysaccharides (LPS) of the isolates demonstrated a difference in O-antigens, thus proposing the existence of two different "O" serotypes (O1 and O2). As for the genetic characteristics, the separate serotypes were almost exclusively divided between eel isolates (O2) and all other isolates included in the analysis (O1).

The concordance observed between the genetic and serologic characteristics culminated in the proposal of two separate clonal lineages for the bacterium (Romalde, 2003). This hypothesis

was further supported by additional genetic analyses by Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) and Repetitive Element Palindromic-PCR (REP-PCR) (López Romalde, 2005). In addition to supporting the existence of two clonal lineages, these genetic techniques demonstrated high discriminating power within these groups. Three genetically distinct groups were established on the basis of these methods of intergenetic DNA fingerprinting (López Romalde, 2005).

Clinical cases of infections involving *P. anguilliseptica* are generally characterized as hemorrhagic septicemias (Berthe et al., 1995; Wakabayashi and Egusa, 1972; Wiklund and Bylund, 1990), though lesions and clinical signs vary between fish species. One of the most common external signs of disease are petechial hemorrhages in the skin, predominantly on the ventral parts of the fish. Eye lesions such as punctures of the cornea (Lonnstrom et al., 1994), keratitis (Berthe et al., 1995) and exophthalmos are observed in several cases. Subcutaneous hemorrhages are also reported from head and mouth regions as well as around the vent among salmonid species (Wiklund and Bylund, 1990). Petechial hemorrhages in the peritoneum and occasional hemorrhages within the liver and adipose tissue of visceral organs are usual findings upon necropsy (Wiklund, 2016). Abdominal distension due to accumulation of ascitic fluid in the abdomen and enlargement of the spleen are typical lesions associated with gram-negative septicemias, also observed in most infections with *P. anguilliseptica* (Berthe et al., 1995; Michel et al., 1992).

Few descriptions have been made regarding the histopathological changes caused by the bacterium (Magi et al., 2009). Histopathological analysis from clinical outbreaks has so far been limited to diseased European and Japanese eel (Ellis et al., 1983; Miyazaki and Egusa, 1977), trout, whitefish (Wiklund and Bylund, 1990), and Atlantic cod (Ferguson et al., 2004). Magi et al., (2009) conducted one of the first extensive infection challenges administrating *P.* anguilliseptica as the infectious agent with aims of describing the pathology induced by the bacterium in turbot, comparing those of previously published studies on naturally infected fish species.

Through intraperitoneal (i.p.) administration of the bacterium, Magi et al., (2009) was able to reproduce clinical disease and pathology associated with systemic infections involving *P. anguilliseptica*. Diffuse multifocal necrosis within the gut, spleen and kidney were highlighted as the most histologically prominent lesions. Bacterial aggregates were present in all areas of the circulatory system examined, confirming the systemic nature of the infection. Magi et al.,

(2009) concluded that the histopathological changes induced by the bacterium under controlled laboratory conditions were similar to those reported from other fish species naturally infected with *P. anguilliseptica*. However, recommendations were made for additional challenge trials studying infection routes replicating natural conditions (i.e. cohabitation, bath challenge plus stressors) in order to confirm this hypothesis.

The pathological changes associated with infections of *P. anguilliseptica* in lumpfish have so far not been described in detail. Nor have there been attempts to fulfill Koch's postulates and reproduce the pathology observed in clinical cases by conducting a challenge study on this fish species.

1.4 Genetic characterization

Pseudomonas anguilliseptica is a member of the *Pseudomonas* genus which is one of the largest and most complex bacterial genera known (Mulet et al., 2012). The first phylogenetic description of the genus was conducted by Migula in 1894 by characterizing the different morphological properties of its members (Mulet et al., 2012). Since then, the genus has been subject to several revisions due to the development of new taxonomic methodologies. Modern bacterial classification now relies heavily upon molecular techniques for genotypic characterization.

1.4.1 16S rRNA

16S rRNA sequencing and DNA-DNA hybridization (DDH) have for a long time been considered the "gold standards" for determining and assigning bacterial species to their respective genera (Martens et al., 2008). The 16S rRNA gene is still a widely used phylogenetic marker as Stackebrand & Goebel (1994) showed its high resolving power for measuring relationship between organisms above the species level (Glaeser and Kämpfer, 2015; Martens et al., 2008).

Among the gene's desirable properties for basic phylogenetic analysis is its evolutionary conservation. It's essential function in protein synthesis across both *Bacteria* and *Archaea* makes the gene less susceptible to genetic mutation (Glaeser and Kämpfer, 2015), as such might have fatal consequences for the organisms viability. Despite its high degree of evolutionary conservation, the gene still displays sufficient sequence variation to discriminate between taxa (Glaeser and Kämpfer, 2015). However, the resolution of 16S rRNA sequencing renders the

gene insufficient as a phylogenetic marker at the intraspecific level. Several studies conducted on different species of bacteria have revealed nearly identical 16S rRNA gene sequences (≥99%) (Amann et al., 1992; Jaspers and Overmann, 2004; Sullivan et al., 1996). An additional disadvantage with this methodology is the presence of several *rrn* operons within single genomes. Combined with the known occurrence of horizontal gene transfer between distantly related *rrn* operons, this may cause sequences heterogeneity not necessarily reflecting the true evolutionary distance between species (Acinas et al., 2004).

1.4.2 Multi-Locus Sequence Analysis/Typing (MLSA/MLST)

In contrast to the genes encoding ribosomal sub-units such as 16S rRNA, protein-coding genes evolve at a slow but constant rate making them suitable markers for phylogenetic analysis with higher resolution (Glaeser and Kämpfer, 2015). Single gene sequence-based phylogenies do not however provide a sufficient representation of the phylogenetic relationship between microorganisms (Glaeser and Kämpfer, 2015). Due to the bias caused by possible distorting effects of recombination at a single loci, it has been regarded as necessary to include multiple protein-coding genes in providing a representative illustration of the taxonomic relationship between microorganisms (Gevers et al., 2005). The application of a phylogenetic analysis based on internal fragments of multiple protein-coding genes is called Multi-Locus Sequence Analysis (MLSA), a term first proposed by Gevers et al., (2005). Its promise as a measure for delineation of genomic relationships at the inter- and intraspecific level was highlighted by the ad hoc committee for re-evaluation of the species definition (Stackebrandt et al., 2002). Since then, MLSA has been a widely accepted and applied tool in creating an extensive overview of the evolutionary relationship between prokaryotes above and below the species level (Martens et al., 2008).

Certain criteria were emphasized by the ad hoc committee regarding the genes to be included in MLSAs in order to develop a global and reliable phylogenetic database (Stackebrandt et al., 2002). Genes subject to sequencing need to be ubiquitous within the actual taxon, present in a single copy as well as distributed at distinct chromosomal loci (Martens et al., 2008). Genes with high mutation frequencies where recombination might pose a selective advantage such as virulence genes should be avoided (Gevers et al., 2005). Instead, conserved protein-coding genes that serve an indispensable function in the organism, termed housekeeping (HK) genes, are desirable as they are thought to reflect the "true" evolutionary relationship between microbiological taxa (Glaeser and Kämpfer, 2015). Stackebrandt et al. (2002) proposed that a

minimum of five HK genes should be included in a MLSA.

The most common approach has been to amplify and sequence internal fragments of 5-12 loci, depending on the resolving power required (Glaeser and Kämpfer, 2015; Maiden, 2006; Martens et al., 2008). The correlation between number of HK genes included and the resolution achieved is however disputed (Glaeser and Kämpfer, 2015). Phylogenetic relationships are measured by concatenating sequences of the genes in question and subsequently subjecting the concatenated sequences to phylogenetic analysis using a suitable evolutionary model (Gevers et al., 2005). Compared to 16S rRNA sequencing and DDH, MLSA has proven superior for identification purposes being capable of discriminating between several taxonomic levels including species and sub-species levels (Gevers et al., 2005; Martens et al., 2008).

The fact that bacterial genomes acquire homologous sequences through horizontal gene transfer renders MLSA somewhat vulnerable to displaying genetic differences not necessarily reflecting the evolutionary relationship between bacteria (Maiden et al., 2013). Maiden et al., (1998) proposed an analytic approach by applying allelic differences, Multi-Locus Sequence Typing (MLST), rather than nucleotide sequences to overcome the possible distorting effects of horizontal gene transfer. In general, recombination by horizontal transmission generate more pronounced genetic polymorphism than single point mutations (Didelot and Maiden, 2010). MLST was originally designed to accommodate such distorting effects by ascribing each allelic difference as a genetic event. Doing so reduces the phylogenetic resolution but eliminates the discrimination between recent point mutations and polymorphism due to recombination (Maiden et al., 1998). MLST also provides a valuable tool to identify specific bacterial strains of epidemiological relevance within certain species (Maiden et al., 1998). Combined, MLSA and MLST constitute valuable analytic tools for determining the phylogenetic relationship and population structure of bacteria at and below the species level.

Intraspecific genotyping of bacterial pathogens associated with emerging diseases are essential for determining a range of epidemiological aspects related to disease outbreaks (Olive and Bean, 1999). Typing of isolates originating from clinical infections enable us to recognize future outbreaks, describe potential differences related to geographic or host specificity, as well as identifying virulent strains of a particular bacteria (Olive and Bean, 1999). These aspects are combined with virulence studies conducted under controlled laboratory conditions among the most important fundaments for vaccine development (Knappskog et al., 2014).

1.5 Vaccine development

Vaccines represents the most important preventive measure against some of the most prevalent and serious pathogens in Norwegian aquaculture. They have provided a vital tool for preventing bacterial diseases and reduced the application of antibacterial therapeutants to a minimum (Gudding, 2014) (figure2).

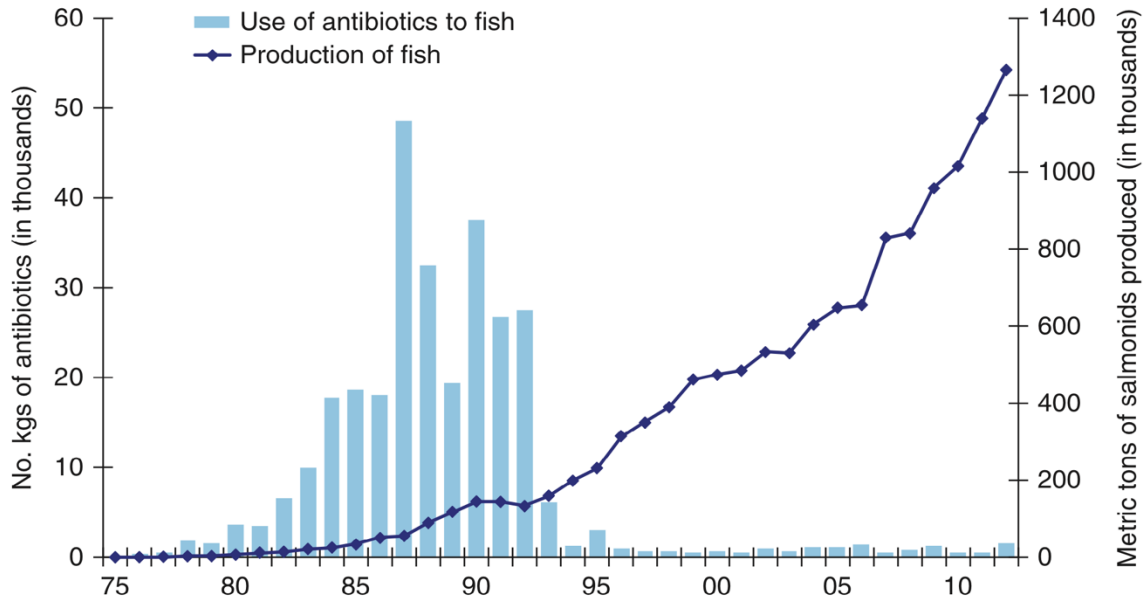


Figure 2: The development in usage of antibiotics in relation to the metric tons of salmonids produced in Norwegian aquaculture during the past decades (Gudding, 2014).

The historic efficiency of vaccines in today's modern aquaculture has made them a field of priority in research on emerging diseases (Sommerset et al., 2005). Sustainability in intensive aquaculture of salmonids is today completely dependent on potent vaccines. Likewise, the introduction and comprehensive use of cleaner fish species demands vaccination programs for novel species and pathogens in order for the industry to remain sustainable (Gulla et al., 2015).

Although knowledge regarding losses of deployed cleaner fish is scarce, bacterial infections are often described as either direct or indirect causes of the elevated mortality observed among cleaner fish (Nilsen et al., 2014). The widespread usage of lumpfish and the emerging bacterial diseases associated with them has facilitated the need for vaccine development. This will be essential if sustainable production and an increased fish welfare is to be achieved. Furthermore, bacteria associated with disease in lumpfish that demonstrate low host specificity could potentially pose a threat to cohabiting salmonids in commercial net pens. Among the bacterial

agents which have been proposed as potential hazards of cross-species transmission is *P. anguilliseptica* (Rimstad et al., 2017).

Understanding the population structure of pathogenic bacteria is therefore crucial for selecting suitable candidate strains for vaccine development (Gulla et al., 2015). Once a representative strain of a bacteria is selected and the pathogenic effects documented, vaccine development can begin (Knappskog et al., 2014). The latter is usually determined by *in vivo* experiments exposing naïve fish to the actual bacteria. Thus, the relationship between pathogen and disease can be established by fulfilling Koch's postulates.

1.6 Koch's postulates

A fundamental requirement for demonstrating that a pathogenic organism is responsible for the observed disease is the fulfillment of Koch's postulates (Byrd and Segre, 2016). Physician and bacteriologist Robert Koch proposed in the late 1800's a set of postulates to serve as guidelines for evaluating the causation of infectious diseases (Blevins and Bronze, 2010). Outlined and published by Loeffler (1884), the following postulates remain to this day the basis for etiological and pathogenetic characterization of microbes (Walker et al., 2006) (direct quotation):

1. *The organism must be shown to be invariably present in characteristic form and arrangement in the diseased tissue.*
2. *The organism, which from its relationship to the diseased tissue appears to be responsible for the disease, must be isolated and grown in pure culture.*
3. *The pure culture must be shown to induce the disease experimentally.*

4[a]. The organism should be re-isolated from the experimentally infected subject.

