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TECHNICAL REPORT

An overview of emerging diseases in the salmonid farming industry

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Cover Photo: Ole Bendik Dale.

Foreword

Dear reader,

Although we are still early in any domestication process, salmon is a relatively easy species to hold and grow in tanks and cages. Intense research to develop breeding programs, feed formulae and techniques, and technology to handle large animal populations efficiently and cost-effectively, are all parts of making Atlantic salmon farming likely the most industrialized of all aquaculture productions today. Consequently, salmon farming is an important primary sector of the economy in producing countries; according to Kontali Analyse[1], global production of Atlantic salmon exceeded 2.3 million tons in 2017 and today salmon is a highly asked-for seafood commodity worldwide.

However, new diseases – emerging diseases - occur "every second year" and health challenges are constantly a most important limiting factor on salmon production. The world organization for animal health (OIE) defines an emerging disease as "a disease, other than listed diseases, which has a significant impact on aquatic animal or public health resulting from a change of known pathogenic agent or its spread to a new geographic area or species; or a newly recognized or suspected pathogenic agent". Over the years, we have experienced such new diseases, emerging diseases, occurring frequently in the various aquaculture sectors. Some of these diseases have shown devastating effects locally, nationally as well as internationally by rapidly spreading through trans-boundary trade and other activities.

In this report we highlight and discuss six important diseases or health challenges affecting farmed salmon. We have identified them as emerging as there is new knowledge on agent dynamics, they re-occur or they are well described in one region and may well become a threat to other regions with the same type of production.

Knowledge sharing on salmonid production, fish health and emerging diseases has become a key prime awareness with dedicated resource and focus from the farming industry through groups such as the Global Salmon Initiative (GSI). Being proactive to prevent an introduction and having early detection systems in place to restrain an unwanted event to develop is necessary to help towards the ultimate goal of controlling emerging diseases.

Tomorrow you may go home from your lab, office or field work where you may just have started working on a completely new disease challenge; an emerging disease. Your knowledge, your competence and professional network will prove vital to move forward and help curb and solve this new situation. This report aims to be helpful for you, and for the global salmon farming industry, in this important work.

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CONTENTS

Sea Lice Resistance

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Introduction

Sea lice are parasitic crustaceans. The two species of sea lice which constitute the greatest health threat towards farmed salmonids are *Lepeophtheirus salmonis* and *Caligus rogercresseyi* in the Northern and Southern hemispheres, respectively (Boxshall and Bravo 2000, Burka et al. 2012). There are two *L. salmonis* subspecies; one in the Atlantic Ocean (*L. salmonis salmonis*) and one in the Pacific Ocean (*L. salmonis oncorhynchi*) (Yazawa et al. 2008, Skern-Mauritzen et al. 2014). *L. salmonis* and *C. rogercresseyi* have direct life cycles with three planktonic and five parasitic life stages, each separated by a moult. The parasitic life stages in *L. salmonis* comprise two chalimus stages, two pre-adult stages and one adult stage (White 1942, Hamre et al. 2013). *C. rogercresseyi* have four chalimus stages and one adult stage (González and Carvajal 2003). The adult females of both species are oviparous and the eggs hatch to planktonic larvae that are spread in the water masses. The development time from egg to adult is reduced with increasing temperature (González and Carvajal 2003, Samsing et al. 2016).

Infestations with sea lice are stressful for the fish (Bowers et al. 2000, Gómez et al. 2016). *L. salmonis* feed on the mucus, skin and blood of their hosts. Their feeding behavior can cause wounds and anemia in the fish which may lead to secondary infection and osmoregulatory problems (Pike 1989). The pathological potential depends on the number and life stage of lice compared to the size of the fish (Wagner et al. 2008). Larval lice spread between wild fish and farmed fish held in open net cages. Transmission of infestation between farmed and migrating wild salmonid populations has been reported in several areas (Krkosek et al. 2007, Gargan et al. 2012, Vollset et al. 2016). Quantification of the effect on wild salmonid populations, has however, been difficult.

Fish farmers need to control lice infestations to protect their fish from lice induced injury. The authorities in many countries have also set maximum permitted numbers of sea lice per fish (Anonymous 2012, Anonymous 2015, Anonymous 2016). These limits are intended to protect farmed fish, and (in some areas) to protect wild fish from lice of farm origin. Traditionally sea lice have been controlled using medicinal treatments. The available chemical classes for medicinal bath treatment of sea lice are organophosphates (active substance applied today: azamethiphos), pyrethroids (active substances applied today: cypermethrin and deltamethrin) and hydrogen peroxide. Avermectins (active substances applied today: emamectin benzoate and ivermectin) and benzoyl phenylureas (active substances applied today: di- and teflubenzuron and lufenuron) are applied as in-feed treatments. The licensing situation for the different classes of chemicals varies between countries (Aaen et al. 2015). With the exception of the benzoyl phenylurea lufenuron released in Chile in 2016 (Pérez 2016), globally the last introduction of a new active substance was emamectin benzoate in 1999. The limited number of available medicines for sea lice control combined with the high frequency of treatment over many years has therefore, led to development of resistance to chemical treatments in sea lice in many salmon producing areas. This is equivalent to resistance development seen in human, animal and plant parasites (Denholm et al. 2002).

Resistant sea lice negatively impact both farmed fish and fish farmers, and potentially also wild salmonids. Increased use of medicines, probably both in terms of treatment frequency and working concentration has been observed in Norway and Chile (Helgesen et al. 2014). More frequent applications of an active substance increases selection pressure towards development of resistant parasites and may increase the risk of treatment associated fish mortality. A number of preventive and curative non-medicinal methods have also been developed. These include cleaner fish, shielding skirts and physical lice removal treatments. Some of the non-medicinal treatment methods may possibly increase the risk of treatment-induced fish mortality compared to medicinal treatments (Hjeltnes et al. 2017). Increased treatment frequency increases the cost of sea lice control for the fish farmer (Liu and Bjelland 2014). Resistance may also have indirectly led to periods and episodes of increased lice number, which represents a health threat for

farmed salmon and possibly also wild salmonids (Hjeltnes et al. 2017).

The present chapter aims to present current knowledge on resistance to chemical treatment in sea lice. Such knowledge can help identify where resistance exists, how and where it is developing and avoid introduction of resistance to new areas. All of this in order to reduce the negative consequences of resistance.

Resistance definition and development

Resistance can be defined at the individual louse level as a genetically based decrease in susceptibility to a pesticide (Tabashnik et al. 2014). This property most likely developed independently of chemical treatments, but has been selected for by such treatments. It has for example been shown that decreased organophosphate susceptibility as a trait in sea lice was most likely present at a low frequency also prior to chemical treatments (Kaur et al. 2017). The degree of resistance and presence of multi-resistance (resistance towards more than one chemical class) may vary between individual sea lice (Jensen et al. 2017). The degree of resistance also varies between developmental stage and gender (Westcott et al. 2008, Whyte et al. 2014, Marín et al. 2015). The resistance patterns towards various chemical classes can, therefore, vary substantially between fish farms in both the degree of resistance and in the frequency of resistant parasites. These patterns can change drastically over a short time period. Infestation of lice from other fish farms or wild fish, with a different resistance pattern, can either increase or decrease the resistance problem on any particular farm.

Identification of resistance

Sea lice treatment efficacy and usage data for sea lice medicines, can, over time, give indications of development of resistance (Jones et al. 2013, Helgesen et al. 2014), but such data cannot be used for identification of resistance at the farm level. Changes in treatment efficacy and in medicinal usage may have other explanations than resistance (Lees et al. 2008).

Resistance at the farm level has traditionally been identified using toxicological tests on live parasites removed from the fish (bioassays). In a bioassay, groups of parasites are exposed to different concentrations of a given chemical for a given period of time. The parasites are then evaluated with respect to their physiological status (healthy, weak or dead). The chemical concentration immobilizing 50% of the parasites (EC 50) is calculated. Bioassays have been developed for resistance testing for pyrethroid, organophoshate, hydrogen peroxide and emamectin benzoate resistance (Tully and McFadden 2000, Sevatdal and Horsberg 2003, Bravo et al. 2008, Westcott et al. 2008, Helgesen et al. 2015). *In vitro* testing for benzoyl phenylurea resistance is difficult as these compounds target the moulting process, and the parasitic sea lice stages must be attached to a fish in order to moult.

Genetic tests are available for detection of organophosphate, pyrethroid and hydrogen peroxide resistance in *L. salmonis salmonis* (Kaur and Horsberg 2015, Nilsen and Espedal 2015, Kaur et al. 2016). Organophosphate resistance is caused by a mutation in the gene coding for the target molecule, acetylcholinesterase, and the test detects this mutation (Kaur et al. 2015). Pyrethroid resistance is detected by identifying a genetic marker that co-varies with resistance. The exact resistance mechanism or mechanisms have not been pin-pointed (Jensen et al. 2017). The hydrogen peroxide resistance test detects increased expression of an enzyme-coding gene. Increased expression of this gene has been found in hydrogen peroxide resistant parasites (Helgesen et al. 2017). The exact genetic signature for hydrogen peroxide resistance is not known. A genetic test for resistance to emamectin benzoate has not yet been developed. The resistance mechanisms in *C. rogercresseyi* have not yet been sufficiently described for development of genetic based resistance tests.

Results from single resistance tests should be interpreted with care. Toxicological tests have several steps in which human error can be introduced. Different laboratories often have different bioassay protocols, making comparison of inter-lab bioassay results difficult. Variation in degree of resistance between individual lice on a fish farm makes representative sampling difficult for both bioassays and genetic resistance tests, and natural variation is therefore also a possible source of error (Robertson et al. 1995).

Occurrence and management of resistance

A complete overview of the resistance situation in different countries is not possible to obtain, as the results from resistance tests are generally not publically available. In addition, treatment and infestation with lice originating from external sources can alter the resistance pattern rapidly. Several reports of resistance are, however, available in the scientific literature. In addition, there has been a national surveillance program on sea lice resistance in Norway since 2013 and a single large-scale surveillance study on resistance was conducted in Chile in 2013 and 2014.

The Canadian situation

In 2008 in Eastern Canada, reduced efficacy of emamectin benzoate was observed, in which only half of the qualifying treatments (17/33) analysed by Jones et al. (2012) were established to be effective. Resistance towards emamectin benzoate has also been detected using bioassays (Igboeli et al. 2012). Reduced treatment efficacy suggests the additional presence of both pyrethroid and organophosphate resistance in Eastern Canada (Whyte et al. 2014, Gautam et al. 2017). Whyte et al. (2014) showed that cage-level reduction in number of adult female and chalimus lice stages, following pyrethroid treatment, varied considerably but could be less than 50%. In the study described by Gautam et al. (2017) the overall effectiveness of bath treatment with organophosphate or hydrogen peroxide rarely reached 80% efficacy, regardless of assessment date (i.e. 1-7 days post treatment).