[a] Additional postulate formulated after Loeffler (1884).

1.7 Objectives

The recent development in numbers of disease outbreaks involving *P. anguilliseptica* in Norwegian farmed lumpfish has raised the need to further understand the genetic and pathogenic nature of this bacterium. Describing the different genotypes originating from clinical infections in Norwegian aquaculture is essential for the assessment of potential candidate strains for vaccine development. Likewise, documenting the pathogenic nature of the bacterium is a major prerequisite for evaluating the need for vaccine development.

The purpose of this study was primarily to perform a phylogenetic analysis by MLSA/MLST to describe the heterogeneity/homogeneity amongst Norwegian isolates of *P. anguilliseptica* from lumpfish. By including isolates originating from a range of different geographic localities, fish species and time of isolation we set out to reconstruct the phylogenetic relationship between all isolates included in this study. Combined with an infection challenge administrating the bacterium in naïve lumpfish in order to fulfill Koch's postulates, the aim was to shed light upon the possibilities and potential need for development of a vaccine against *P. anguilliseptica*.

2. Material and methods

2.1 MLSA

2.1.1 Bacterial isolates, storage and culture

A total of 55 isolates were included in the analysis. The isolates were sampled from a range of different geographic origins, fish species and time of isolation (table 3). All isolates were either archived or sent to the laboratories at NVI in Bergen on blood agar. Cryopreservation of the isolates was performed by inoculation of approximately 10 µl of bacterial cells to Pro-Lab Diagnostics Microbank vials containing a cryopreservative medium and glass beads. The material was mixed within the vial to achieve a 3-4 McFarland suspension before removing the cryopreservative by sterile disposable Pasteur pipettes. Samples were also inoculated into vials containing liquid Ordals medium with 25% fetal bovine serum (FBS) and 10% glycerol as a backup measure. Both sets of vials were stored at -80°C.

Table 3: Isolates of *Pseudomonas anguilliseptica* included in this study.

Isolate number	Year of isolation	Strain	Fish species	Origin of isolate	Source
NCIMB 1949[a]	1998	NCIMB 1949	European eel	Japan	NCIMB
NVIO 11299	2018		Lumpfish	Ireland	Dr. Felix Scholz
NVIO 11300	2018		Lumpfish	Ireland	Dr. Felix Scholz
NVIO 11301	2018		Lumpfish	Ireland	Dr. Felix Scholz
NVIO 11302	2018		Lumpfish	Ireland	Dr. Felix Scholz
NVIO 11303	2018		Lumpfish	Ireland	Dr. Felix Scholz
NVIO 9942	2015		Lumpfish	Faroe Islands	Dr. Debes Christiansen
NVIO 10973	2017		Lumpfish	Faroe Islands	Dr. Debes Christiansen
NVIO 11158	2017		Lumpfish	Faroe Islands	Dr. Debes Christiansen
NVIO 11159	2017		Lumpfish	Faroe Islands	Dr. Debes Christiansen
NVIO 11160	2017		Lumpfish	Faroe Islands	Dr. Debes Christiansen
NVIO 11161	2017		Lumpfish	Faroe Islands	Dr. Debes Christiansen
NVIO 11162	2017		Lumpfish	Faroe Islands	Dr. Debes Christiansen
NVIO 8180	2011		Lumpfish	Norway	NVI diagnostics
NVIO 8227	2012		Lumpfish	Norway	NVI diagnostics
NVIO 9976	2015		Lumpfish	Norway	NVI diagnostics
NVIO 10039	2015		Lumpfish	Norway	NVI diagnostics
NVIO 10341	2016		Lumpfish	Norway	NVI diagnostics
NVIO 10449	2016		Lumpfish	Norway	NVI diagnostics
NVIO 10550	2016		Lumpfish	Norway	NVI diagnostics
NVIO 10726	2016		Lumpfish	Norway	NVI diagnostics
NVIB 50-927	2015		Lumpfish	Norway	NVI diagnostics
NVIB 50-1353	2016		Lumpfish	Norway	NVI diagnostics
NVIB 50-1579	2016		Lumpfish	Norway	NVI diagnostics
NVIB 50-1705	2016		Lumpfish	Norway	NVI diagnostics
NVIB 50-1763	2017		Lumpfish	Norway	NVI diagnostics
NVIB 50-1825	2017		Lumpfish	Norway	NVI diagnostics
NVIB 50-1846	2017		Lumpfish	Norway	NVI diagnostics
NVIB 50-1895	2017		Lumpfish	Norway	NVI diagnostics
NVIB 50-1910	2017		Lumpfish	Norway	NVI diagnostics
NVIB 50-1914	2017		Lumpfish	Norway	NVI diagnostics
NVIB 50-1952	2017		Lumpfish	Norway	NVI diagnostics
NVIB 50-2015	2017		Lumpfish	Norway	NVI diagnostics
NVIB 50-2095	2017		Lumpfish	Norway	NVI diagnostics
NVIB 50-2040	2017		Lumpfish	Norway	NVI diagnostics
NVIO 11313	2018		Lumpfish	Norway	NVI diagnostics
NVIO 11370	2019		Lumpfish	Norway	NVI diagnostics
NVIO 8905	2013		Wolffish	Norway	NVI diagnostics
NVIB 50-2084	2017		Ballan wrasse	Norway	NVI diagnostics
NVIB 50-2255	1992	STR-6	Baltic herring	Baltic sea	Dr. Tom Wiklund
NVIB 50-2260	1992	STR2-1	Baltic herring	Baltic sea	Dr. Tom Wiklund
NVIB 50-2256	1991	P57B/91	Rainbow trout	Finland	Dr. Tom Wiklund
NVIB 50-2257	1993	0506-F05	Brown trout	Finland	Dr. Tom Wiklund
NVIB 50-2258	2000	P19/00	Atlantic salmon	Finland	Dr. Tom Wiklund
NVIB 50-2259	2008	P33-6/08	Whitefish	Finland	Dr. Tom Wiklund
NVIB 50-2262	2015	P17-3/15	Whitefish	Finland	Dr. Tom Wiklund
NVIB 50-2261	2009	P30-5/09	Rainbow trout	Finland	Dr. Tom Wiklund
NVIO 11214	2018	AZ/210-1	Turbot	Spain	Dr. Jesus Romalde
NVIO 11215	2018	AZ/210-2	Turbot	Spain	Dr. Jesus Romalde
NVIO 11216	2018	AZ/211-1	Turbot	Spain	Dr. Jesus Romalde
NVIO 11217	2018	AZ/211-2	Turbot	Spain	Dr. Jesus Romalde
NVIO 11219	2018	TW47/L1	Sea Bream	Spain	Dr. Jesus Romalde
NVIO 11220	2018	TW75/L3	Sea Bream	Spain	Dr. Jesus Romalde

[a] Type strain NCIMB 1949.

Phenotypic characterization, Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF) and 16S sequencing were performed to positively confirm the species identity of the isolates included in the MLSA. All 55 isolates were analyzed by MALDI-TOF while 16S rRNA sequencing was performed on 39 of the isolates to test the concordance between the two methods. Phenotypic characterization was conducted on a representative selection of six isolates (NVIO 10973, NVIO 8905, NVIO 11214, NVIB 50-2255, NVIB 50-1910 and NVIO 9976). Characteristics examined were: colony morphology, gram staining, motility, anaerobic respiration and the ability to cause hemolysis on blood agar. Biochemical properties such as oxidative/fermentative (O/F) production of acid from glucose, as well as the ability to produce enzymes (decarboxylases) which decarboxylate the amino acids arginine, lysine and ornithine (ALO), was also tested. An oxidase test was also performed to examine the bacterium's ability to produce certain cytochrome c oxidases.

2.1.2 Isolation of DNA template

Initial DNA template was isolated by making a suspension of approximately 1µl bacterial cells in 200µl of phosphate buffered saline (PBS) for each isolate. The suspensions were heated to 98°C for 20 minutes before being centrifuged at 16 000G for 3 minutes. The supernatant was transferred to a 1.5ml microtube and frozen at -23°C to be used as template for the following PCR assays. This way of isolating DNA template proved sufficient for amplification of 16S rRNA sequences. However, the quality of the template was not adequate for amplification of the HK genes. DNA template was thereafter isolated using the High Pure PCR Template Preparation Kit (Roche Applied Science, Penzberg, Germany) in accordance with its protocol. Concentration and purity of nucleic acid in the final template was assessed with the NanoDrop 2000 Spechtrophotometer (ThermoFisher Scientific, Waltham, USA). All samples were diluted to 2 ng/µl before being frozen at -23 °C.

2.1.3 Primer design:

The following housekeeping (HK) genes were included in the analysis: *rpoB* (RNA polymerase beta subunit), *atpD* (ATP synthase F1 beta subunit), *gyrB* (DNA gyrase beta subunit), *rpoD* (RNA polymerase, sigma factor), *ileS* (isoleucyl-tRNA synthetase), *aroE* (shikhimate dehydrogenase), *carA* (carbamoyl-phosphate synthase small chain), *glnS* (glutaminyl-tRNA synthetase), and *recA* (recombinase A).

In order to design primers for each of the nine housekeeping genes, homologous sequences from two species within the genus *Pseudomonas* were aligned with a reference strain of *P. anguilliseptica* (DSM 12111). By aligning a distant related species (*P. aeruginosa*) and a more closely related species (*P. orzihabitans*) with the reference strain, we were able to uncover well conserved regions within the genes that made suitable regions for primers that consequently was thought to be conserved across all isolates included in the analysis. Sequences for the reference strain DSM 12111 was imported from the NCBI database, while sequences for *P. aeruginosa* and *P. orzihabitans* were retrieved from the *Pseudomonas* database www.pseudomonas.com. The sequential software used for the alignment and primer design was Geneious (Biomatters, Auckland, New Zealand). Primers are listed in Table 2.

A standard 16S PCR program including an annealing step with a temperature gradient ranging from 48°C to 60°C for 1 min was run to find an optimal annealing temperature for all nine primer sets. Five isolates of different geographic origin were included in the PCR as a representative selection. Reaction mixture and PCR setup (excluding gradient annealing step) is described in section 3.4. An additional M13 primer sequence (Thermo Fisher Scientific, Waltham, USA) was added to each primer set following the initial tests of optimal annealing temperature. M13 Forward: 5′d[GTAAAACGACGGCCAG]3′, M13 Reverse: 5′d[CAGGAAACAGCTATGAC]3′. By doing so, sequencing of all HK genes was made possible using only the M13 primers.

Table 4: Primers (without M13) for each of the nine housekeeping genes included in the analysis.

Primer	Sequence (5´- 3´)	Gene product	Size of target sequence (bp)	PCR product post trim (bp)	Annealing temp (°C)	Referance
aroE-42 F	CAAGTCGCCGCTGATTCATC	Shikimate dehydrogenase	653	505	56°C	This study
aroE-761 R	GTTCGACCAGCATGCCCAG					
atpD-114 F	ACCCTGGAAGTTCAGCAGC	ATP synthase F1, beta subunit	808	645	56°C	This study
atpD-965 R	TACAACGGTGGCGTCCAAG					
carA-143 F	CCGATCCTTCCTATGCCCAG	Carbamoyl-phosphate synthase	762	526	56°C	This study
carA-956 R	GTTCTGGCTGGTGATCATCAC					
glnS-238 F	CGCCAAGGAAGACCAGGAG	Glutaminyl-tRNA synthetase	587	480	56°C	Andreani et. al 2015
glnS-863 R	CTTGCGCTTGCTGGTAATCG					
ileS-43 F	TTTCCGATGAAGGCCGGC	Isoleucyl-tRNA synthetase	708	645	56°C	Andreani et. al 2015
ileS-788 R	GGTAAACTCCGGGTGAACGT					
rpoB-3,307 F	TGTGGTCTCGGTGATCATGC	RNA polymerase, beta subunit	529	507	56°C	Andreani et. al 2015
rpoB-3,878 R	GAACTGCGCCTTACCACCC					
rpoD-294 F	GACCCAGTGCGCATGTACAT	RNA polymerase sigma factor	766	735	56°C	This study
rpoD-1,204 R	ATGCGACGGTTGATGTCCTT					
recA-136 F	CTCGCTTGGTCTGGACATCG	Recombinase A	740	641	56°C	This study
recA-959 R	CCTTGCCTTGGCCGATCTT					
gyrB-329 F	ACAGCTACAAGGTTTCCGGC	DNA gyrase, subunit B	703	646	56°C	This study
gyrB-1,089 R	CTTGCCCATTTCCTGCTCGA					

2.1.4 PCR and sequencing of HK genes

Amplification of the HK genes by PCR was based on a standard reaction mixture containing (per reaction) 4 µl 5xGreen GoTaq Flexi buffer, 1.5 µl MgCl12 Solution (25 mM), 0.4 µl dNTP (10 mM), 1 µl of forward and reverse primers (10 µM), 0.1 µl Go Taq G2 Flex DNA polymerase, and 8 µl nuclease-free water. Final volume of the mastermix was 16 µl. All reagents were supplied by Promega (Madison, USA). 4 µl of DNA template was added to each reaction, making the final reaction volume 20 µl. The PCR was performed at 95°C for 3 min, 30 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, elongation at 72°C for 1 min, followed by 72°C for 5 min in an Agilent SureCycler 8800 Thermal Cycler (Santa Clara, USA). PCR-product of desired size was confirmed performing a 1% agarose gel electrophoresis using the GelPilot® 100bp Plus Ladder (QIAGEN, Venlo, Netherlands) as a reference ladder. All PCR products were documented with the Bio-Rad Gel Doc XR+ (Hercules, USA). Amplified PCR-products were sent to NVI in Oslo for sequencing by Sanger-sequencing.

Prior to sequencing, PCR-products were purified with AMPureXP® (Beckman Coulter, Pasadena, USA) using the Biomek4000 pipetting robot (Beckman Coulter, Pasadena, USA). Sequencing of HK genes was based on a reaction mixture containing (per reaction) 0.5 µl BigDye v3.1, 2.0 µl 5X Sequencing Buffer, 2.0 µl M13 primer (2.5 µM), 2.0 µl purified PCR-product and 3.5 µl MilliQ-water. Final reaction volume was 10 µl. The sequencing PCR program was performed at 96°C for 1 min, 25 cycles of 96 °C for 10 seconds, 50°C for 5 seconds and 60°C for 4 min in an Applied Biosystems® 3500xl Genetic Analyzer (ThermoFisher Scientific, Waltham, USA) Sequence products were purified using the BigDye® XTerminator Purification Kit (ThermoFisher Scientific, Waltham, USA) in accordance with its protocol.

2.1.5 Sequence processing and tree generation

Forward and reverse strands of the HK genes were sequenced for all isolates and deposited in the local database at NVI. The sequences were retrieved and imported to Geneious 11.1.5 (Biomatters, Auckland, New Zealand) for further processing. The two strands from each reaction were thereafter assembled into contiguous sequences (contigs). Ambiguities within strands of the contigs were manually edited by applying the corresponding strand as a template, given that the quality of the chromatogram was adequate. Sequences of poor or insufficient

quality were re-sequenced until desired quality was achieved. Once all contigs were edited they were trimmed at the 5′and 3′-ends to exclude primer sequences and the poor quality of the chromatogram usually achieved at both primer ends. All sequences were temporarily translated and trimmed within the first and final codon to preserve the reading frame. Final processed sequences were imported and concatenated in Microsoft Excel. Concatenated sequences were subsequently re-imported to Geneious to construct the final alignment. All sequences were stored in the local database at NVI awaiting pre-publication deposition in GenBank.

The alignment was exported from Geneious in a MEGA-alignment file format and subsequently imported into Molecular Evolutionary Genetics Analysis (MEGA, version 7). A maximum likelihood model analysis was run on the imported alignment, using the model test module in MEGA, in order to determine the optimal substitution model for the phylogenetic analysis. The model analysis regarded TN93 (Tamura and Nei, 1993) as the optimal substitution model and was consequently chosen as the model for phylogenetic reconstruction. Number of bootstraps were set to 1000, while all other inputs were set to default. Finally, the SVG file was imported to Microsoft Word where brackets and complementary annotations were added to the finalized tree.

2.5 Infection challenge

2.5.1 Cultivation of challenge material

Three isolates of epidemiological relevance for Norwegian aquaculture of lumpfish (NVIO 10039, NVIO 10973 and NVIB 50-1353) were selected for potential use in the challenge trial. The isolates were prepared by inoculation 1µl of available material to plates of BA and incubated at 20°C for three days. Approximately 100 µl of colony-forming material of the chosen isolates was thereafter inoculated from the plates of BA to 100 ml tryptic soy broth (TSB) and incubated at 20°C. Optical density (OD_{595}) was measured for each culture 48 hours post start of incubation using the Eppendorf BioPhotometer Plus (Hamburg, Germany) and applying uninoculated TSB as reference medium (OD=0). Documenting optimal nutrient composition for bacterial growth was performed by inoculating 10 ml of the homogenized culture to two separate growth medias (TSB and TSB w/1.5% NaCl (100 ml). OD was measured at T=0 and the cultures were incubated at 20°C for 48 hours. 10 ml of homogenized inoculum was then transferred to parallel sets of TSBs (100 ml) after having documented the bacterium's enhanced ability to multiply within TSB without added salt. These were then incubated at 15 and 20°C to monitor bacterial growth rate at the two given temperatures. OD was measured at

T=0 and 48 hours post incubation. The following measurements confirmed the bacterium's preference for 20°C.

Isolate NVIO 10039 demonstrated in general a superior growth rate compared to the two other isolates in question. NVIO 10039 was thereby chosen as the infectious agent to take part in the challenge trial while the other isolates were discarded. A final inoculum of 20 ml was transferred from NVIO 10039 incubated at 20°C to new sets (n=2) of TSBs (200 ml). OD was measured at T=0 and 48 hours post incubation. One of the cultures was subject to documentation of growth characteristics (cfu-count+OD_{595}) for titer calculations, while the other served as the final challenge material. The latter was transferred to 50 ampules of 1 ml with 20% glycerol added and cryopreserved at -80°C.

2.5.2 Preparation of challenge material

Challenge material from an ampule of cryopreserved concentrate was thawed and homogenized for dilution of infection dosages. The challenge material was serially diluted in pre-cooled (4°C) physiological saline (0.9% NaCl) to obtain the desired concentrations. Theoretical concentrations were calculated based on titer estimates prior to cryopreservation. A sample of the challenge material was diluted 10-fold, plated on Tryptic Soy Agar (TSA) and incubated at 20°C. The actual concentration of the challenge dosages (cfu/ml) was determined by counting colony-forming units of bacteria on the plates in question five days post incubation. Diluted challenge material was kept cool in order to prevent bacteria from multiplying within the solutions before being administrated within three hours post preparation.

2.5.2 Pilot study

A pilot challenge model was designed to test the dose-response by i.p. administration of *P. anguilliseptica* in lumpfish. Measures were taken to ensure the absence of any pathogens present in the fish submitted for the experiment. Bacteriological samples were inoculated from head kidney of 15 fish and plated on BA and BA w/2% NaCl and incubated respectively at 20°C and 15°C. Tissues for histopathological analyses (gills, heart, liver, pyloric caeca, intestine, spleen, kidney and skeletal musculature), and real-time (rt) qPCR (head-kidney) were also sampled to comprehensively document the health status of fish prior to the experiment. A total of 80 unvaccinated fish were included in the experiment and divided into four groups of 20, each group i.p. injected with separate dosages. All fish subject to injection were sedated and

weighed prior to administration of challenge material. The four fish groups were marked uniquely by intracutaneous injection of Visible Implant Elastomer (VIE) (Northwest Marine Technologies, UK) before being injected with 0.05 ml of challenge material. The four dosages applied were: $4.3x10^8$, $4.3x10^6$, $4.3x10^4$ and $4.3x10^2$ cfu/fish (table 5).

Table 5: Pilot challenge model. † **ant.** anterior, **d.l.** dorsolateral.

Group no.	No. of fish	Route of challenge	Dose volume	Challenge dose (cfu/fish)	Tagging †
1	20	Intraperitoneal injection	0.05 ml	$4.3x10^8$	Green d.l left
2	20			$4.3x10^6$	Red d.l left
3	20			$4.3x10^4$	Green ant.
4	20			$4.3x10^2$	Red ant.

Average fish weight at challenge was 17.7 ± 3.4g, and all 80 fish were held in one tank of 150L throughout the experiment. Environmental parameters are listed in table 6. Deceased fish were collected and registered daily and twice a day at times with increased mortality. Moribund fish at humane cut-off point, at which fish were unable to keep its orientation in the water column, were euthanized by anesthetic overdose (tricain mesylate 100%) and registered as mortalities at the time given.

Table 6: Environmental parameters during challenge.

Parameter	Value
Salinity	>33‰
Temperature	12°C
Flow	≥ 300 l/h
Oxygen saturation	≥ 75%
Fish density	9.44 kg/m^3
Feeding regime	*ad libitum*

Bacteriological samples from head-kidney of all deceased fish were plated on BA and BA w/2% NaCl and incubated respectively at 20 and 15°C. Colony morphology compatible with *P. anguilliseptica* served the basis for species identification of cfu forming a week post incubation. MALDI-TOF was performed on a representative number of isolates to confirm the bacterial species identification. Five moribund fish from each group were sampled for histopathological analysis during the challenge. Tissues sampled were: gills, heart, pyloric caeca, intestine, liver, kidney, spleen and skeletal musculature. All tissue samples were preserved in formalin (10%).

2.5.3 Cohabitant challenge study

Two challenge models were designed to test the possible horizontal infection route of the bacterium from i.p. and i.m. infected fish through naïve cohabiting fish. A total of 740 unvaccinated fish were included and divided into the two challenge trials. Each challenge model

consisted of three fish groups of 30 fish (n=90) subject to three separate infection dosages (high, medium and low) as well as a cohabitant group of 90 naïve fish (table 7-10). The total number of fishes per challenge model was 180 and each model was replicated with two parallels. The three dosages administered by intraperitoneal injection were: $4.0x10^3$, $1.8x10^3$ and $4.0x10^2$ cfu/fish, while dosages administered by intramuscular injection were: $4.0x10^3$, $4.0x10^2$ and 4.0x10 cfu/fish (table 7-10).

Table 7: Intraperitoneal challenge model, parallel 1. **ant.** anterior, **d.l.** dorsolateral.

Tank ID	Group no.	No. of fish	Route of challenge	Dose volume	Challenge dose (cfu/fish)	Tagging
1	1A	30	Intraperitoneal injection	0.05 ml	$4.0x10^3$	Red ant.
	1B	30			$1.8x10^3$	Red d.l left
	1C	30			$4.0x10^2$	Red d.l right
	1D	90	Cohabitation	N/A	N/A	Green ant.

Table 8: Intraperitoneal challenge model, parallel 2. **ant.** anterior, **d.l.** dorsolateral.

Tank ID	Group no.	No. of fish	Route of challenge	Dose volume	Challenge dose (cfu/fish)	Tagging
2	2A	30	Intraperitoneal injection	0.05 ml	$4.0x10^3$	Red ant.
	2B	30			$1.8x10^3$	Red d.l left
	2C	30			$4.0x10^2$	Red d.l right
	2D	90	Cohabitation	N/A	N/A	Green ant.

Table 9: Intramuscular challenge model, parallel 1. **ant.** anterior, **d.l.** dorsolateral.

Tank ID	Group no.	No. of fish	Route of challenge	Dose volume	Challenge dose (cfu/fish)	Tagging
3	3A	30	Intramuscular injection	0.05 ml	$4.0x10^3$	Green ant.
	3B	30			$4.0x10^2$	Green d.l left
	3C	30			4.0x10	Red d.l right
	3D	90	Cohabitation	N/A	N/A	Red ant.

Table 10: Intramuscular challenge model, parallel 2. **ant.** anterior, **d.l.** dorsolateral

Tank ID	Group no.	No. of fish	Route of challenge	Dose volume	Challenge dose (cfu/fish)	Tagging
4	4A	30	Intramuscular injection	0.05 ml	$4.0x10^3$	Green ant.
	4B	30			$4.0x10^2$	Green d.l left
	4C	30			4.0x10	Red d.l right
	4D	90	Cohabitation	N/A	N/A	Red ant.

Samples for bacteriology, histopathology and qPCR were sampled from 20 fish 24 hours prior to challenge to confirm the absence of any systemic infection upon start of the experiment. Desired challenge doses were estimated on the basis of cumulative mortality of the separate fish groups in the pilot study (figure 6). Average fish weight at challenge was 42.7 ± 9.1 g. Each challenge parallel of 180 fish were kept in separate tanks of 150 L (n=4). Environmental parameters during cohabitant challenge were identical to the pilot challenge (table 6) with the exception of fish density which was approximately 25.6kg/m^3 of shedder-fish at the start of challenge. Moribund and deceased fish were euthanized and registered in accordance with the protocol applied in the pilot study (see section 3.5.2).

Bacteriological samples from head kidney of approximately 200 deceased fish were plated solely on BA w/2% NaCl and incubated at 15°C. Tissues for histopathological analysis were sampled from 10 moribund fish from each challenge parallel (n=40). As with the pilot challenge, tissues sampled were gills, heart, pyloric caeca, intestine, liver, kidney, spleen and skeletal muscle, as well as the brain. Colonies of *P. anguilliseptica* were primarily identified by compatible colony morphology, though analyses by MALDI-TOF were performed for accurate species identification. Colonies other than *P. anguilliseptica* isolated in mixed cultures were also subject to analysis by MALDI-TOF. Surviving fish following termination of the challenge study were euthanized by anesthetic overdose (tricain mesylate 100%). Bacteriological samples from head-kidney were plated on BA w/2% NaCl in order to examine the presence of *P. anguilliseptica* in any of the surviving fish.

3. Results

3.1 MLSA

3.1.1 Confirmation of isolate identity

MALDI-TOF analysis of samples submitted to the study positively identified 53 out of 55 isolates as *P. anguilliseptica*. One of the deviant isolates was identified as *Francisella philomiragia*, while no positive identification was possible for the second isolate in question. 16S rRNA sequencing of 39 of the 55 isolates demonstrated concordance between MALDI-TOF and 16S rRNA results as well as identifying the deviant isolates as *F. philomiragia* and *Psychrobacter sp.* Both isolates in question were discarded from further study. A general compliance between all isolates was registered with regard to phenotypic characteristics except for production of arginine dihydrolase and vibriostatic sensitivity. Isolates NVIO 10973, NVIO

11214 and NVIB 50-1910 were positive for production of arginine dihydrolase while the remaining isolates proved negative. The majority of the isolates were vibriostat resistant, though NVIO 8905 and NVIO 9976 were sensitive. All phenotypic characteristics examined and results for each isolate are listed in table 11.

Table 11: Phenotypic characteristics of six isolates of *P. anguilliseptica* included in the analysis.

Test	NVIO 10973	NVIO 8905	NVIO 11214	NVIB 50-2255	NVIB 50-1910	NVIO 9976
Gram	–	–	–	–	–	–
Motility	+	+	+	+	+	+
Cytochrome oxidase	+	+	+	+	+	+
O/F	–	–	–	–	–	–
Anaerobe respiration	–	–	–	–	–	–
Arginine dihydrolase	+	–	+	+	–	–
Lysine decarboxylase	–	–	–	–	–	–
Ornithine decarboxylase	–	–	–	–	–	–
Vibriostatic sensitivity	–	+	–	–	–	+
Haemolysis on blood agar	–	–	–	–	–	–

3.1.2 MLST/MLSA

The phylogenetic analysis by MLSA reveals five clusters and two singletons with branch support values ranging from 62-98. Norwegian lumpfish isolates are distributed between two of the major phylogenetic groups resulting from the analysis (figure 3). One of these groups (group 1) constitutes a dominant collection of isolates originating from the majority of fish species and various geographic origins, while the other (group 2) solely consists of isolates from Norwegian lumpfish. The type strain of NCIMB 1949 constitutes one of the two singletons revealed and is situated within group 3. So are also all Irish lumpfish isolates except for NVIO 11302 which is situated within group 1. All isolates from The Faroe Islands and Finland/Baltic Sea displays identical genotype situated within group 1. Among these are isolates originating from several salmonid species such as Atlantic Salmon, Rainbow Trout, and Whitefish. There was no clear sign of host specific genotypes, except for the clustering of the two Sea Bream isolates submitted from Spain, located within group 1 (figure 3).