Organophosphate bioassays described by Whyte et al. (2016), conducted on lice from 2009-2012, showed increasing EC 50 values throughout the time period. All bioassay results showed values above those observed in sensitive lice collected in Norway in 2011 (Helgesen and Horsberg 2013). Van Iderstine (2017) conducted a limited hydrogen peroxide bioassay assessment on Bay of Fundy *L. salmonis* populations, and found that the EC 50s for this population were in a similar range to those described from resistant populations in Norway (Helgesen et al. 2015).

The mutation known to cause azamethiphos resistance was found in sea lice from farms in Atlantic Canada sampled in 1999, 2002 and 2009 (Kaur et al. 2017). Genetic studies have shown that the mechanism(s) underlying emamectin benzoate resistance is/are most likely similar for the entire Atlantic Ocean (Besnier et al. 2014). Both studies indicate a certain degree of lice exchange between Europe and Canada. If the reduced pyrethroid treatment efficacy observed in Eastern Canada was due to resistant parasites, the resistance mechanisms involved could therefore be expected to be identical to those found in sea lice in Norway (Nilsen and Espedal 2015).

Studies from British Columbia (BC) on emamectin benzoate efficacy have so far not found an established population of resistant sea lice (Saksida et al. 2013). However a novel genotype emerged in *L. salmonis oncorhynchi* in 2013 from the Klemtu region, which coincided with the first loss of efficacy of emamectin benzoate in Klemtu. In 2014, this genotype cluster was reduced in Klemtu and associated with a return of emamectin benzoate efficacy, whereas first observations of reduced efficacy were observed in another region also coinciding with the first presence of the novel genotype. With removal of emamectin benzoate treatment (in 2015 hydrogen peroxide was first introduced in BC, for use in rotation) and inbreeding of the local population with the dominant wild type genotype, this novel genotype associated with reduced emamectin benzoate efficacy has not returned, nor has the phenotype of reduced efficacy of emamectin benzoate (Messmer et al., *In press*). Of further interest is that 748/778 single nulceotide polymorphisms that make up this novel genotype were located on chromosome/linkage group 5, which was also associated with emamectin benzoate resistance in the Atlantic Ocean (Besnier et al. 2014). Figure 1 shows the amount of fish treated with avermectins in BC. The increased use in 2014 despite reduced production of salmon could be explained by reduced treatment efficacy. Assumptions for Figure 1, 2, 4 and 5 are given in Grave et al. 2004. Ivermectin calculations were based on Johnson and Margolis 1993. Lufenuron calculations (for Chile) were based on Joint FAO/WHO food standards program codex committee on residues of veterinary drugs in foods (http://www.fao.org/fao-who-codexalimentarius/sh-proxy/fr/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FMeetings%252FCX-730-23%252FWD%252Frv23_10e.pdf)

In Atlantic Canada, from 2010, the aquaculture bay management areas in New Brunswick developed a coordinated treatment plan to coordinate treatment strategies, maintaining lice thresholds in each area, product rotations and synchronized treatments (http://www.atlanticfishfarmers.com/publications/). This has been continued through 2017. In BC, infestations involving more than 3 mobile lice per fish between March 1 and June 30 require compulsory treatment or harvest, whereas infestation intensities over this threshold between July 1 and February 28, require elevated monitoring, alternative treatment or harvest (Anonymous 2016).

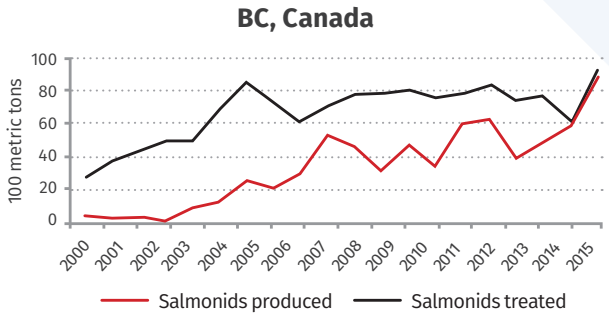

Figure 1: Production of Atlantic salmon in Canada (black line) and the biomass of salmon treated against sea lice using avermectins (ivermectin and emamectin benzoate) (red line). Data on hydrogen peroxide usage (unknown) is not included. The data were collected from Fisheries and Oceans Canada (http://www.pac.dfo-mpo.gc.ca/aquaculture/reporting-rapports/therapeut/index-eng.html?wbdisable=true).

The Chilean situation

Resistance has been described towards emamectin benzoate, pyrethroids, and organophosphates in Chile (Bravo et al. 2008, Helgesen et al. 2014, Marín et al. 2015).

In the large-scale spatial study performed in 2013 and 2014, sensitivity was evaluated simultaneously towards pyrethroids and organophosphates (Marín et al. 2015). Reduced sensitivity towards pyrethroids was observed in both the Los Lagos and the Aysén region, in a total of three different areas/macrozones. Reduced sensitivity towards azamethiphos was observed in several farms in one area, with occasional observations in the two other areas. The observed sensitivity varied between farms within each area.

Figure 2 shows the amount of fish treated with sea lice medicines in Chile. Although there may be multiple explanations for the increase in number of medicinal treatments in 2006, resistance towards emamectin benzoate is probably an important explanatory factor (Bravo et al. 2008). The massive increase in use indicates a widespread resistance. Pyrethroids were then introduced to the market and the total usage of medicines fell, only to increase again from 2011, most likely caused by the development of resistance towards pyrethroids (Helgesen et al. 2014). This resistance was parried by the introduction of organophosphates and again, the total usage dropped.

The Specific Sanitary Program of Surveillance and Control of Caligidosis (PSEVC) describes Chilean regulations concerning *C. rogercresseyi* (Anonymous 2015). The program regulates mandatory farm level surveillance of lice, but does not include a specific resistance management program. It defines control measures if the abundance of ovigerous females is three parasites or greater per fish. These measures include intensified surveillance, coordinated treatments, rotation of active ingredients applied for bath treatment (a given active ingredient can only be consecutively applied three times during a production cycle), dissemination control, and early harvest of the infested biomass. The authority (National Service of Fisheries and Aquaculture) has defined high-risk management areas. The farms in these areas must have a common lice control strategy, which may include surveillance of lice sensitivity. To date there is no information available on the results of measures instigated following identification of reduced levels of sensitivity in any particular farm or area.

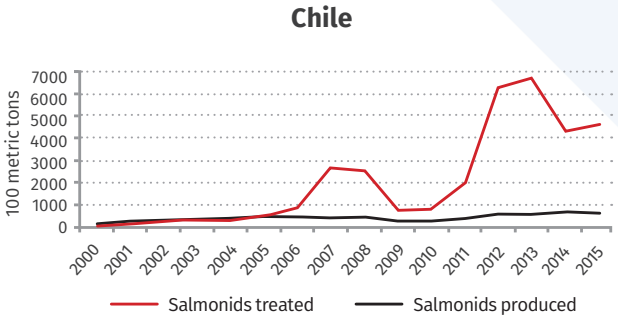

Figure 2: Combined production of Atlantic salmon and rainbow trout in Chile (black line) and the biomass of these species treated against sea lice, with available treatments: organophosphates, benzoyl phenylureas, pyrethroids and avermectins (red line). Data on hydrogen peroxide usage (unknown) are not included. The data were provided by Sernapesca. Data up till 2012 are published in Helgesen et al. 2014.

The PSEVC opens up for the use of non-medicinal lice control methods. The farmers must notify the authority before implementation of such methods. Until now alternative strategies have not been commonly used in the Chilean salmon industry.

The Norwegian situation

Resistance has been identified towards pyrethroids, organophosphates, emamectin benzoate and hydrogen peroxide (Sevatdal et al. 2005, Helgesen and Horsberg 2013, Helgesen et al. 2015).

The resistance surveillance program, which started up in 2013, is based on bioassay monitoring of resistance towards pyrethroids, organophosphates, emamectin benzoate and hydrogen peroxide (included from 2014) (Grøntvedt et al. 2014, Grøntvedt et al. 2015, Grøntvedt et al. 2016, Helgesen et al. 2017). During these years, resistance towards all included chemical classes has spread in both northerly and southerly directions. However, resistance was present along most of the coast already when the program started. Genetically based resistance towards organophosphates has also been detected in sea lice from wild salmonids caught in Norwegian fjords (Fjørtoft et al. 2017). In 2016, resistance towards all four chemical classes was observed all along the Norwegian coast, although with some regional differences (see Figure 3). The frequency of hydrogen peroxide resistance was lower than the level of resistance towards the other medicines (Helgesen et al. 2017). During a study of 56 fish farms performed in 2012-2014 the mutations responsible for organophosphate resistance were found in all coastal regions of Norway. The frequency was shown to be lower in the far south and far north of Norway (Kaur et al. 2016).

There was an increase in the use of medicinal treatments in Norway in 2008, as shown in Figure 4. Although there may be several explanations for this increase, resistance towards emamectin benzoate, pyrethroids and organophosphates, are probably important explanatory factors (Sevatdal et al. 2005, Kaur et al. 2015). The total medicine usage was reduced from 2015, probably due to a combination of widespread resistance and available alternatives to medicinal treatments (Grøntvedt et al. 2016).

Management of resistance is covered by three different Norwegian legislations: the general legislation relating to management of sea lice and in two regional legislative documents relating to salmon production in two specific zones in Norway (Anonymous 2010, Anonymous 2010b, Anonymous 2012). According to the general legislation, resistance surveillance is mandatory in coordinated sea lice plans, medicines may only be used when good efficacy can be expected, measures against resistant lice on a farm must be implemented, and suspected resistance must be reported to the authorities. According to the zoning regulations, fish farms within a zone are obliged by law to share information and coordinate medicinal treatments and resistance testing.