Epidemiological resolution achieved by MLST demonstrated a concordance with the MLSA by describing seven different sequence types (STs) with a dominant ST (ST2) making up more than 50% of all isolates included in the analysis (figure 4 and 5). Few substitutions per HK gene were discovered and only five (*rpoD, rpoB*, *atpD*, *ileS* and *carA*) of the nine genes sequenced produced more than one unique allele (table 12). The remaining HK genes (*recA*, *glnS*, *aroE*

and *gyrB*) displayed identical sequences in all isolates. The ratio between non-synonymous and synonymous (dN/dS) substitutions for the genes producing more than one unique allele were below 1 for all genes except for rpoD (1.5) (table 12).

Table 12: Number of unique alleles and ratio between non-synonymous (dN) and synonymous (dS) substitutions discovered for each HK gene following the sequencing of all 53 isolates of *P. anguilliseptica.*

HK gene	rpoD	rpoB	atpD	ileS	carA	recA	glnS	aroE	gyrB
No. of alleles	5	3	3	3	2	1	1	1	1
dN/dS	1.5	0	0	0.5	0	0	0	0	0

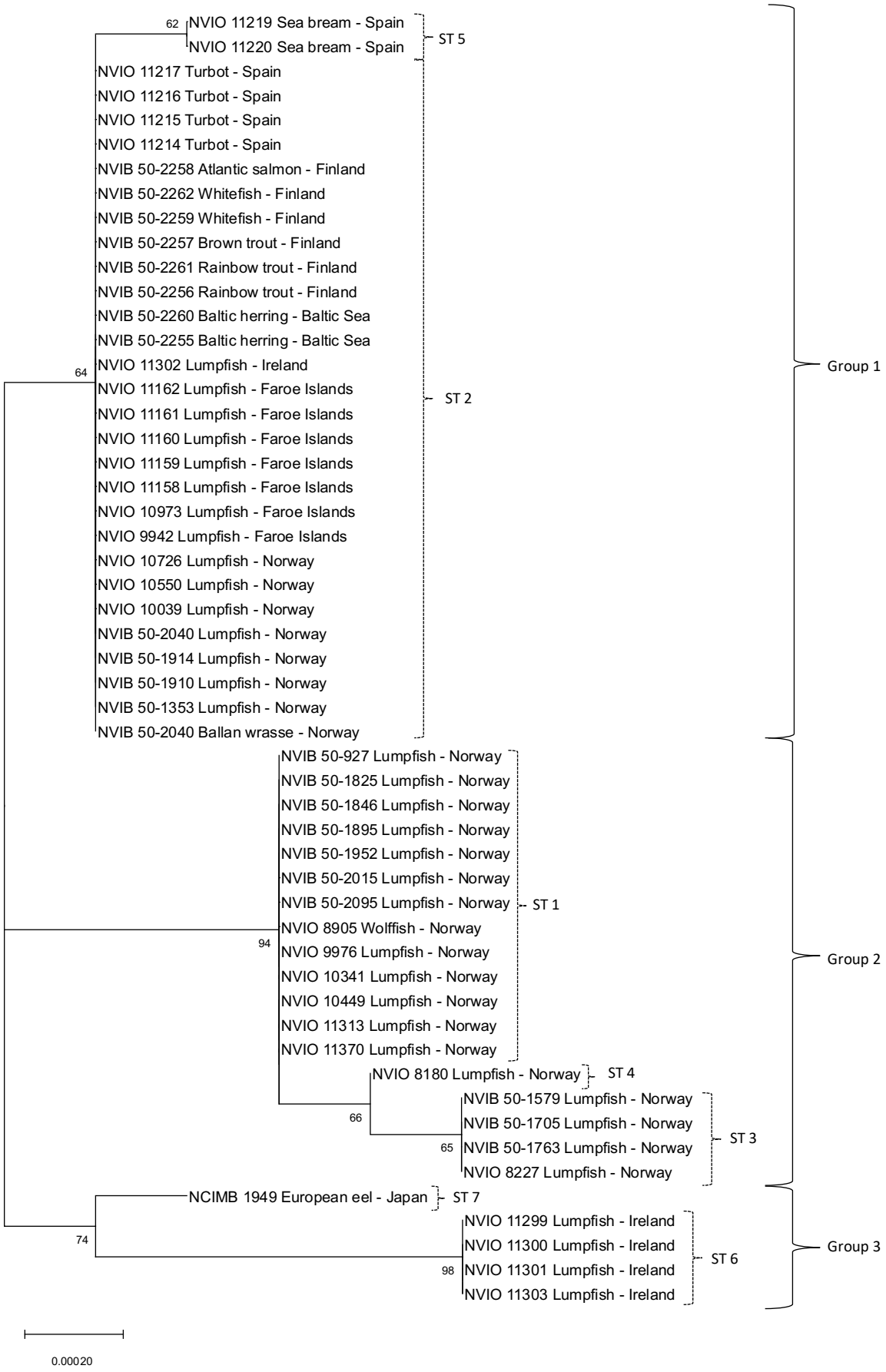


Figure 3: Phylogenetic reconstruction of 53 isolates of *P. anguilliseptica* based on the concatenated *aroE, recA, rpoB, ileS, rpoD, glnS, atpD, gyrB* and *carA* gene sequences. Annotation: Norwegian Veterinary Institute Bergen/Oslo (NVIB/NVIO), isolate number, fish species. Stapled brackets refer to STs identified by MLST (figure 4 and 5) Unstapled brackets refer to the major groups formed following the phylogenetic reconstruction.

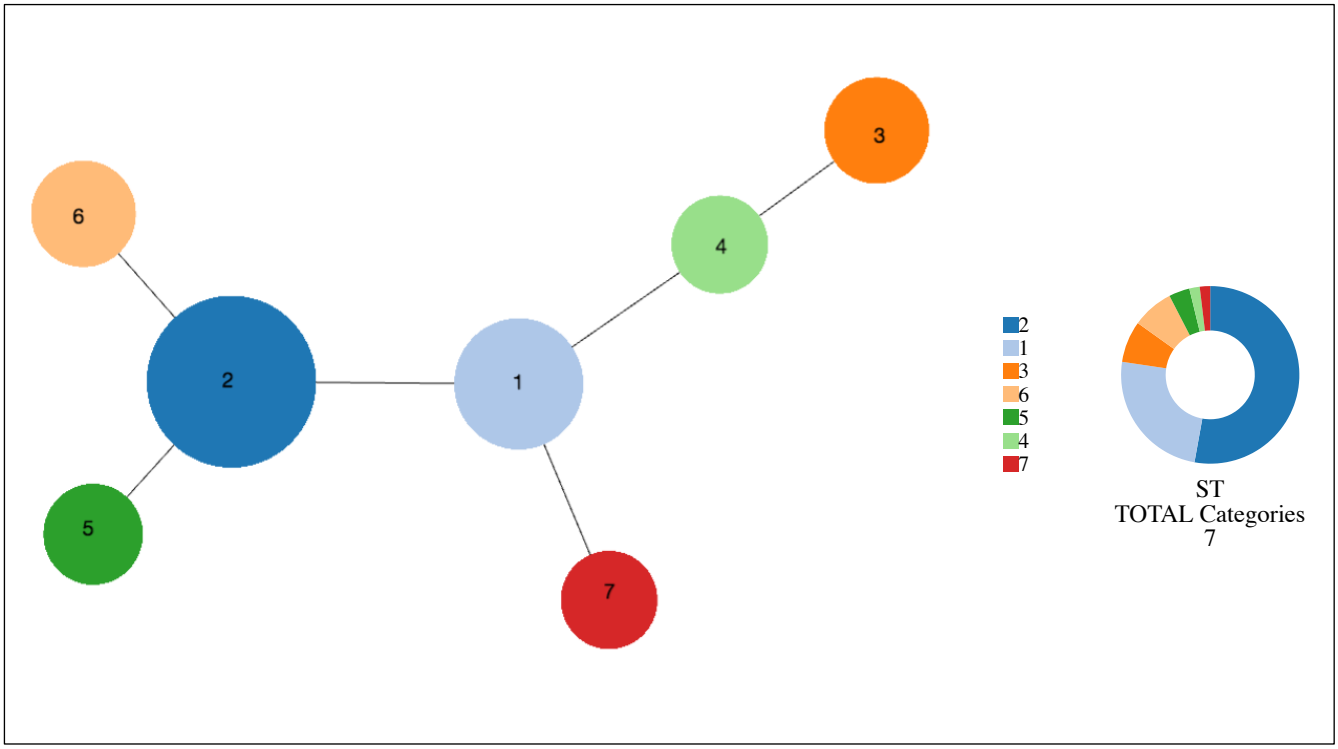


Figure 4: Visualization of the 7 different STs at a tree cut-off value off 4, at which all links between nodes will be deleted at differences above 4. The size of the nodes is representative of the relative counts of STs among all isolates included in the analysis, also illustrated by the pie chart at the right of the figure.

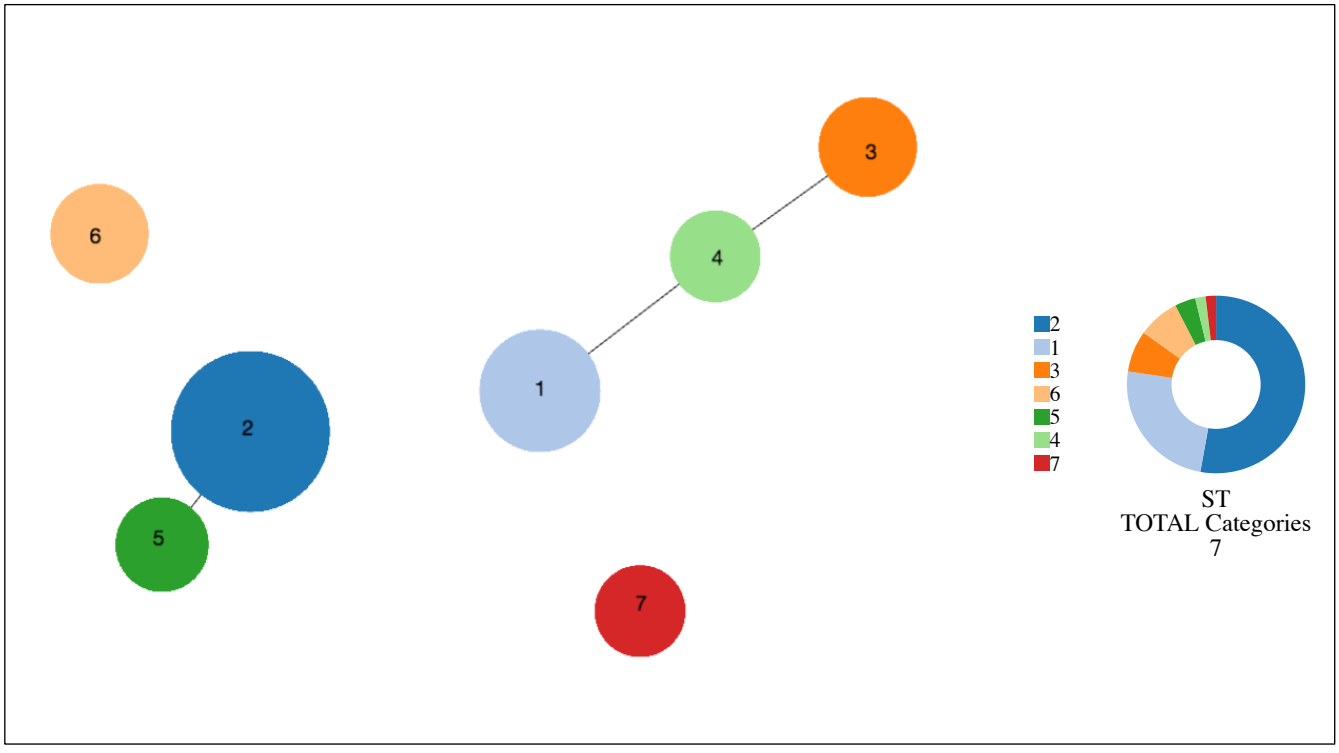


Figure 5: Visualization of the 7 different STs at a tree cut-off value off 2, at which all links between nodes will be deleted at differences above 2. The size of the nodes is representative of the relative counts of STs among all isolates included in the analysis, also illustrated by the pie chart at the right of the figure.

3.5 Infection challenge

3.5.1 Pilot study

A definitive dose-response effect was observed among the separate fish groups with regards to the correlation between concentration of challenge material, registration of the first fatalities and partly the cumulative mortality. An exponential increase in mortality was observed following the first fatalities seven days post challenge (DPC) among the two fish groups infected with the highest concentrations of challenge material. Three of the four challenge dosages ultimately induced a cumulative mortality of 100%. The lowest challenge dose of $4.3x10^2$ cfu/fish was the only dose which caused a cumulative mortality below 100% of challenged fish, at a respective level of 65% (figure 6). The mortality ceased 16 DPC and persisted the following days. The experiment was terminated 19 DPC.

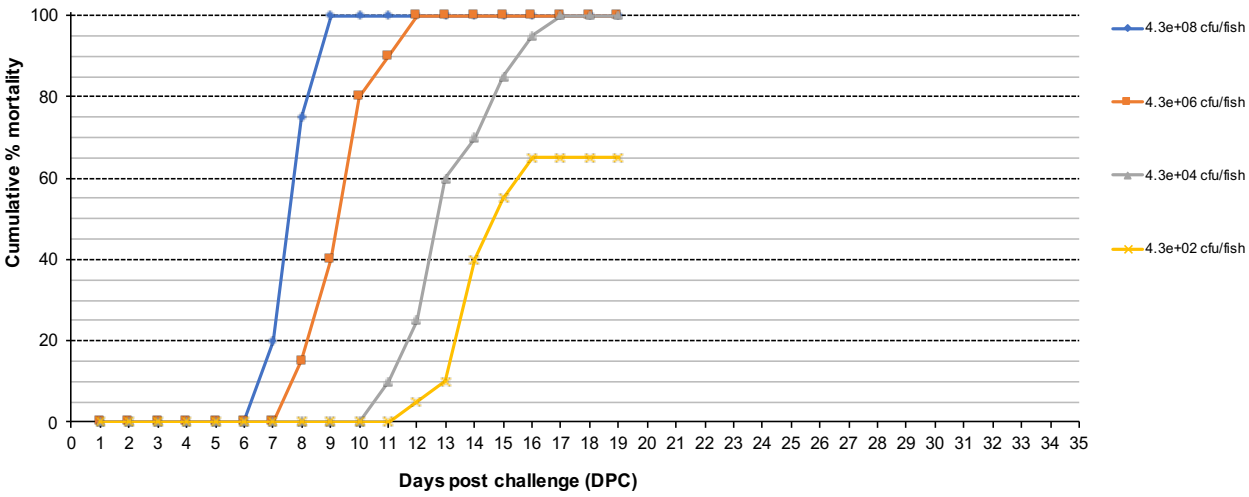


Figure 6: Pilot study. Cumulative mortality for fish infected by intraperitoneal injection. Four groups of fish (n=20) each infected with separate dosages (cfu/fish).

P. anguilliseptica, confirmed by MALDI-TOF was re-isolated in pure culture from kidney of all challenged fish. Clinical signs of disease were few, though general change in pigmentation, swollen vent and exophthalmia were common (figure 7). Occasional cases of cataract and hemorrhagic eye lesions were registered. Fish at terminal stages of disease were observed hyperventilating and apathetic with a definitive loss of appetite. Significant ascites, swollen spleen and pale kidneys were among the limited pathological findings upon necropsy. Histological examination revealed septicemia involving large numbers of slender rod-shaped bacteria within all parts of the circulatory system (figure 8). The most prominent

histopathological changes were observed in the spleen and kidneys of the fish. Diffuse necrotic areas in the spleen, and multifocal hemorrhagic necrosis in the kidneys were common findings across all fish groups (figure 8). Little or no excessive infiltration of inflammatory cells was found within the circulatory system, nor in the surrounding tissues. Few if any histopathological distinctions were made between the four fish groups.

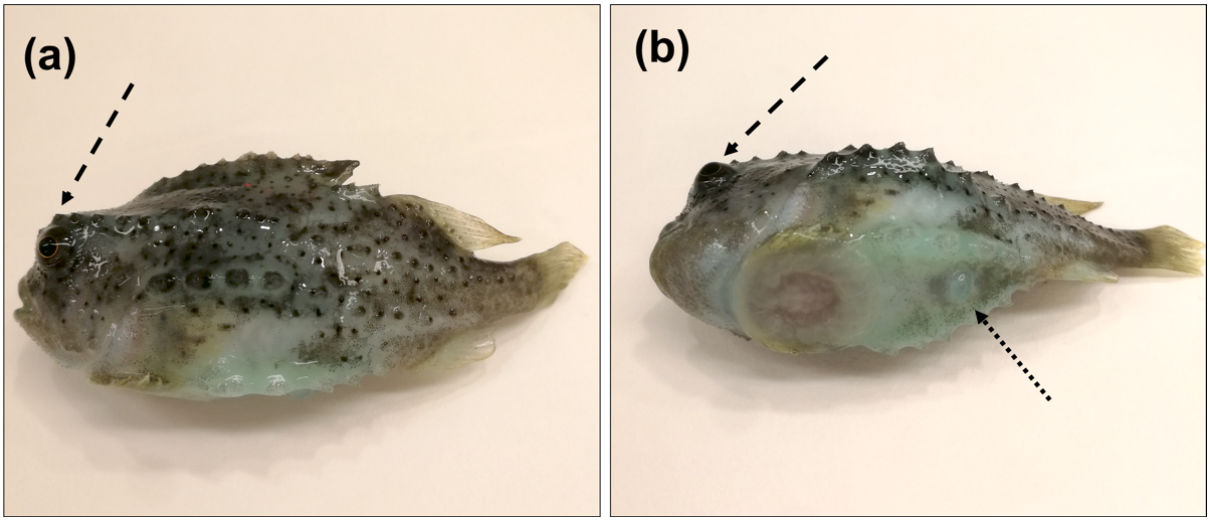


Figure 7: Macroscopic lesions associated with i.p. infection of *P. anguilliseptica* under controlled laboratory conditions. a,b: fish showing moderate signs of exophthalmos (big stapled arrows) and swollen vent (small stapled arrow) due to accumulation of ascites fluid in the abdomen. Fish also displaying a general pigmental discoloration across its body.

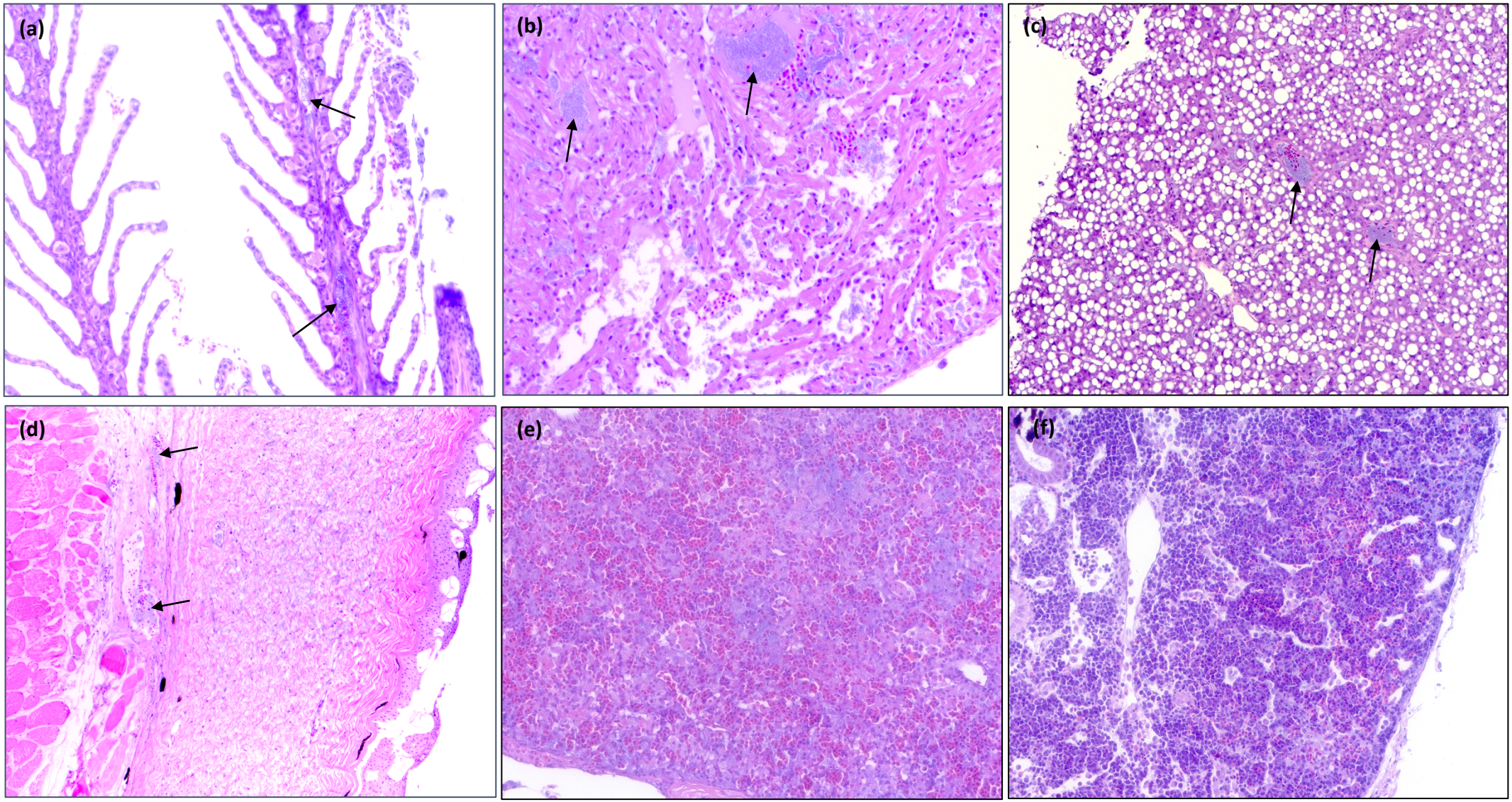


Figure 8:Histopathological lesions induced by i.p. injection of $4.3x10^2$ cfu/fish under controlled laboratory conditions (20X). Large aggregates of slender rod-shaped bacteria (black arrows) within the circulatory system of the gills (a), heart (b), liver (c), dermis and musculature (d). Diffuse multifocal hemorrhagic necrosis observed in the spleen (e) and multifocal hemorrhagic necrosis in the kidney (f).

3.5.2 Cohabitant challenge study

Only minimal differences in dose-response effect were observed between i.p./i.m. challenged fish groups as the first fatalities were registered 11±2 DPC followed by an exponential increase in mortality rate. All challenge doses administered by i.m. injection ultimately induced a cumulative mortality of 100 %. The i.p. challenge dose of $4.7x10^2$ cfu/fish was the only dose administered by injection, which caused a cumulative mortality below 100% in the two challenge models at levels of 93 and 97% (figure 9 and 10).

Initial signs of horizontal infection route were observed as the first fatalities in all cohabitant groups occurred approximately three weeks post challenge. An almost identical development in cumulative mortality was observed among all cohabitant fish groups in the two challenge models. A rapid increase in mortality rate was observed before reaching a plateau effect at an average of 47.5±2.5% cumulative mortality. The mortality persisted at a low but constant rate following the plateau effect and did not cease before the experiment was terminated 49 DPC. The final cumulative mortality of the cohabitant fish groups was an average of 55.8% for the i.p. challenge model and 61% for the i.m. challenge model (figure 9-12).

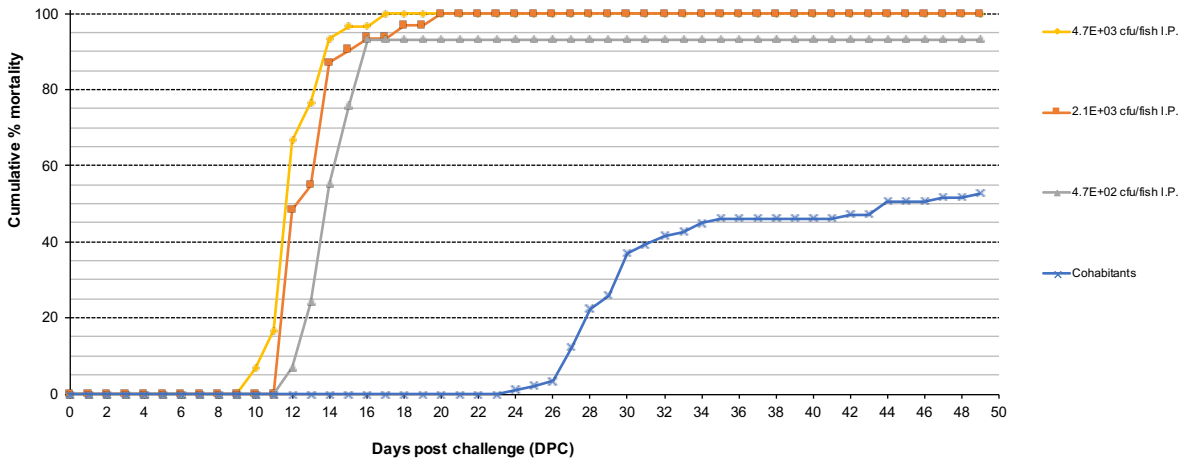


Figure 9: Intraperitoneal challenge model, parallel 1. Cumulative mortality for i.p. challenged fish and naïve cohabiting fish.

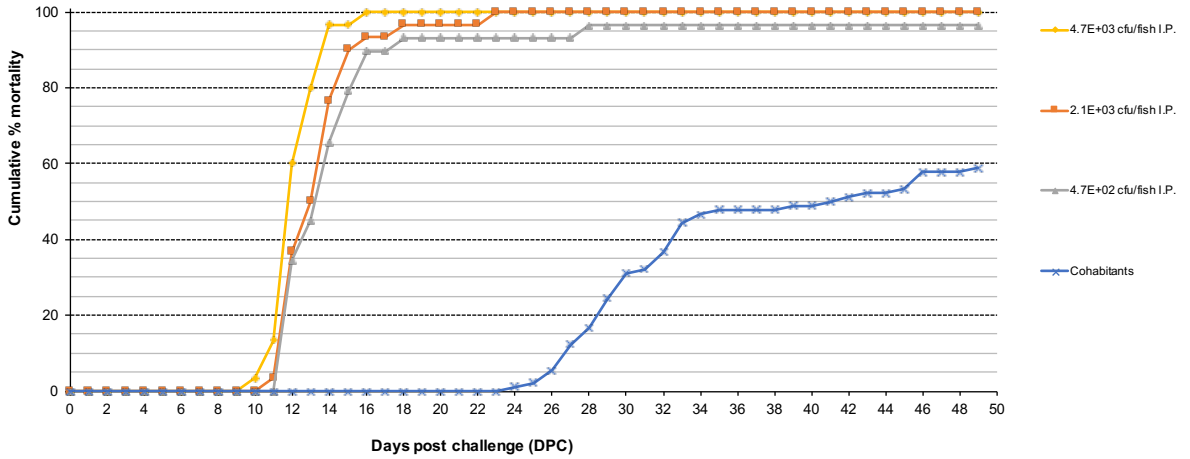


Figure 10: Intraperitoneal challenge model, parallel 2. Cumulative mortality for i.p. challenged fish and naïve cohabiting fish.