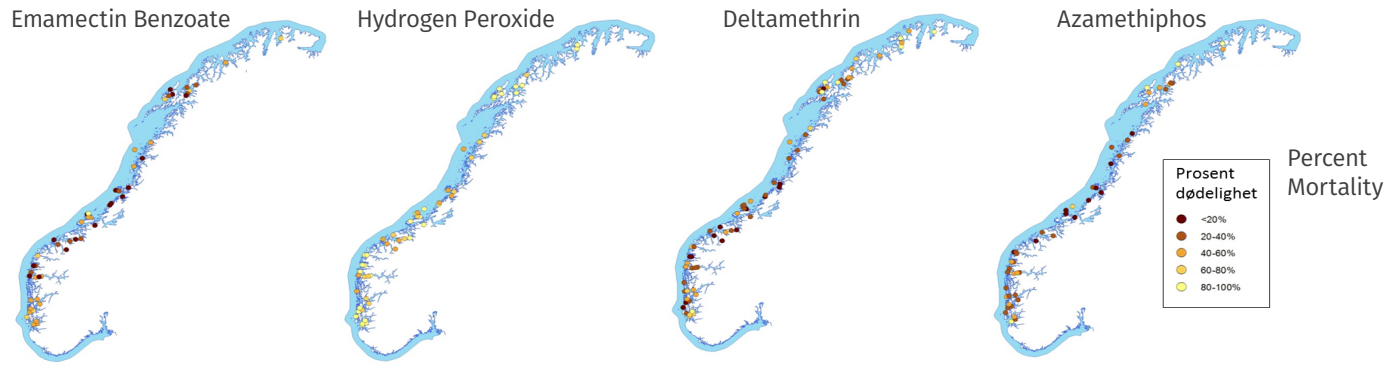

Figure 3: Categorical louse mortalities in bioassays with given concentrations of emamectin benzoate, hydrogen peroxide, deltamethrin and azamethiphos. The colors of the dots indicate a category of mortality. The darkest colors are indicative of lowest mortality and thereby highest frequency of resistant parasites. The results are from the surveillance programme for resistance to chemotherapeutants in sea lice in Norway 2016 (https://www.vetinst.no/overvaking/lakselus-resistens).

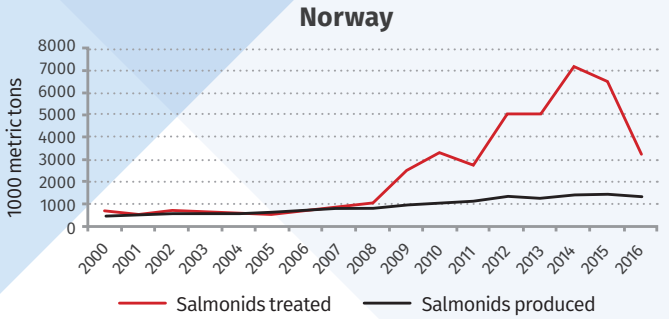

Figure 4: Combined production of Atlantic salmon and rainbow trout in Norway (black line) and the biomass of these species treated against sea lice, with all available treatments: organophosphates, benzoyl phenylureas, pyrethroids, avermectins and hydrogen peroxide (red line). The data were collected from the Norwegian Institute of Public Health (https://www.fhi.no/hn/legemiddelbruk/fisk/2016-salg-av-lakselusmidler-er-synkende/)and Statistics Norway (https://www.ssb.no/jord-skog-jakt-og-fiskeri/statistikker/fiskeoppdrett/aar).

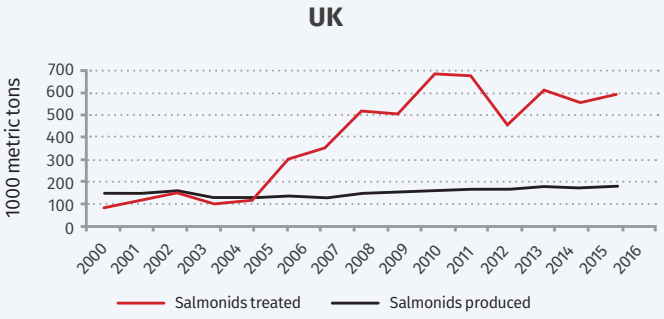

Figure 5: Combined production of Atlantic salmon, rainbow trout and artic char in the UK (black line) and the biomass of these species treated against sea lice, with available treatments: organophosphates, benzoyl phenylureas, pyrethroids and avermectins (red line). Data on hydrogen peroxide usage is not included (unknown amount). The data were aggregated from data available from Scotland's aquaculture (http://aquaculture.scotland.gov.uk/) and Food and Agriculture Organization of the United Nations (www.fao.org). Production data from 2016 was estimated (1.02 X production in 2015).

During the period these regulations have been effective, the resistance problem has increased in Norway (Jansen et al. 2016). The exact effect of the legislation on development of resistance, has not, however, been evaluated.

Other possible measures to combat resistance have been described in the Norwegian government's action plan against resistance in sea lice, which was published in 2017 (Anonymous 2017). This plan advocates the use of non-medicinal treatments. In 2016, use of non-medicinal treatments based on fresh water, temperate water or mechanical treatments increased more than six times compared to 2015 (Helgesen et al. 2017

The Scottish situation

Resistance has been shown towards pyrethroids, organophosphates, emamectin benzoate and hydrogen peroxide (Jones et al. 1992, Treasurer et al. 2000, Sevatdal et al. 2005, Lees et al. 2008, Heumann et al. 2012). There was an increase in the use of medicinal treatment in 2006 in Scotland, as shown in Figure 5. Although there may be several explanations for this increase, resistance towards emamectin benzoate, pyrethroids and organophosphates, are probably important explanatory factors.

Discussion

Resistance management aims to slow the development and/or reduce the spread of resistance. Available knowledge on this topic is based on the results of specific studies and practical experiences from other parasitic species (Denholm 2013, McEwan et al. 2015, McEwan et al. 2016). The goals of resistance management can be both in accordance with and in contradiction to the overall goal of reducing the number of sea lice. Management of resistance in sea lice requires cooperation between industry, authorities and research partners (Denholm et al. 2002).

The speed of resistance development in a farm or an area depends on several factors, such as the intensity of medicinal treatments, the selective power of each treatment, the availability of sensitive genotypes amongst external populations of lice, wild fish or neighboring farms, and the rotation regimes for medicinal treatments. The selective power of a single pyrethroid or organophosphate treatment has been shown under laboratory conditions. Pyrethroid treatments removed more than 70% of the sensitive lice, while organophosphate treatments removed more than 80% of the fully or partly sensitive lice (Jensen et al. 2017). A modelling study showed that larger numbers of wild hosts compared to the number of farmed hosts reduced the speed of resistance development in sea lice (McEwan et al. 2015). This is one of the possible explanations for the favorable resistance situation in British Columbia, Canada (Saksida et al. 2013). The model assumes that sensitive parasites spread from wild fish to farmed fish. This is however not always the case. The mutation giving rise to organophosphate resistance was found in sea lice collected in 2014 from wild salmonids along the entire

Norwegian coastline (Fjørtoft et al. 2017).

Rotational use of medicines with different modes of action is mandatory according to Chilean sea lice regulations, and is recommended in the Norwegian sea lice therapy guidelines of 2012 (Anonymous 2012b, Anonymous 2015). In a modeling study on the effect of different rotation and combination regimes for medicinal treatments, combination of two efficient and chemically unrelated substances postponed resistance development the longest (McEwan et al. 2016). New medicines have however not been introduced for sea lice treatments in the Atlantic area for many years, and resistance has developed. Rotation and/or combinations of fully effective drugs is/are therefore difficult to obtain.

Reduced intensity of medicinal treatment will slow development of resistance (Denholm et al. 2002). Many non-medicinal preventive or treatment methods have been developed and commercialized over the last several years. The number of medicinal treatments can also be reduced by optimising sea lice management strategies. A Norwegian study showed that counting lice on more fish per cage, counting lice on fish from all cages instead of half the cages, managing lice on a cage level instead of farm level and monitoring of all mobile stages instead of the adult female stage, reduces the number of lice treatments necessary to maintain the lice level below maximum limits (Aldrin and Huseby 2017). The number of sea lice treatments depends on the local density of farmed salmonids (Jansen et al. 2012).

Principles of the Bay management area plan for New Brunswick Canada have included reduced fish or rearing density on the farms since 2008 and show a reduction from 41,000 tons in 2006 to 26,000 tons in 2008 (Chang et al. 2011). The reduction in biomass in the Bay of Fundy in Atlantic Canada could therefore be expected to reduce the need for sea lice treatment and thereby reduce selection pressure towards resistance.

In one area in the Los Lagos region and two areas in the Aysén region in Chile, only sensitive parasites were found in the 2013/2014 survey (Marín et al. 2015). These areas were completely or mainly located on the continental coast and experienced lower salinities than those located closer to the ocean (the remaining areas). The Aysén region had lower sea temperatures compared to the Los Lagos region. Given this, it is possible to infer that parasite abundance may be maintained at lower levels in the areas with an estuarine water influence and lower temperatures. A greater number of sea lice treatments are likely to be applied in the areas with greater parasite abundance than in the areas with low parasite abundance (Jansen et al. 2012). Frequent treatment in an area has been shown to explain the appearance of more resistant parasites (Jansen et al. 2016). This may explain some of the differences in sensitivity observed between geographical areas. Taking advantage of the environmental conditions that maintain low parasite abundance on fish could delay the development of resistance, and thereby maintain sensitive lice populations.

When resistance is present in an area, there is no evidence that it will disappear completely. This has been shown on a large scale in Norway for organophosphate resistance. Despite non-usage over a period of nine years (Helgesen et al. 2014), the same resistance mutation was found in lice collected both prior to and after this nine year period (Kaur et al. 2015). Resistance genes can therefore persist in the sea lice population under field conditions and without selection pressure for at least nine years and this may be exacerbated in areas with small wild salmon populations. Thus, one should try to avoid introduction of resistant lice to a new area through transport of lice infested fish.

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Amoebic Gill Disease

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Etiology

Amoebic gill disease (AGD) is a gill pathology mainly associated with farmed Atlantic (*Salmo salar*) and Pacific Salmon (*Oncorhynchus* spp.; Mitchell & Rodger, 2011) although other species of farmed marine fish may also be affected. The etiological agent for AGD was initially thought to be *Neoparamoeba pemaquidensis* (Kent et al., 1988), but attempts to induce AGD using cultured *N. pemaquidensis* in Atlantic Salmon failed (Morrison et al., 2007). Young and coworkers (2007) subsequently identified a new amoebic species, *N. perurans*, associated with AGD. Twenty years after the first description, *N. perurans* was confirmed as the etiological agent of AGD by Crosbie and coworkers (2012) who fulfilled Koch's postulates and induced the disease in Atlantic salmon. Besides the fact that *N. perurans* is a ubiquitous free-living protozoan parasite, little information is available on the biology of this organism. *Neoparamoeba* spp. belong to the family of Vexilliferidae which contain an endosymbiont, *Perkinsela amoebae* (parasomes) (Young et al., 2014). *N. perurans* colonizes the gills and induces epithelial cell proliferation, which leads to the fusion of gill filaments and inter-lamellar inclusion of eosinophils (Lovy et al., 2007). These ultrastructural modifications may cause a loss of main gill functions such as respiration and osmoregulation which may compromise growth and ultimately survival.