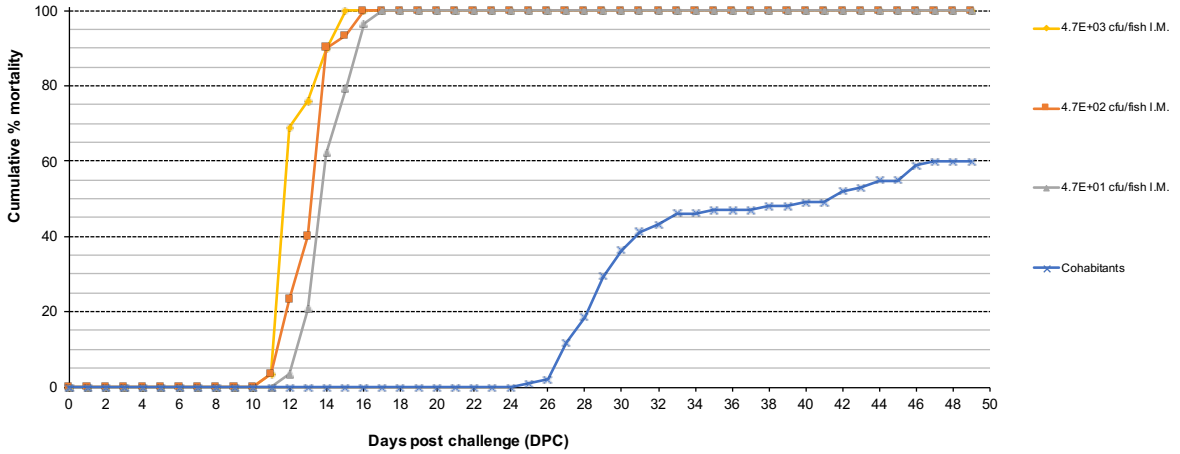
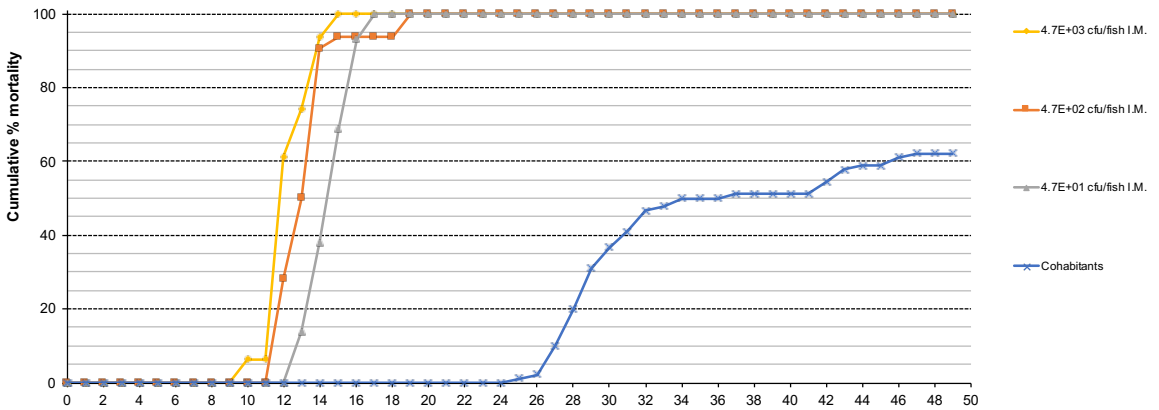


Figure 11: Intramuscular challenge model, parallel 1. Cumulative mortality for i.m. challenged fish and naïve cohabiting fish.



Figur 12: Intramuscular challenge model, parallel 2. Cumulative mortality for i.m. challenged fish and naïve cohabiting fish.

Distinct clinical signs of disease were observed among the naïve cohabitant fish in both challenge models. Eye lesions including cataract, puncture of the cornea and/or hemorrhage were among the first and most common signs of disease. Fish suffering from eye lesions gradually developed hyperemia/hemorrhages within the operculum as well as at the fin bases and on the anterior parts of the suction cup. In addition to the hemorrhagic lesions, the fish also suffered from swollen lips (figure 13). The swelling of the lips gradually decreased in severity during the course of the disease and was not observed among deceased fish. General skin discoloration, swelling of the vent and exophthalmia was also among i.p. and i.m. infected fish. As in the pilot study, fish at terminal stages of disease were observed hyperventilating and were apathetic with a definitive loss of appetite.

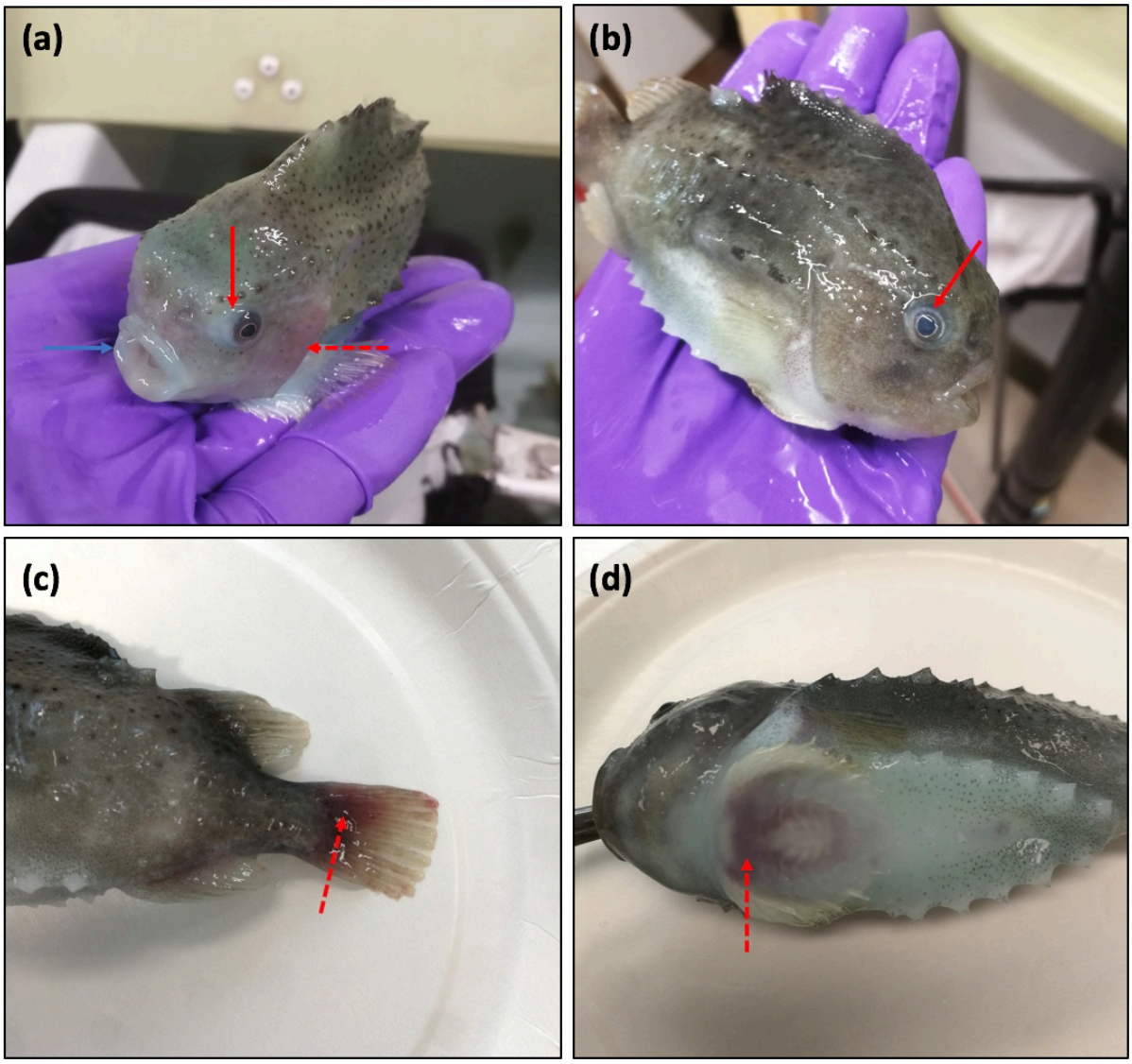


Figure 13: Clinical signs of disease observed on naïve cohabiting fish. Hemorrhagic lesions (red stapled arrows), eye lesions (red whole arrows) and swollen lips (blue arrow) displayed on moribund (a-b) and deceased fish (c-d).

Distinctions were also made between deceased cohabitant fish and i.p./i.m. challenged fish with regards to the pathological changes observed upon necropsy. Sampling from deceased cohabiting fish revealed petechial hemorrhages in the kidney, skeletal muscle, peritoneum and occasionally in the adipose tissue surrounding visceral organs (figure 13). The most prominent lesions involved large amounts of ascites within the abdomen and swelling of the spleen. A fibrinous coat covering the liver was also observed in several fish. A common pathological feature observed in both cohabitant fish and i.p./i.m. challenged fish was hemorrhage within the brain (figure 14).

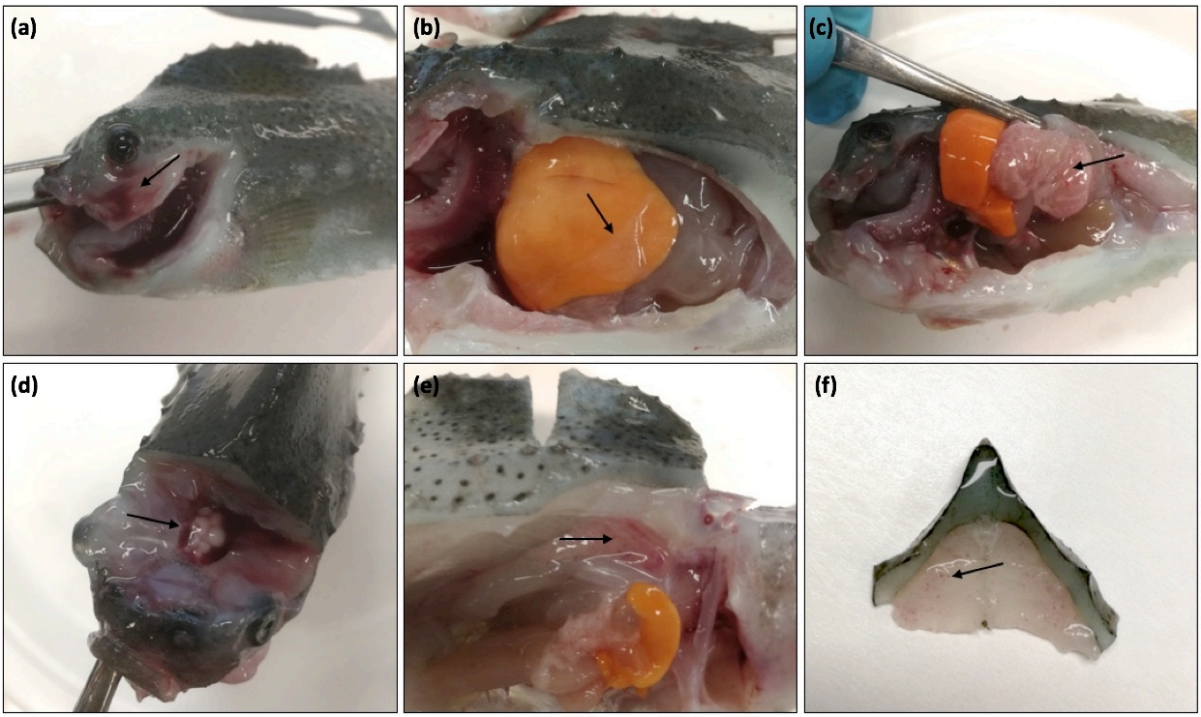


Figure 14: Pathological changes observed upon necropsy of deceased cohabitant fish. a: hemorrhages within the operculum, b: fibrinous coat covering the liver, c: petechial hemorrhages in the adipose tissue surrounding visceral organs, d: hemorrhages within the brain, petechial hemorrhages in the head-kidney (e) and skeletal musculature (f).

P. anguilliseptica, confirmed by MALDI-TOF, was predominantly re-isolated in pure culture from kidney of cohabitant fish subject to bacteriological analysis. Mixed cultures were dominated by colonies of *P. anguilliseptica. Vibrio logei* confirmed by MALDI-TOF, was also sporadically found (figure 15). Head-kidney tissues from 10 surviving fish sampled from each challenge parallel (n=40) following the termination of the experiment proved negative for the culture/presence of *P. anguilliseptica.*

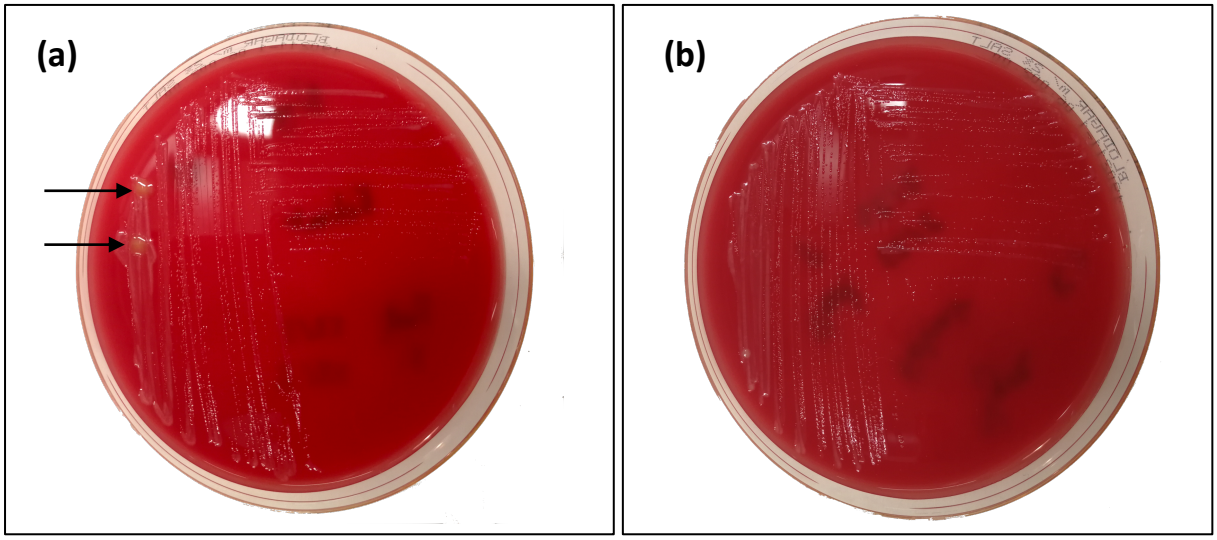


Figure 15: Primary bacteriology isolated from head-kidney of moribund cohabitant fish. Mixed culture dominated by colonies of *P. anguilliseptica* with sporadic colonies of *Vibrio logei* (arrows)(a) and pure culture of *P. anguilliseptica* (b).

Histopathological analyses of moribund cohabitant fish revealed a systemic infection involving large numbers of slender rod-shaped bacteria in all organs examined. As with the pilot study the most prominent lesions were located in the kidneys and spleen of the fish. An enlargement of the kidney capsule with a moderate sub-epithelial infiltration of inflammatory cells was observed in several fish. Substantial numbers of bacteria were observed in the sinusoids as well as widely distributed within the hematopoietic tissue of the kidney. Scattered, poorly demarcated areas of necrotic tissue were seen in association with the bacteria present in the interstitium (figure 16). The secretory tissues remained apparently unaffected. Diffuse hemorrhagic necrosis was observed within the spleen associated with a total infiltration of bacteria in the hematopoietic tissue (figure 17).

Analyses of heart tissues revealed aggregates of bacteria present in the lumen of the ventricle and atrium as well as occasionally within the myocytes of both compartments. Sporadic foci of degenerated and necrotic myocardium were observed in the atrium whereas the myocardium of the ventricle remained mostly unaffected (figure 17). An increase in circulating leucocytes was observed compared to heart tissue examined from the pilot study.

The livers of all fish showed large numbers of bacteria within the sinusoids and central veins, though little or no infiltration of bacteria was observed in the surrounding tissue. Nor was there any sign of inflammation or necrosis associated with the intravascular bacteria. A fibrinous-

like coat with excessive amounts of bacteria within the coat as well as the parenchyma in close proximity was observed in several cases (figure 16).

Examining the skin and skeletal musculature of the fish showed an enlargement of the basal membrane in the epidermis due to large accumulations of bacteria (figure 16). Aggregates of erythrocytes was also found in relation to bacteria surrounding the brain tissue of several fish (figure 17).

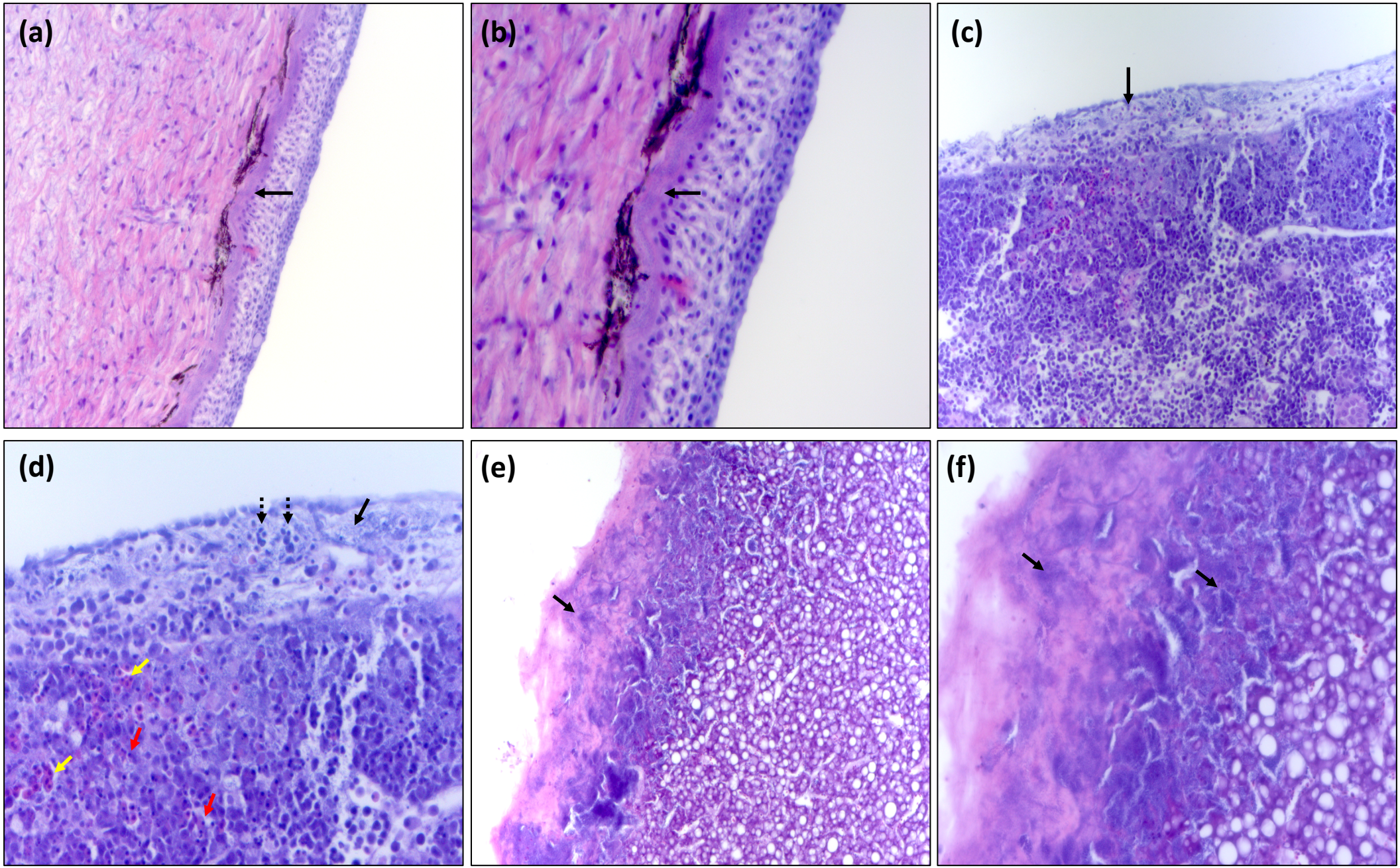


Figure 16: Histopathological changes observed in the skin (a-b), kidney (c-d), and liver (e-f). a-b: Infiltration of bacteria (black arrows) within the basal membrane of the epidermis. c-d: Sub-epithelial infiltration of inflammatory cells (black stapled arrows) in the kidney capsule, surrounding bacteria (black arrow), single cell necrosis (red arrows) and hemorrhages (yellow arrows) within the interstitium of the kidney. e-f: Fibrinous coat surrounding the liver with massive amounts of bacteria (black arrow) inside the coat as well as within the parenchyma in close proximity.

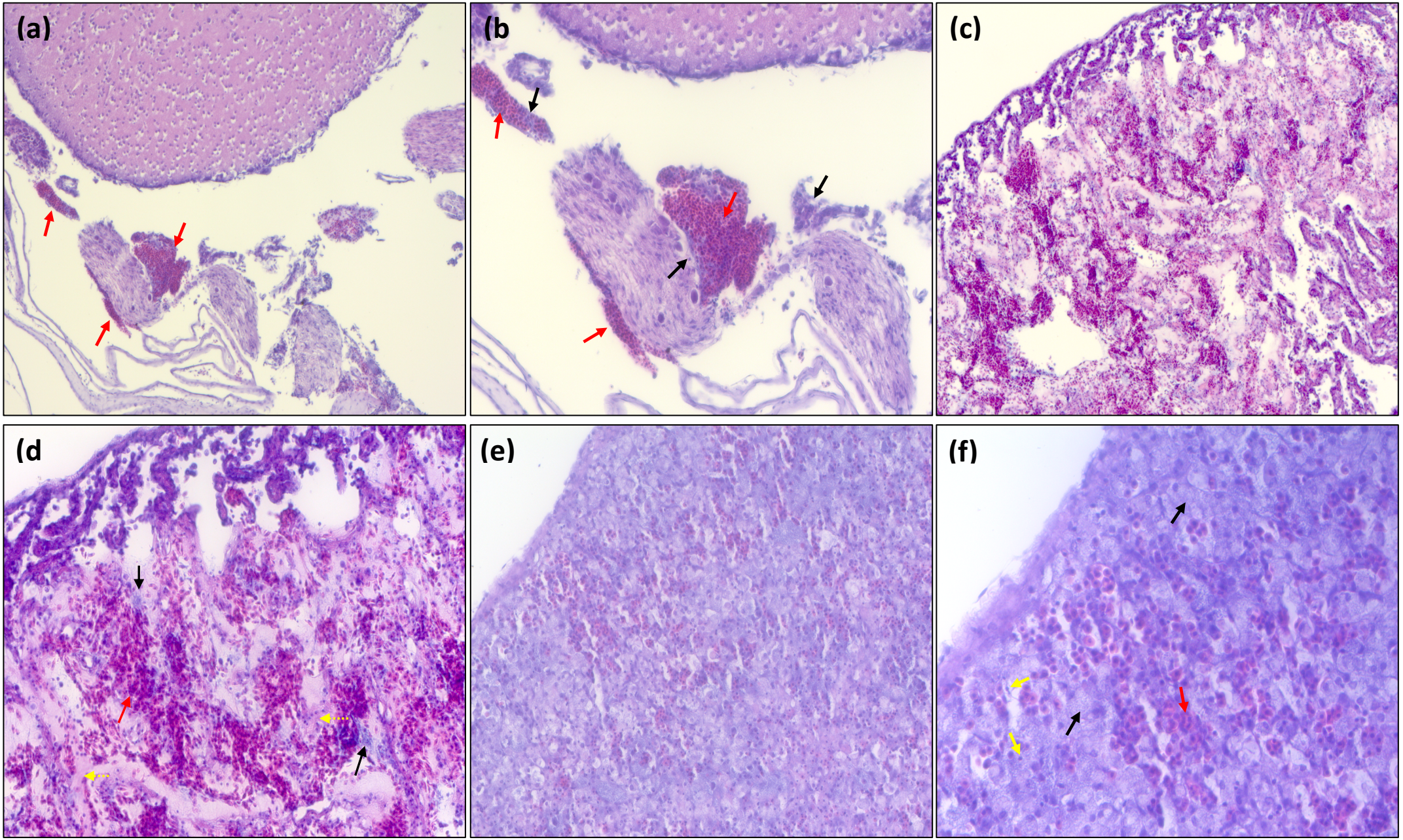


Figure 17: Histopathological changes observed in the brain (a-b), heart (c-d) and spleen (e-f). a-b: Aggregates of erythrocytes (red arrows) within the brain surrounding accumulated bacteria (black arrows). c-d: Diffuse necrosis of the myocytes in the atrium of the heart, bacteria (black arrows), degenerated and necrotic tissue (yellow arrows) and aggregates of erythrocytes (red arrows). e-f: Bacteria (black arrows), single cell necrosis (yellow arrows) and aggregates of erythrocytes) observed within the spleen of the fish.

4. Discussion

The present work represents the first phylogenetic study by MLSA/MLST of the fish pathogenic bacterium *P. anguilliseptica* originating from a range of different geographic localities, fish species and time of isolation. Reconstructing the phylogenetic relationships between all isolates subject to analysis revealed a considerable degree of genetic homogeneity, though several differences were ultimately identified. An isolate of epidemiological relevance for Norwegian lumpfish aquaculture was administered as the infectious agent in the first cohabitant challenge study this bacterial species. The highly virulent properties of the bacterium were documented and Koch's pustulates fulfilled. Elucidation of the population structure, and the pathogenic nature of the bacterium associated with production of lumpfish in Norway, has provided valuable information of relevance for future vaccine development.

The phylogenetic resolution achieved by MLSA reveals a high degree of genetic similarity regardless of their different origins. Alignment of the final concatenated sequences (5 364bp) of all isolates identified an average sequence identity of ≥99.9%. Such lack of genotypic heterogeneity amongst the isolates demonstrates the conserved nature of the bacterium and indicates that it may be adapted to its niche as a fish pathogen. All isolates included in the analysis originate from clinically infected fish involving *P. anguilliseptica*. No environmental isolates were available or included. Had such isolates been available they may have revealed a greater intraspecific diversity. There is to date no literature published regarding the existence of environmental strains of the bacterium. All studies published so far have been conducted on isolates from clinically infected fish species. The possibility of the conserved genetic being the result of a pathogenic clone of the bacterial species specially adapted as a fish pathogen, cannot be ruled out.

Evolvement of niche-specialized genotypes is a well-known phenomenon observed within members of the *Pseudomonas* genus (Spiers et al., 2000). Rainey & Travisano (1998) demonstrated the adaptive radiation of niche-specialist genotypes of *Pseudomonas fluorescence* by cultivating strains of the bacterium in the heterogenous environment of a static broth culture. Among the factors which could help explain the enhanced evolutionary rate in pseudomonads is the sheer size of their genomes (Spiers et al., 2000). Bacterial genomes evolve (amongst other mechanisms) by acquiring and conserving sequences of ecological value through lateral gene transfer (Spiers et al., 2000). Likewise, sequences of non-ecological

significance are lost by deletion (Lawrence, 1999). The considerable size of genomes within *Pseudomonas* is therefore proposed to reflect the diversity of ecological niches encountered by most strains (Spiers et al., 2000). The genome size of *P. anguilliseptica* at 5.2 million base pairs is to be considered a large bacterial genome which could facilitate the evolvement of a conserved niche-specialized clone of the bacterium.

Although the isolates in general demonstrated a low degree of genotypic diversity, several key differences were ultimately identified. The phylogenetic reconstruction by MLSA of all 53 isolates revealed five clusters and two singletons (figure 3). Concordance between MLSA and MLST was also observed as seven different STs were identified by MLST (figure 4 and 5). Phylogenetic patterns identified by MLSA appear to be somewhat related to geographic origin with little or no discrimination between fish species or time of isolation registered. The Norwegian isolates were distributed between two of the major phylogenetic groups (group 1 and 2) (figure 3) formed following the reconstruction. Group 1 is the most dominant of the three groups with isolates originating from most geographic localities and fish species. Approximately a third of all Norwegian isolates are situated within this group while the rest are situated in group 2. This group consists solely of Norwegian isolates and seem to constitute a lone group of genotypes associated with clinical infections of lumpfish in Norway. Although he Norwegian isolates were distributed within two phylogenetic groups, no distinct differences were registered between these isolates related to geographic origin or time of isolation.

Interestingly, the phylogenetic patterns seem to reflect some of the few differences observed among isolates with regards to phenotypic characteristics. A general compliance was registered between all isolates subject to phenotypic characterization except for the production of arginine dihydrolase and vibriostatic sensitivity. Three isolates (NVIO 11214, NVIO 50-2255 and NVIO 10973), of a total of five proved positive for production of arginine dihydrolase (table 11). These isolates are originally derived from lumpfish, Baltic herring and turbot in Spain, the Baltic sea and Faroe Islands. All three isolates belonged to the same sequence type (ST2) situated in group 1 (figure 3). Likewise, isolate NVIO 8905 and NVIO 9976 derived from wolfish and lumpfish in Norway were the only isolates to demonstrate vibriostatic sensitivity (table 11). These isolates clustered together within group 2 also constituting identical sequence type (ST 1). Thus, proposing the possible distinct phenotypic characteristics attributed certain sequence types.