Risk factors

Currently the disease is pan-hemispheric and reported in most countries that farm salmon: Norway, Chile, Scotland, Ireland, Tasmania and recently, Canada and the USA. Although the parasite affects several host species such as Turbot (*Psetta maxima*), Rainbow Trout (*Oncorhynchus mykiss*) and Sea Bass (*Dicentrarchus labrax*), Atlantic salmon remains the most susceptible species (Mitchell & Rodger, 2011). The disease is a complex multifactorial pathology involving physical and biological environmental parameters. AGD can affect the physiology of the fish but additional stress factors such as high temperature and/or algae blooms can exacerbate the morbidity and mortality. High temperatures ranging between 12 and 20°C are considered the main stressor for fish affected by *N. perurans* although AGD outbreaks have been reported in Scottish farmed Atlantic Salmon at a water temperature as low as 7.5°C (Rodger, 2014). Conversely, increasing sea water salinity is considered a key risk factor for AGD (Rodger, 2014), as the gill is a major organ of ion regulation and damaged gills contribute as a stress element through osmoregulatory imbalance. To complicate matters, recent studies have shown that *N. perurans* can adapt to lower salinity environments through osmoregulation (Lima et al., 2016a, 2016b). A transitional pseudocystic phase may allow *N. perurans* to survive freshwater treatments and re-emerge when conditions are optimal (Lima et al., 2016b).

Diagnosis

AGD is diagnosed by routine macroscopic screening of gills and comparing the observed 'patches' with gill score cards in addition to microscopic examination of direct gill preparations (Taylor et al., 2009). Monitoring the presence of the parasite in the marine environment currently represents the best tool for mitigation of outbreaks and guide treatments. Treatments are based on the abundance and presence of white mucoid patches on the gill structures indicating presumptive parasite infestation. However, these methods are nonspecific and need to be confirmed by more specific diagnostic tests.

Molecular testing should be considered complementary to gill scoring as an early detection method prior to the infestation becoming macroscopically visible. The recent development of molecular testing such as PCR and qPCR methods to detect *N. perurans* on fish tissues provides fast and more specific results compared to visual assessment. These molecular tests can help detect the parasite at the early stage of the infection before the spread of the disease. Early detection will therefore allow a better control of the disease and help deliver more efficient plans for mitigation by managing the level of infection. Several methods have been developed for testing (Young, et al., 2008; Bridle et al., 2010; Fringuelli, et al, 2012; J. K. Downes et al., 2015;).

Recently, a comparative study evaluated available qPCR assays and selected a preferred method as the best assay to detect *N. perurans* from gill swab samples (Downes et al., 2017).

The British Columbian situation

On the Pacific coast of North America, gill disease related to *N. pemaquidensis* was observed in sea water reared US Coho Salmon in 1985 (O. *kitsutch*; Kent et al., 1988). Since then, no AGD-related outbreaks have occurred that could be considered a threat to fish farming along the Pacific coast of North America. However, a high prevalence of low-level infestation has been observed recently on farmed fish in coastal BC waters. These observations are putatively related to the warmer water now experienced on the Pacific West Coast of Canada where temperatures and salinity have risen above recorded norms in the past few years. The first clinical signs of the disease were observed in the late spring and summer of 2014 and achieved a peak during the fall. Stemming from these observations, a project was initiated involving local salmon farming companies, the BC Centre for Aquatic Health Sciences (BC CAHS) and the University of Tasmania to investigate the incidents, identify detection methods and possible mitigation strategies. This work included a focus on isolation and culture of *N. perurans* from farmed fish from the west coast of Vancouver Island (BC). Phylogenetic analysis of 18S ribosomal RNA confirmed that the British Columbian and Washington State isolates belong to the species *N. perurans*. Further sequences analysis revealed a high degree of similarity with 18S rRNA sequences from Canadian, American, Norwegian, Tasmanian and Chilean isolates (personal communication, A Siah 2018).

The Norwegian situation

In Norway, AGD was observed for the first time in farmed Atlantic salmon at four sites in the autumn of 2006 (Steinum et al., 2008). For the next five years, AGD was not detected in Norwegian fish farms but in the late autumn 2012 AGD was again diagnosed at five sites on the Southwest coast of Norway. In 2013 and 2014, AGD made a major impact in Norwegian salmon farming and led to significant mortalities. Even though AGD is a relatively new disease to the Norwegian fish farming industry, outbreaks in 2015 and 2016 were controlled quite well. The amoeba was detected early due to good sampling routines, and good management decisions were made regarding the necessity and timing of treatments. The environmental conditions (e.g. water temperature and salinity) were also more favourable in 2015 and 2016 compared to 2013 and 2014.

Specific prophylactic measures against AGD are not known, but early detection of *N. perurans* and initiation of treatment is important in relation to control of AGD. The longer the disease is allowed to progress, the more difficult it is to implement effective treatment. Treatment stress may also aggravate mortality. In Norway, AGD is controlled by bath treatment using either hydrogen peroxide or freshwater. None of the treatments appear to be 100% effective, and repeated treatments are often conducted within the same production cycle. Treatment with freshwater is the milder form of treatment for salmonid fish and appears to be more effective than hydrogen peroxide. Tasmanian research has shown that different strains and breeding lines of Atlantic salmon have different susceptibility or resistance to *N. perurans* (Taylor et al., 2007; Kube et al., 2012). Breeding for increased resistance is, therefore, also relevant for the Norwegian Atlantic salmon farming industry.

AGD is not a notifiable disease in Norway, thus the precise number of annual outbreaks is not known. However, reports from the fish health services and the Norwegian Veterinary Institute indicate that the amoeba is now endemic in the Norwegian fish farming industry. Despite the detection of *N. perurans* by PCR in some farms throughout the year, AGD remains a seasonal disease associated with periods of warmer water. Outbreaks usually occur from August to December from the south west coast as far north as the county of Nord–Trøndelag. By the end of 2016, *N. perurans* has been detected in gills of farmed Atlantic salmon as far north as Troms County (Steinum et al., 2015).

The Chilean situation

AGD was first recorded in Chile in 2007 in Atlantic salmon farmed in waters off Chiloé Island (Bustos *et al.*, 2011). It is likely that simultaneous infections occurred in all species of salmonids farmed in Chile, including Coho salmon and rainbow trout. Rozas and coworkers noted that in Atlantic salmon the disease occurs mainly between the summer and autumn months while in Coho salmon and rainbow trout peak infections occur

between autumn and winter. Currently, the disease has a wide geographical distribution affecting salmonid farming in the Tenth and Eleventh Regions of the country.

Recently, a case of AGD has been reported in fresh water with pathological characteristics similar to those described in sea water. There is, however, no specific data about the etiology to date. AGD related mortality in salmonid farming in Chile between 2015 and 2016 corresponds to 1.18% and 2.11% of the total disease related mortality experienced in the industry, respectively, with a significant increase in losses in 2016 to 1.23% and 3.33%. On the contrary, in farmed Coho salmon mortality was 2.34% in 2015 and 0.16% in 2016. Mortality attributed to AGD in rainbow trout was 0.14% in 2015 and 0.05% in 2016 (Table 1). Losses associated with this condition may be underestimated due to concomitance with other diseases such as Salmonid Rickettsial Septicaemia (*Piscirickettsia salmonis*) and specifically in 2016, massive mortality due to harmful algae blooms.

Table 1: Percentage of accumulated mortality associated with Amoebic Gill Disease in relation to the total losses due to infectious disease between 2015 and 2016.

Year	*S. salar*	*O. kisutch*	*O. mykiss*	Total
2015	1.23%	2.34%	0.14%	1.18%
2016	3.33%	0.16%	0.05%	2.11%

Source: Aquabench, unpublished data, cited with permission 2018.

AGD associated mortality in Chilean salmon presents a seasonal pattern associated with water temperature (> 12°C) and salinity (> 32ppt) (Douglas-Helders et al., 2001; Munday *et al*. 2001; Nowak *et al*., 2012).

Another unquantified impact of AGD in Chile is that it constitutes a risk factor for the presentation of one of the main bacterial diseases affecting salmonid farming i.e. Salmonid Rickettsial Septicaemia (*Piscirickettsia salmonis*).

Reservoirs

N. perurans is a ubiquitous organism in open and coastal marine environments. Few studies have, however, investigated the distribution and potential reservoirs of *N. perurans* within various subsections of the environment. With the development of sensitive molecular techniques, surveillance of *N. perurans* was pursued by Bridle and coworkers (2010) at different depths of sea water in salmon farming and non-farming sites in Tasmania. Results showed that only the farmed sites tested positive for *N. perurans* (Bridle et al., 2010). In addition, recent studies showed that *N. perurans* was highly abundant in surface water to 10 m depth at cage sites in Tasmania (Wright et al., 2015).

Investigations have shown that free swimming *N. pemaquidensis,* a species closely related to *N. perurans*, can survive for up to three weeks without feeding, indicating that this parasite may survive unattached for a long period of time (Martin, 1985). Investigations on the marine reservoir of *N. perurans* in Norwegian sites showed that *N. perurans* could be detected in biofouling organisms, biofilm, plankton and wild fish during episodes of AGD in farmed Atlantic salmon (Hellebo et al., 2016). In 2008, researchers tested for reservoirs of amoeba in farmed Atlantic salmon, sea lice (*Lepeophtheirus salmonis*), invertebrates (blue mussels, anemones, urchin and sponges), macroalgae and sediment from Puget Sound Washington State (US) and Vancouver Island (Canada; Nowak et al., 2010). *N. perurans* was detected in Puget Sound Atlantic salmon whereas no detection of *N. perurans* or clinical signs of AGD were observed in fish from British Columbia. However, the sensitivity of the assay was subsequently questioned as re-testing of some negative samples provided positive results (Oldham et al., 2016). Sea lice were the only other environmental organisms to test positive during this survey, which indicates an environmental distribution of *N. perurans* (Nowak et al., 2010).