Previous studies conducted on *P. anguilliseptica* indicate that the patterns identified might also be related to the existence of several serotypes. Seven of the isolates included in the studies of López-Romalde et al., (2003a) and López-Romalde et al., (2003b) are also represented in the present study. NVIO 11214, NVIO 11215, NVIO 11216, NVIO 11217, NVIO 11219 and NVIB 50-2258, previously characterized by López-Romalde et al. (2003) as serotype O1, were all situated within the same phylogenetic group (group 1)(figure 3). Consequently, it is reasonable to believe that all isolates within this group also belong to serotype O1. Additionally, the singleton of NCIMB 1949 is the same type strain used in the serological study which was found to possess antigenic characteristics compatible with serotype O2.

Noticeably, all but one of the Irish lumpfish isolates included in this study clustered closely to the type strain situated within group 3 (figure 3). The putative serological differences between isolates in group 1 (O1) and NCIMB 1949 (O2) suggest that these Irish isolates might potentially also belong to serotype O2. Future antigenic characterization is required to determine whether this is the case or not. The Irish isolate NVIO 11302 deviates however, from this pattern and is situated within group 1 (figure 3). The distinct clustering and identical genotype of all other Irish isolates questions the placement of NVIO 11302. Such an anomaly could be due to the existence of several genotypes associated with disease in Irish lumpfish. The identical genotype shared with several Norwegian lumpfish-isolates and the known export of lumpfish roe from Norway to Irish producers (Tande (Editor), 2018), opens for the possibility of this genotype being introduced via Norwegian lumpfish roe. However, the likelihood of a potential error related to the submission and/or processing of the isolate cannot be out ruled. Control measures will be implemented to ensure the phylogenetic relationship between these isolates prior to future publication.

As for the Norwegian isolates, eight out of a total of 26 isolates were situated within group 1, thought to solely consist of isolates of serotype O1. The remaining, and thereby majority of Norwegian isolates were situated in group 2 approximately equally, if not slightly more distant related to group 1 than the type strain of NCIMB 1949 within group 3 (figure 3). This raises the question whether the isolates of group 2 represents either of the two serotypes or if they constitute a completely different serotype. Characterizing the serological properties of the Norwegian isolates situated within this group is therefore necessary with respect to the potential development of a vaccine against the bacterium. The fact that all Norwegian isolates are distributed between two of the major phylogenetic groups (1 and 2) complicates the identification of a suitable candidate strain for vaccine development. Future antigenic studies

could help determine the protective abilities of a monovalent vaccine or if development of a multi-genotype vaccine is required.

Interestingly, isolates of identical genotype were identified from lumpfish in Norway and diseased Atlantic salmon in Finland (figure 3). One of the Norwegian isolates in question is NVIO 10039 which was administered as the infectious agent in both challenges. Given the identical genotype and the pathogenic nature of the bacterium this suggests that *P. anguilliseptica* might potentially pose a threat to cohabiting salmonids in commercial net pens. This supports the risk assessment conducted by Rimstad et al. (2017). However, considering the ever-increasing number of outbreaks among lumpfish (table 1) and the lack of reported infections in cohabiting salmonids indicates that Norwegian salmon are not at high risk of cross-species transmission. Furthermore, these are only assumptions based on the phylogenetic reconstruction performed with this actual MLSA scheme. An improved MLSA scheme or application of analyses of higher resolution such as WGS is required to achieve satisfactory support values to determine the true phylogenetic relationship between these isolates. Nonetheless, establishment of a challenge model to test salmon susceptibility to a Norwegian lumpfish strain is necessary to evaluate the putative risk of cross-species transmission

Like the work of López Romalde (2005), an effort was made in the present study to try and describe any intergenetic heterogeneity amongst the three major phylogenic groups formed following the MLSA. A set of primers were designed to amplify rapidly evolving loci of repeating sequences called Variable-Number of Tandem Repeats (VNTRs). By amplifying several loci for each bacterial isolate, the goal was to describe any intra-MLSA-clade heterogeneity observable by conventional gel-electrophoresis of the PCR products. A 2% agarose gel run at low voltage (60V) for two hours was able to distinguish between amplicons from the selected test group of isolates. Thus, proposing the existence of additional genomic differences not describable by the actual MLSA scheme. Unfortunately, time and financial limitations made the analysis inapplicable on all isolates in this study at this stage. Initial tests proved promising and several loci displayed a higher degree of strain resolution than what was achieved by MLSA. It remains to be tested in full scale to determine whether it can achieve an increased discriminatory power to further distinguish the isolates located in the major phylogenetic groups.

The disease associated with a systemic infection involving *P. anguilliseptica* was successfully reproduced by administrating an isolate of the bacterium originating from a clinical outbreak related to production of lumpfish in Norway. Combined with the subsequent re-isolation of the bacterium from diseased individuals, this fulfills Koch's postulates.

Data from the pilot challenge gave early indications that the bacterium might constitute a primary pathogen associated with disease in lumpfish. The i.p. dosages applied in the experiment spanned a wide range of bacterial concentrations with all but the lowest infection dose of $4.3x10^2$ cfu/fish causing a cumulative mortality of 100% (figure 6). This dose-response effect was further supported by data from the cohabitant challenge. An almost indistinguishable effect was registered between the two challenge models with a cumulative mortality of 100% registered at dosages down $2.1x10^3$ cfu/fish (i.p) and 4.7x10 cfu/fish (i.m.) (figure 9-12). Such bacterial concentrations are considered as low compared to similar challenge models established for other pathogenic bacteria associated with disease in lumpfish (Ellul et al., 2018; Rønneseth et al., 2017). Cumulative mortality induced by infection dosages administered by i.p. and/or i.m. injections are only indicative of the virulent properties of the bacterium. The establishment of a horizontal infection route through naïve cohabiting fish displays the truly pathogenic nature of the bacterium.

A definitive cohabitant effect was achieved in all parallels established for the two separate challenge models (figure 9-12). The first onset of disease and subsequent mortality were registered simultaneously across all cohabitant fish groups. An almost identical development in cumulative mortality supports the hypothesis of the bacterium being the ultimate causative agent of the disease. This is further supported by the re-isolation of *P. anguilliseptica* moribund fish. The average cumulative mortality between 50-60% among cohabitant fish groups suggests that the bacterium is to be regarded as a primary pathogen associated with disease in lumpfish.

Distinct clinical and pathological differences were observed between fish challenged by injections and horizontally infected cohabitant fish. Externally, moribund and deceased fish injected with challenge material displayed few signs of disease in each of the two challenges. The pathological changes observed upon necropsy were also scarce, with accumulation of ascites fluid and splenomegaly being the most prominent lesions. In contrast, moribund cohabitant fish displayed a greater clinical and pathological repertoire both externally, upon necropsy and by histology.

Reproducing the disease by mimicking natural conditions through cohabitation, as recommended by Magi et al., (2009), resulted in a clinical state of disease characterized as a hemorrhagic septicemia. Abdominal distension, eye lesions and hemorrhages observed at the fin basis and ventral parts of the fish, are all generally in compliance with previous pathological descriptions made from other naturally infected fish species (Berthe et al., 1995; Magi et al., 2009; Wiklund and Bylund, 1990). So are also the splenomegaly, accumulated ascites and petechial hemorrhages at and within the adipose tissue of visceral organs (figure 14) (Wiklund, 2016). Gross pathological findings not previously described in other fish species are the hemorrhages observed within the brain and operculum, as well as the fibrinous-like coat surrounding the liver (figure 14).

Histopathological lesions were generally characterized by large numbers of bacteria within all tissues examined. Few signs of inflammatory responses were observed considering the substantial amounts of bacteria present intravascularly and throughout the surrounding tissues. A moderate increase in circulating leukocytes and sub-epithelial infiltration of inflammatory cells in the kidney capsule were among the only observable immune responses registered. The fibrinous-like coat surrounding the liver observed upon necropsy displayed histologically as a slightly eosinophilic and homogenous matrix with excessive amounts of bacteria within (figure 17). The underlying cause of such a formation is uncertain, though several diseases characterized as circulatory disorders are known to form a fibrinous “pseudo-membrane” surrounding the liver. Fibrin-specific staining remains to be tested to determine the composition of the matrix. Still, the most prominent lesions were observed within the kidney and spleen of the fish which revealed multifocal to diffuse hemorrhagic necrosis predominantly in the hematopoietic tissue. These major alterations are considered probable causes of death.

Hemorrhages occur due to damages inflicted on the vascular endothelium. They are usually caused by trauma, neoplasia, necrosis, infection or inflammation (Bruno et al., 2013). The hemorrhagic lesions observed are thought to be related to the massive numbers of intravascular bacteria throughout the fish (figure 8). Although the toxic properties of the bacterium have not been described in detail, proposals have been made regarding the bacterium’s ability to produce exotoxins such as exo-enzymes (Austin and Austin, 2007). Despite the general lack of metabolic reactivity, the bacterium has demonstrated a variable ability to metabolize protein (gelatin) and lipids (Tween 80) (López-Romalde et al., 2003a). Thus, suggesting that production of proteases and lipases might constitute virulent mechanisms capable of damaging the vascular endothelium when bacteria are abundant (Austin and Austin, 2007).

No distinct tissue tropism has previously been reported associated with clinical infections involving *P. anguilliseptica*. Nor has there been observed in this study. Differences in pathology induced by the bacterium are most likely restricted to the different host species rather than an organ/tissue specific manifestation of disease.

The different routes of infection are known to impact the onset of disease and pathology induced in infection challenges (Magi et al., 2009). Challenge material administered by i.p and/or i.m. injections bypasses the fish' physical immunologic barriers. Thus, high concentrations of bacteria are deposited within the fish at once enabling a systemic infection in a relatively short time frame. The lack of distinct clinic and pathology among these fish groups is probably due to the rapid onset of disease and subsequent death of the fish. Naïve cohabiting fish are in contrary exposed to relatively low concentrations of bacteria over an extended period of time. By replicating a natural infection route through horizontal transmission, all parts of the fish' innate immune system are able to respond to the infection in a timely manner. These factors are thought to reflect the differences in clinic, gross pathology and histopathological changes observed between the separate fish groups.

Although all dosages applied during the cohabitant challenge were similar to the dose inducing LD_{65} in the pilot challenge, all but one i.p administered injection dose ultimately induced a cumulative mortality of 100%. Differences in fish size and familial sensitivity are both factors known to impact the ability to reproduce the dose-response effect in challenge studies conducted on fish (Magi et al., 2009). The average weight of the fish taking part in the cohabitant challenge was 141.2% that of the fish in the pilot challenge. Combined with slightly increased biomass per tank compared to the pilot challenge, this caused a 171.4% difference in fish density. Fish for each of the two challenges were also obtained from different production facilities with most probably different familiar descent. These factors could help explain the somewhat deviant relationship between dosages applied and cumulative mortality achieved across the two challenges.

Administrative and economic limitations have influenced the experimental setup for the infection challenge which might have caused systemic errors to influence the outcome of the results. Among these errors is the lack of a control group to rule out any influence inflicted by the agent administration itself. Administrating challenge material on individual fish through a syringe could potentially cause mechanically inflicted injuries or compromise the fish making

it more susceptible for infectious agents. A control group added to the pilot and each of the two cohabitant challenge models would help reveal any influence on the cumulative mortality not solely attributed the challenge material. Another potential systemic error with the challenge setup is the inclusion of multiple fish groups injected with separate dosages kept within the same tanks. Preferably, each injected fish group should have been kept within separate tanks with naïve cohabiting fish to eliminate the possible altering effect they may have on each other. Fish shedding bacteria due to the infection might cause a cohabitant effect among the injected fish groups themselves.

5. Future perspectives

The high degree of genetic relatedness observed among isolates of *P. anguilliseptica* may be indicative of the bacterium being well adapted to its niche as a fish pathogen. The fact that all isolates originate from clinically infected fish species could however have facilitated the genetic homogeneity observed. The lack of environmental isolates questions whether the isolates included in the present study represents a clone of the bacterium specially adapted as a fish pathogen or not. Future studies regarding the existence of environmental isolates of *P. anguilliseptica* could potentially reveal a greater intraspecific diversity than that revealed in this study.

The documented pathogenicity of *P. anguilliseptica* associated with disease in lumpfish proposes a potential need for future vaccine development. Though, the isolate administered as the infectious agent only represents one of two phylogenetically distinct groups identified from Norwegian isolates. Genotypic heterogeneity observed among Norwegian lumpfish isolates complicates the selection of a suitable candidate strain. Putative serological differences between the major phylogenetic groups revealed by MLSA calls for complementary antigenic studies to investigate the serological characteristics of these isolates. Potential differences in virulent properties between the phylogenetic groups can also not be ruled out. A future infection challenge administrating an isolate representing the dominant group of genotypes from lumpfish in Norway could reveal potential differences in pathogenicity. Identical genotypes originating from diseased lumpfish in Norway and salmonids in Finland propose a potential risk of cross-species transmission. The establishment of a challenge model to evaluate the susceptibility of salmon to a Norwegian lumpfish strain is therefore also recommended.

6. Conclusion:

Phylogenetic resolution achieved by MLSA/MLST displayed a high degree of genetic similarity among isolates of *P. anguilliseptica*, yet ultimately described seven different genotypes. Several genotypes were identified from Norwegian lumpfish isolates, whereas the majority made up a single genogroup from clinically infected lumpfish. Thus, proposing the existence of a dominant group of closely related genotypes associated with disease in production of lumpfish in Norwegian aquaculture. The cumulative mortality of i.p/i.m. infected fish and the establishment of a cohabitant effect suggest that the bacterium probably constitutes a primary pathogen in diseased lumpfish. The ever-increasing numbers of outbreaks involving *P. anguilliseptica* in Norwegian aquaculture and the pathogenic nature of the bacterium, demonstrates a necessity for vaccine development. Future studies remain however to determine a suitable candidate strain with regards to putative antigenic differences among Norwegian isolates from lumpfish.

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