BC salmon farmers have adopted a harvest-fallow practice. There are concerns, however, relating to possible persistence of pseudocysts under unfavourable environmental conditions. The past three summers along the BC coast have been warm and dry – conditions that favour the presence of the amoeba. Will changes in the environmental conditions away from El Niño summers mean a decrease in the number of amoeba? And most of all, if the numbers of amoeba and pseudocysts have increased to a critical threshold, will AGD become endemic in BC?

Because of the recent detection of *N. perurans* in British Columbia, little knowledge of the biology and ecological

distribution of the parasite in the local environment is available. *N. perurans* is a free-swimming parasite that can be passively transported, survive unattached and therefore horizontally transmit between locations. To address the issue of distribution and compartmentalization of amoeba, the development of a molecular method to detect *N. perurans* in the sediment was investigated at BC CAHS in collaboration with the University of Tasmania and industry partners. A DNA extraction method was performed on sediment spiked with different numbers of cultured *N. perurans*, and the qPCR method optimized to select the best conditions for detection. Once the methodology was developed, screening for *N. perurans* was performed on sediment collected from different locations of British Columbia aquaculture farms as a routine surveillance. Results showed that *N. perurans* could be detected at very low levels in sediments in farms experiencing clinical AGD (personal communication, A Siah 2018).

Future directions

Clinical Amoebic Gill Disease was first described in farmed fish 30 years ago and the etiological agent identified 10 years later (see: Oldham et al., 2016 for review). At that time the disease was restricted to a few countries and until recently, not regarded as a concern in the northern hemisphere. However, AGD is now detected worldwide as a new emergent disease in several countries where Atlantic salmon are farmed.

In their review, Oldham and coworkers (2016) suggested several mitigation strategies such as lower stocking density, optimal cage environment and early treatment for AGD. They also identified several knowledge gaps including the biology, environmental distribution of *N. perurans*, the interactions of environmental factors with the parasite, the need for alternative control strategies such as vaccines or selective breeding of resistant fish (Oldham et al., 2016). In addition to these gaps, *N. perurans* genome is still unsequenced. Sequencing and annotation of the genome will yield insight into further treatment and mitigation strategies highlighted by Oldham and coworkers (2016). However, with no reference genome for the amoeba, and the genome input of the symbiotic endosymbiont *Perkinsela amoebae* that lives within *N. perurans*, whole genome sequencing and annotation of the amoeba genome presents analytic challenges. However, sequencing of the BC *N. perurans* genome is currently ongoing at BC CAHS in collaboration with Elanco. The outcome of this investigation will hopefully provide more insight into some genomic characteristic of *N. perurans* inhabiting British Columbia water.

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Infectious salmon anaemia (ISA)

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Infectious salmon anaemia (ISA) is a serious and contagious viral disease of Atlantic salmon, *Salmo salar L.* The disease was first described in juvenile Atlantic salmon in a hatchery on the west coast of Norway in 1984 (Thorud and Djupvik 1988). Since then outbreaks have occurred every year with a peak in 1990 when close to 100 outbreaks were recorded. The disease was reported in Canada in 1996 (Mullins *et al.* 1998), in Scotland in 1998 (*Rodger et al.* 1998), in the Faroe Islands in 1999 (Lyngøy 2003), in Chile in 1999 (Kibenge *et al.* 2001) and in Maine, USA, in 2000 (Bouchard *et al.* 2001). Around 1990, a series of management and hygienic measures were implemented in Norway, including fallowing of infected sites, regulation and control of movement of fish, year class separation, and regulatory zones. These measures had a remarkable effect, and in 1994 only two new ISA outbreaks were recorded. Now, however around 10-15 outbreaks are diagnosed yearly in Norway (ranging from one to twenty annual outbreaks during the last 25 years). ISA is now widespread, and outbreaks have been recorded in most countries with intensive Atlantic salmon aquaculture. In Norway, ISA has occurred either as smaller epidemics, or as single outbreaks with unknown source.

Infectious salmon anaemia virus (ISAV)

ISA is caused by the infectious salmon anaemia virus (ISAV), an aquatic orthomyxovirus belonging to the genus Isavirus, thus the virus belongs to the same virus family as the influenza viruses. The virus is an enveloped virion with a diameter of 90-140 nm (Dannevig et al. 1995, Nylund *et al.* 1995). Two glycoproteins are embedded in the envelope, the hemagglutinin esterase protein (HE) and the fusion protein (F). Both proteins are important for virus uptake and cell tropism. ISAV uses 4-O-acetylated sialic acids, expressed on endothelial cells (the cells that cover the inside of the blood vessels), red blood cells and some epithelial cells in the skin, gut, gill and conjunctiva as receptors (Krossøy *et al.* 2001, Falk et al. 2004, Hellebø *et al.* 2004, Aspehaug *et al.* 2005, Aamelfot *et al.* 2012).

The viral genome consists of eight negative-sense single-stranded RNA segments encoding 10 or 11 proteins. RNA polymerases PB2, PB1 and PA are coded by segments 1, 2 and 4 respectively, whereas segment 3 encodes a nucleoprotein. Segment 5 and 6 encode for the viral surface proteins, fusion (F) and haemagglutinin-esterase (HE), involved in functions such as fusion and receptor binding in a similar fashion to the influenza virus family. Segment 7 and segment 8 encode the matrix protein, a nuclear export protein, and an RNA-binding protein (Rimstad *et al.* 2011).

The segmented ISAV genome is highly conserved. Phylogenetic analysis showed two main genogroups of ISAV: European and North American clusters (Devold *et al.* 2001, Krossøy *et al.* 2001). ISAV variants may be differentiated based on the highly polymorphic region (HPR) of segment 6 encoding the HE protein. Sequence analysis of segment 6 suggests that ISAV pathogenicity is related to a deletion in this segment (known as HPR-del) combined with mutation in the F-protein (Fourrier *et al.* 2014, Fourrier *et al.* 2015, Christiansen *et al.* 2017). All ISAV isolates from disease outbreaks have deletions in the HPR region (Rimstad *et al.* 2011). The non-virulent non-deleted (HPR0) variant is considered the ancestor to all virulent variants of ISAV (HPR-del), and is thus considered a potential risk factor for development of infectious salmon anaemia. This ancestry was recently confirmed during examination of a new ISA outbreak on the Faroe Islands. The factors that favour development of this deletion and the risks associated with the development of virulent ISAV HPR-del from HPR0 remain, however, unknown (Christiansen *et al.* 2017). Our current hypothesis is that the development of virulence is a step by step process, starting with low-virulent ISAV intermediates that may eventually develop into fully virulent ISAV (Figure 1). This hypothesis is supported by observations related to ISAV field diagnostics. The new Faroese virus, which as HPR0 was initially limited to epithelial cell infection, subsequently evolved the capability to cause systemic infection. (Christiansen *et al.* 2017). Christiansen et al. (2017) concluded that deletion in HE HPR, combined with mutations in the F protein are the minimum requirement for a non-virulent HPR0 virus to change tissue tropism and evolve into a virulent ISAV.

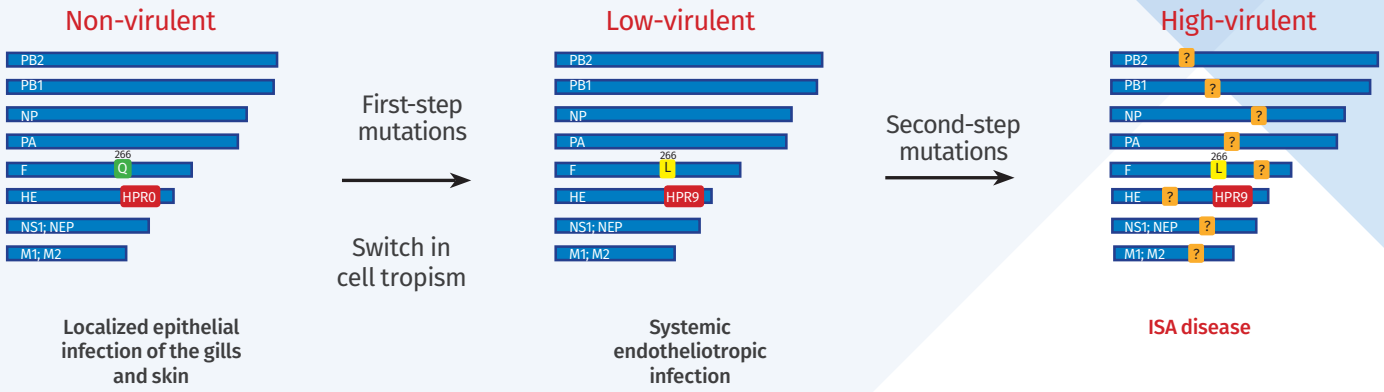


Figure 1. Additional second-step mutations are most likely required for the evolution from a non-virulent HPR0 to a highly-virulent HPR-del ISAV (Figure: Maria Aamelfot).

Virus spread, transmission and survival

Smaller ISA epidemics or local outbreaks suggest horizontal transmission (Lyngstad *et al.* 2008, Lyngstad *et al.* 2011). Horizontal waterborne transmission of viruses as shown by cohabitation challenges, suggests that this is important for the spread of viruses. Horizontal transmission occurs within a cage or within a farm, but also between different farming sites. An important risk factor for development of ISA is proximity to an already infected farm. The risk of vertical transmission of ISAV (transmission of virus from parent to offspring though eggs or sperm) is considered very low, but cannot be excluded (Rimstad *et al.* 2011, Anonymous, 2012).

Virulent ISAV mainly infects endothelial cells. As new virus particles are then released into the bloodstream, blood will thus contain a lot of virus and is therefore highly infectious (Aamelfot *et al.* 2012, Aamelfot *et al.* 2014). An important feature of ISAV is the ability to attach to and cross-bind red blood cells. ISAV is shed in all natural secretions including mucus, stools, urine and blood. Skin and skin mucus from infected fish contain large numbers of virus particles (Aamelfot *et al.* 2016), and fish that survive ISAV infection can excrete viruses probably for a month or more.

ISAV survival time outside the host is very difficult to assess, as it will depend on numerous factors, including available substrate (i.e. water vs organic material), temperature, time and most probably virus type and strain. In general, ISAV survives (retains the ability to infect cells) longer in cold water compared to warmer water (the ability decreases as the temperature increases). In addition, the virus appears to survive or retain infective capacity longer in freshwater compared to salt water (Rimstad *et al.* 2011). Very little information about survival in substrates like bio-filters, protein foam, biofilm etc. is available.

While virulent HPRdel variants target endothelial cells and red blood cells, HPR0 appears to replicate only in epithelial cells of the gills and the skin (Aamelfot *et al.* 2016).

Outbreaks of ISA have only been detected in farmed Atlantic salmon; however the causative agent has been detected in several other wild salmonid fish species, including rainbow trout and sea trout. Further, a number of additional species have been demonstrated experimentally to support replication of ISAV (Rimstad *et al.* 2011). ISA may occur in most stages of Atlantic salmon production from hatcheries to brood stock farms, however the majority of the cases occur in the ongrowing stage at sea. Non-virulent HPR0 variants are present in most salmon producing countries and lead to frequent and transient infections in farmed fish without observable signs of disease (Christiansen *et al.* 2011). Based on available and anecdotal data, it may be assumed that all Atlantic salmon populations, in countries that have had ISA, will experience an HPR0 infection during their life cycle.

Clinical signs and histological findings

Outbreaks of ISA vary greatly in regard to clinical signs, disease development and histological findings. The main pathological signs are more or less specific, and are related to circulatory disturbances, blood vessel damage and a significant anaemia. The fish are often lethargic with abnormal swimming behaviour. At the beginning of an outbreak, fish with mild to moderate clinical signs are usually only found in one or two cages and daily mortality is typically low (0.05-0.1%) (Rimstad *et al.* 2011). However, if measures are not taken to limit disease development, the disease will spread to other cages, and accumulated mortality may reach more than 80% over a period of several months. Episodes of high and rapid mortality are rare, but are observed, often related to stressful events such as handling of the fish (i.e. for transport or treatments). In later stages of the disease, severe anaemia with haematocrit values below 10% are regularly found. A variable set of pathological changes may or may not be observed in individual fish suggesting circulatory disturbances. These may include petechial haemorrhages in the skin and on abdominal surfaces, oedema in various organs, and ascites (Aamelfot *et al.* 2012, Aamelfot *et al.* 2014, Dannevig and Falk 2017). Histological findings often include hepatocellular necrosis and/or renal tubular necrosis with bleeding in the surrounding tissue (Thorud and Djupvik 1988, Evensen *et al.* 1991, Byrne *et al.* 1998, Mullins *et al.* 1998, Godoy *et al.* 2008). Haemorrhages may also be associated with the gut wall. Because of these variations in clinical presentation, diagnosis based on post mortem findings alone can be difficult. However, ISA should always be considered in Atlantic salmon displaying signs of circulatory disturbances and anaemia.

Disease development

ISA is a systemic disease affecting the circulatory system of salmon. Investigation of blood samples during the later stages of an ISA outbreak reveals anaemia (i.e. low haematocrit), and leukopenia (low levels of white blood cells), especially lymphocytopenia and thrombocytopenia, and an increase in red blood cell fragility.

Using immunohistochemistry, we recently found that the primary target cells for ISAV are the endothelial cells coating the inside of blood vessels in all organs of the fish. However, the link between the infected endothelial cells and the severe anaemia and necrosis is currently unknown. ISAV isolates from field outbreaks have varying virulence. While virulence may be estimated by observation of disease development, mortality and clinical signs during an outbreak, the precise degree of virulence is difficult to determine. Experience from diagnostic work suggests that ISA outbreaks caused by low virulent ISAV may be difficult to detect on the farm as several recent diagnosed cases involved fish that were not initially suspected to have ISA.

Incubation time (time from infection to clinical disease) in natural outbreaks varies from a few weeks to several months. Fish experimentally infected by intraperitoneal injection, cohabitation or bath/immersion develop ISA usually within 10-12 days at 12°C, maybe longer depending on the fish and the virus virulence.

Diagnostics

Diagnosis of ISA is performed according to procedures outlined in the OIE Manual of Diagnostic Tests for Aquatic Animals (2017) and is based on clinical signs, macroscopic lesions, and histological findings supplemented with immunohistochemical investigations for endothelial infection (Aamelfot *et al.* 2012). Positive immunohistochemical findings are confirmed by qPCR testing (Plarre *et al.* 2005, Snow *et al.* 2006) and virus isolation in cell culture. The ISAV HE gene is sequenced to determine HPR type and for epidemiological investigations.

The Norwegian situation

Over the past few years, a high proportion of Norwegian ISA outbreaks have been located in northern Norway. This trend continued in 2016, especially in the area around Rødøy in Nordland. In 2016, ISA was confirmed on 12 sites compared to 15 sites in 2015. The majority (eight) of the sites were located in Nordland, and several of these outbreaks appear to be associated with closely related viruses. In 2017, the number of outbreaks stayed more or less the same (14 outbreaks), however this year, more equally distributed along the entire coast line. Delayed discovery of the disease and delayed harvest of infected fish may have contributed to the

development of a local epidemic. Fallowing and systematic monitoring of all salmon and rainbow trout sites within defined zones in northern Norway in 2015 and 2016 is expected to lead to an improvement in the infectious situation in the region.

ISA is a listed notifiable disease in Norway (List 2), in the EU, and by the World Organisation for Animal Health (OIE). Outbreaks of ISA are strictly controlled in Norway. Following an outbreak, both an inner combat zone, and an outer surveillance (observation) zone are established around the outbreak site. Control measures will vary depending on whether or not the outbreak is in an ISA-free zone or not. After a period of two years without further detection of new cases within the observation zone, the Norwegian Food Safety Authority may lift the zone(s). Successful combat of ISA and prevention of further spread are based on early detection of the disease and rapid elimination of infected fish. Since autumn 2015 in Norway, in a cooperation between the industry, fish health services and the Norwegian Food Safety Authority, systematic monitoring has been carried out in ISA control areas. The monitoring involves monthly inspections and sampling for ISA virus detection to uncover ISA at an early stage.

As monitoring within ISA free zones and segments and ISA control zones are based on detection of infection with ISAV HPR-del and presentation of clear clinical and pathological signs, low virulent ISAV may exist for a long time without being discovered. In combination with the fact that evolution of increased virulence is likely to happen over time, it is of the uttermost importance that efficient biosecurity practices are implemented, including strict separation of generations, and populations in general.

The Canadian situation

In Canada, ISA was first reported in farmed Atlantic salmon in the Bay of Fundy (New Brunswick) in the summer of 1996 (Mullins *et al.* 1998). On the west coast of Canada, ISAV has not been detected from several thousands of samples analysed during various screening programmes performed by either federal/provincial agencies or independent third party laboratories. Kibenge *et al.* (2016) reported 'non-negative' ISAV test results from market-bought, farm-raised Atlantic salmon, and wild salmon using molecular tests. These reported results are, however, highly controversial, and none were confirmed by additional testing, and/or cell-culture. Also, no clinical signs consistent with ISA were ever reported.

The British Columbia Ministry of Agriculture tested 4,726 freshly harvested farmed salmonids between 2003 and 2010 and ISAV was not detected either by molecular assay or cell culture. In 2012 and 2013, a large survey was performed by a Federal authority, the Canadian Food Inspection Agency (CFIA), who screened 8,006 wild Pacific salmonids for ISAV and all samples tested negative (CFIA 2014). In addition, the National Aquatic Animal Health Program in Canada and the US, screened returning wild anadromous salmonids for ISAV between Alaska and the Washington-Oregon border including the Columbia River in 2012-2013. ISAV RNA was not detected in 923 fish from Washington State or in 1,431 fish from the Alaska regions (Amos *et al.* 2014). Based on this testing, British Columbia is considered as an ISAV free zone by the Canadian Regulatory Authorities.

The Faroese situation

In the Faroe Islands, the first ISA outbreak was reported in 2000. During the following five years a devastating ISA epidemic almost completely destroyed the industry. A total of 33 ISA outbreaks were recorded, with all but two of Faroese salmon-farming sites affected. New regulations regarding increased biosecurity, reduction in production intensity, year class separation, scheduled fallowing and mandatory vaccination against ISA were implemented. In addition, an intensive surveillance and screening program for ISAV was established by the Faroese authorities. During the following years (2006 – 2013) more than 50,000 fish were screened for the presence of ISAV. Only non-virulent ISAV-HPR0 was detected in this period (Christiansen *et al.* 2017).

However, in 2014, a new ISAV-HPR-deleted strain was identified following routine screening of harvest ready Atlantic salmon. Clinical signs consistent with ISA were not observed and the site was emptied within 3 weeks of initial detection. The new ISAV-HPR-deleted virus was not detected in any of the epidemiologically linked marine or freshwater farms. Based on these findings, we recently presented the first evidence for the evolution of an ISAV-HPR0 to a low-virulent ISAV-HPR-deletion (Christiansen *et al.* 2017).

Another ISAV-HPR-del strain was identified in 2016 at a marine farming site recently stocked with Atlantic salmon smolts. Despite intensive surveillance and sampling over the following five months, no ISAV was detected and clinical signs consistent with ISA were not observed. However, following a period with bad weather and sea lice treatment, the ISAV-HPR-del strain re-appeared and the site was subsequently diagnosed with ISA disease. To prevent further spread of ISAV all fish were removed from the site within a month in the spring of 2017. In addition, a systematic monitoring and screening for ISAV has been carried out in the ISA control zone. So far, no ISAV-HPR-del strains have been detected.

The Chilean situation

After two decades of strong growth and financial results, the Chilean industry started to experience increased problems with various diseases, including salmon rickettsial syndrome (SRS) caused by *Piscirickettsia salmonis* from 2005-2006. The average harvest weight dropped from 4.5 kg in 2004 to 2.7 kg in 2007 (Alvial et al. 2012). The first clinical report of ISA in Atlantic salmon was made in 2007, caused by ISAV belonging to the European genotype (or genotype I), HPR7b (Kibenge *et al.* 2005, Godoy *et al.* 2008). The outbreak site was located in an area with many other farms. The site had recently recovered from an outbreak of SRS (Godoy et al. 2008). This outbreak is, however, unlikely to represent the first outbreak of the Chilean epidemic. Thus, the timing of introduction of the virus remains uncertain (Plarre *et al.* 2005, Kibenge *et al.* 2009, Alvial *et al.* 2012). The ISA epidemic had a huge impact on the production of Atlantic salmon in Chile.

Biosecurity and sanitary measures introduced by the Chilean government in 2007-2008 included implementation of an ISAV surveillance program for both fresh and salt water facilities, and implementation of an ISAV control program (Alvial *et al.* 2012).

Figure 2 shows the evolution of the ISAV situation, ISAV-HPR-del and ISAV-HPR0 in farmed Atlantic salmon during the grow-out stage. Following the first reports during the winter of 2007, a significant increase was observed, reaching a peak of 24 cases in November 2008. Subsequently, the number of cases decreased significantly due to the reduction of farmed biomass

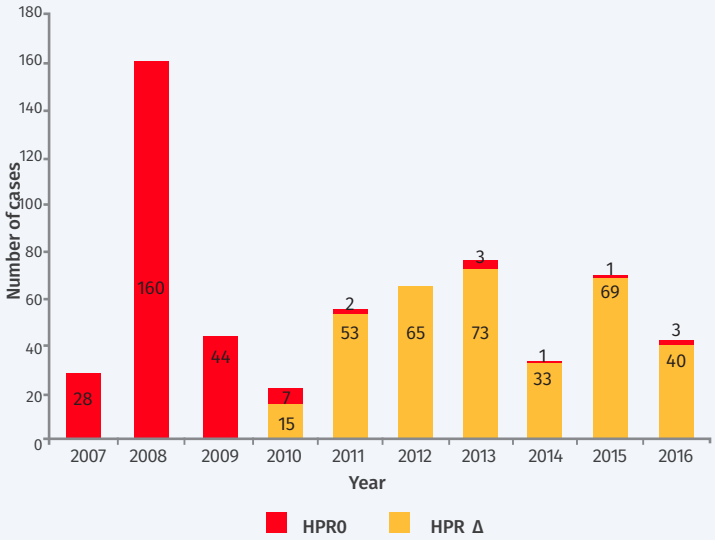


Figure 2. Evolution of the ISA situation in Chile, both virulent ISAV-HPRdel and ISAV-HPR0, in on-growing farmed Atlantic salmon, July 2007 to December 2016 (Sernapesca, 2017).

and the implementation of biosecurity measures. As of 2010, sporadic detections of ISAV-HPRdel have been observed, with 3 outbreaks in 2016.

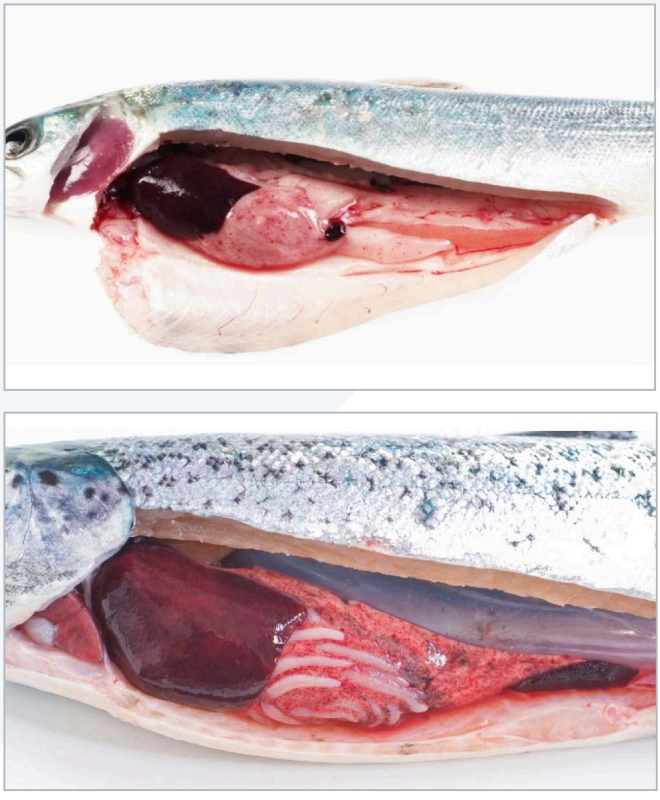
Initially 79.7% of the cases of ISAV-HPRdel were associated with ISAV-HPR7b strains (Kibenge *et al.* 2009). As of 2013, outbreaks have not been associated with the specific predominance of any single HPR-del group. Table 1 shows the types of virulent ISAV-HPRdel associated with outbreaks recorded since 2013.

Table 1. Year, month, neighbourhood and type of HPR corresponding to ISA outbreaks in farmed Atlantic salmon, from 2013 to 2016 (Sernapesca, 2017)

Year of detection	Month of dedection	Neighborhood	HPR variant
2013	April	18d	HPR3
2013	April	20	HPR14
2013	December	18B	HPR7a
2014	January	9a	HPR7b
2014	November	25a	HPRs3
2015	February	25a	HPR3
2015	March	25a	HPR3
2015	June	9b	HPR14
2015	June	22b	HPR8
2015	August	25a	HPR3
2015	November	21c	HPR2d
2016	April	21b	HPR8
2016	September	21b	HPR8
2016	December	33	HPR7b

The first case of ISAV-HPR0 was reported in 2009 (Kibenge *et al.* 2009), after which an increase in the frequency of ISAV-HPR0 cases was observed with 40 cases in 2016 in sea water sites. Genetic analyses of the ISAV-HPR3 and ISAV-HPR14 cases link these isolates to the presence of ISAV-HPR0 (Godoy *et al.* 2013).

Regardless of the type of HPR, the outbreaks that have occurred since 2013 present the classic clinical signs described for ISA (Godoy *et al.* 2013), characterized by petechial haemorrhages on the skin, pale gills, black liver, petechial haemorrhage in visceral fat, haemorrhagic enteritis among others (Figures 3 and 4).



Figures 3 and 4. Atlantic salmon with ISA. Dark livers, petechial haemorrhages in peripyloric fat and absence of intestinal contents can be observed (photos: Marcos G. Godoy).

Research activity, knowledge gaps and challenges

A major challenge with regards to ISA is the lack of knowledge on the risk of evolution of ISAV HPR0 to virulent HPR-del. Knowledge gaps include the mechanisms and drivers of this evolution. Such knowledge would make an important foundation for detection and prevention of emergence of new virulent HPR-del ISAV. Added to this, we hypothesise that the evolution from HPR0 to highly virulent HPR-del ISAV is a stepwise transformation via one or more low virulent stages of virus. If this is the case, we need to identify virulence markers that may indicate the evolutionary status of detected virus. It is also of uttermost importance to be able to prevent this evolution. A key measure here is to prevent contact between salmon populations in general and in particular between generations. Such measure will also help prevent development of new and unknown infectious diseases.

Brood stock farms and smolt farms aim to produce ISAV HPR0 free products, however we do not know if this removal of HPR0 is beneficial to the fish, or whether these non-virulent infections of the mucosal system may have a vaccine-like effect.

Current ongoing research on ISA in Norway focuses on pathogenesis and virulence, in particular aimed at understanding the evolution of ISAV HPR0 to HPR-del. In addition, studies are conducted to better understand ISAV dissemination, and to make surveillance more efficient. One current study is investigating the relationship between the virus and red blood cells and the implications this interaction has for disease development. Another study focuses on uptake of virus and the early stages of infection, comparing low virulent and highly virulent isolates of the virus. Research on HPR0 is somewhat hampered by the lack of an *in vitro* cell culture system, but it is still possible and feasible to increase knowledge about mechanisms associated with the evolution from HPR0 to HPR-del ISAV through laboratory experiments. Added to this, the project aims to provide a better foundation and improved procedures for virus screening and control, as well as an increased ability to identify ISA risk sites, and the determination of ISAV virulence through a standardised fish experimental model.

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Heart and skeletal muscle inflammation and Piscine orthoreovirus

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General introduction

The disease heart and skeletal muscle inflammation (HSMI) was first described in Norway in 1999 on a number of Atlantic salmon sea farms on the Norwegian west coast (Kongtorp, Kjerstad *et al.* 2004). Initial experimental trials performed at the Norwegian Veterinary institute clearly indicated that this was an infectious disease, since injection of homogenized tissue from diseased fish into healthy individuals caused HSMI, and naïve cohabiting fish in the same tank developed the disease (Kongtorp and Taksdal 2009). A viral etiology was proposed when antibiotic treatment had no effect on disease development (Kongtorp, Kjerstad *et al.* 2004, Kongtorp and Taksdal 2009) and viral particles were detected (Watanabe, Karlsen *et al.* 2006). It took, however, a next generation sequencing revolution before the associated pathogen was identified as a reovirus in 2010 (Palacios, Lovoll et al. 2010), later officially named *Piscine orthoreovirus* (Kibenge, Iwamoto et al. 2013, Markussen, Dahle *et al.* 2013, Nibert and Duncan 2013). The final proof of concept that a Norwegian isolate of *Piscine orthoreovirus* (PRV) is a causative agent of HSMI was recently demonstrated using highly purified PRV in experimental trials (Wessel, Braaen *et al.* 2017).

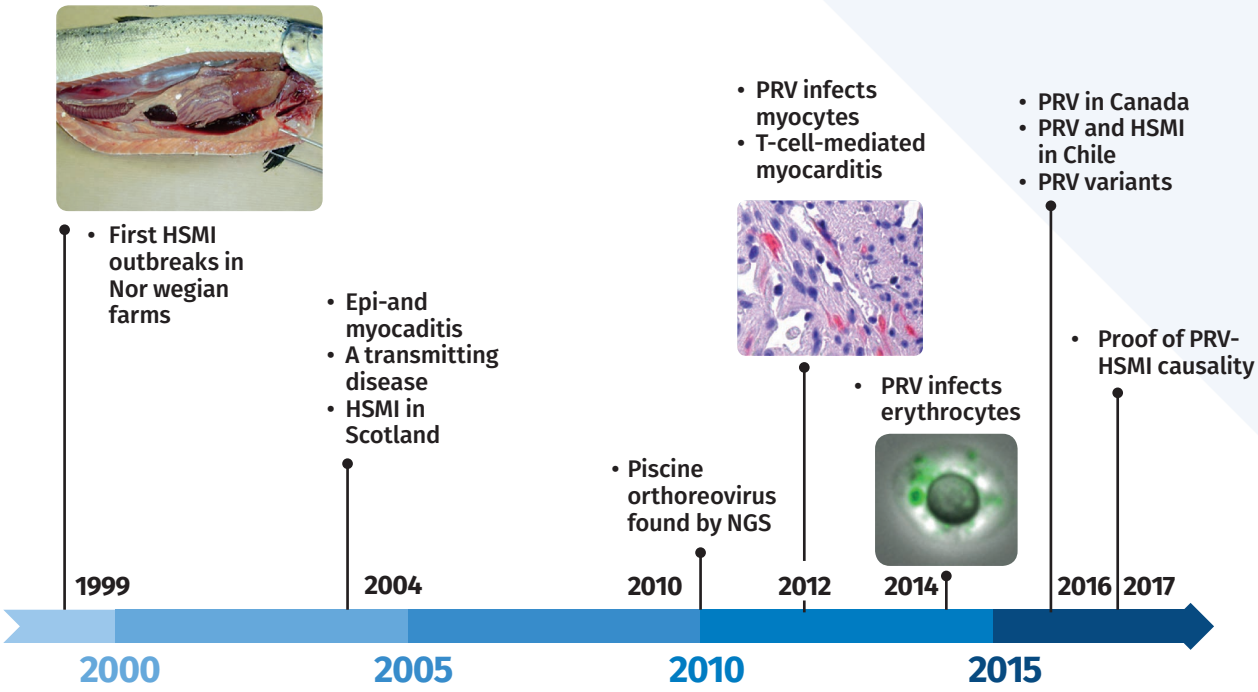

Figure 1. The timeline of some important findings linked to heart and skeletal muscle inflammation (HSMI) and Piscine orthoreovirus (PRV). NGS: Next generation sequencing. Photos: Trygve Poppe and Øystein Wessel.

As the name of the disease indicates, HSMI is primarily characterized by an inflamed heart (epi- and myocarditis), often in combination with red skeletal muscle affection (Kongtorp, Taksdal et al. 2004, Di Cicco, Ferguson et al. 2017). HSMI is today one of the most frequently occurring diseases in farmed Atlantic salmon in Norway (Hjeltnes, Bornø et al. 2017). The importance of PRV and HSMI outside Northern Europe is currently unfolding. PRV is found in farmed and wild salmonids in North America and Chile (Marty, Morrison et al. 2015, Siah, Morrison et al. 2015, Godoy, Kibenge et al. 2016), but no HSMI diagnoses were reported until 2016/2017 (Godoy, Kibenge et al. 2016, Di Cicco, Ferguson et al. 2017). New PRV variants adapted to other salmonid species, responsible for diseases similar but not identical to HSMI (Olsen, Hjortaas et al. 2015, Takano, Nawata et al. 2016, Hauge, Vendramin *et al.* 2017) have been described in the last two-three years. It is likely that more PRV variants will be identified in years to come.

Description

Piscine orthoreovirus - Virus structure

When the sequence of the PRV genome was described and compared to other reoviruses in 2010 (Palacios, Lovoll et al. 2010), PRV was found to most closely resemble the mammalian and avian orthoreoviruses (Kibenge, Iwamoto et al. 2013, Markussen, Dahle et al. 2013, Nibert and Duncan 2013). *The* orthoreoviruses differ from aquareoviruses in the number of genomic segments, capsid structure, cell attachment proteins and the fact that several orthoreoviruses have fusogenic properties (Nibert and Duncan 2013).

Orthoreoviruses are spherical icosahedral, non-enveloped double stranded RNA viruses, with an approximate size of 60–85 nm (Nibert 1998). The orthoreovirus genome is encapsulated by an inner and an outer protein shell, consisting of eight different virus-encoded proteins. Although the sequence identity between PRV and mammalian orthoreovirus (MRV) is low (Kibenge, Iwamoto et al. 2013, Markussen, Dahle et al. 2013, Nibert and Duncan 2013), the PRV structure, protein function and replication processes share many features with MRV. This has been demonstrated through electron microscopy imaging (Finstad, Dahle et al. 2014, Wessel, Braaen et al. 2017), protein functional studies (Key, Read et al. 2013, Markussen, Dahle et al. 2013, Wessel, Nyman et al. 2015, Haatveit, Nyman et al. 2016), and the structure of viral factory inclusions in infected cells (Haatveit, Nyman et al. 2016).

The Reo in *Reoviridae* is derived from "respiratory enteric orphan," since MRV was isolated from respiratory and gastrointestinal tracts and considered "orphan" in the sense that the viruses were initially not associated with disease symptoms (Sabin 1959). Since then, reoviruses have been proven pathogenic in many species, including PRV in Atlantic salmon. The lack of disease symptoms from MRV in humans has also been questioned, as MRV was recently associated with intestinal inflammation and autoimmunity (Bouziat, Hinterleitner et al. 2017).

Genomic analysis

The PRV genome consists of 10 double stranded RNA segments divided into three large segments (L1, L2, L3) encoding λ-proteins, three intermediate (M1, M2, M3) encoding μ-proteins, and four small segments (S1, S2, S3, S4) encoding four σ-proteins plus two smaller proteins encoded by internal open reading frames (Palacios, Lovoll et al. 2010, Markussen, Dahle et al. 2013). PRV is related to the aquareovirus group through nine of its segments, but differs in the proteins σ3/p13 and σ2/p8 encoded by the bicistronic genes (Nibert and Duncan 2013). So far, at least ten PRV genomes have been fully sequenced and used to investigate links with HSMI and geographical origin. Genome analysis of isolated sequences from Norway, Chile and Canada has revealed differences mainly in segments M2 and S1. The PRV S1 segment has an internal open reading frame (ORF) that encodes the p13 protein with cytotoxicity features (Key, Read et al. 2013). Alignment of the part of segment S1 encoding p13 protein from PRV from salmonids collected in western North America, Chile and Norway showed amino acid differences that altered the predicted secondary structure, but no frame shifts were detected (Siah, Morrison et al. 2015). The p13 coding sequences from samples of HSMI-diseased fish are 100% similar to sequences from healthy fish, indicating that the differences are not directly linked to virulence. Recent studies on PRV in farmed salmonids from BC also concluded that there were no significant differences between the genome sequences (Marty, Morrison et al. 2015).

HSMI

Myocardial infection and inflammation

Macroscopic signs of HSMI frequently include haemorrhagic, enlarged and pale hearts, loss of cardiac texture, and haemorrhage of the pericardium. Histopathological findings are mainly found in the heart and red muscle (Kongtorp, Kjerstad et al. 2004, Yousaf, Koppang et al. 2012, Yousaf, Koppang et al. 2013). An inflammatory response is induced in the heart, leading to infiltration of mononuclear cells, involving both the compact and the spongy layers of the ventricle, and epi- and endocarditis. In advanced stages of the disease, inflammation, degeneration and necrosis of the muscle fibers are also observed. In the red muscle, findings similar to those described in the heart are found, and in severe cases signs of degeneration such as loss of striation, eosinophilia, vacuolization and karyorrhexis can be seen.

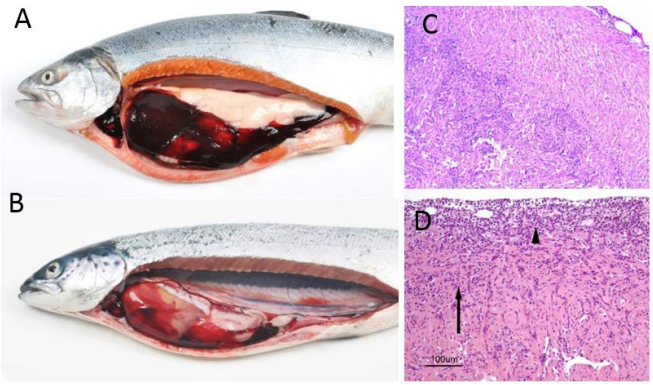

Figure 2. A) Coho Salmon (*Oncochynchus kisutch*) and B) Atlantic Salmon (*Salmo salar*) from Chile affected by heart and skeletal muscle inflammation (HSMI). Haemopericardium and clotting can be observed in the abdominal cavity. Heart sections (H&E) with heart and skeletal muscle inflammation (HSMI) from C) Coho Salmon from Chile and D) Atlantic Salmon from Norway show infiltration of mononuclear cells in the epicardium (short arrow), compactum and spongiosum (long arrow). Photos: Marcos G. Godoy (A-C) and Øystein Wessel (D).

HSMI was first recognised as a transmissible disease following transmission utilizing heart tissue homogenates, clearly indicating that the heart tissues contained the pathogen (Kongtorp, Kjerstad et al. 2004). When the PRV genome sequence was characterised (Palacios, Lovoll et al. 2010), its predicted proteins could be cloned and expressed recombinantly and used to produce virus-targeting antibodies, which further allowed visualization and localization of the virus in the infected hearts. As a result of this, PRV was shown to infect cardiomyocytes (Finstad, Falk et al. 2012).

In an attempt to characterize the type of immune response elicited in infected hearts, antibodies detected cytotoxic immune cells (CD8+), and gene expression analyses indicated cytotoxic activity in the heart (Mikalsen, Haugland et al. 2012, Johansen, Thim et al. 2015). When comparing heart transcriptome responses to HSMI and to pancreas disease (PD) caused by salmonid alphaviruses, the latter show less induction of genes related to cytotoxic T-cells (Johansen, Thim et al. 2015). Another observation typical for HSMI is that the number of virus particles in the heart clearly drops as the inflammation proceeds, indicating that the immune response may be targeting the virus-infected cells (Finstad, Falk et al. 2012, Mikalsen, Haugland et al. 2012). This is in line with a cytotoxic antiviral immune response, and points to a contrasting role of the HSMI response, It is effective in the eradication of PRV infection from the heart, hence beneficial, but at the same time pathological to the host.

Erythrocyte infection

A new step towards understanding PRV infection was made when the virus was shown to infect salmon red blood cells early in the infection (Finstad, Dahle *et al.* 2014). Immunofluorescent staining and electron microscopy of infected erythrocytes revealed intracellular inclusions containing virus protein and virus-like particles (Finstad, Dahle *et al.* 2014, Wessel, Olsen *et al.* 2015). The erythrocyte infection can be massive for a limited time period after infection and represents the acute phase of the disease (Haatveit, Wessel *et al.* 2017). The infection in erythrocytes has a defined peak phase, during which up to 50% of the erythrocytes can be infected (Finstad, Dahle *et al.* 2014, Haatveit, Wessel *et al.* 2017). The erythrocytes respond to infection by a characteristic interferon-mediated antiviral immune response (Dahle, Wessel *et al.* 2015, Haatveit, Wessel *et al.* 2017). Erythrocytes can also be experimentally infected with PRV in culture, and studied separately from the host (Wessel, Olsen *et al.* 2015). When PRV was first visualized in erythrocytes, a puzzling resemblance with another salmonid disease with unknown etiology was found, the erythrocyte inclusion body syndrome (EIBS) (Rodger 2007, Finstad, Dahle *et al.* 2014, Wessel, Olsen *et al.* 2015). Indeed, a couple of years later, EIBS in Japanese Coho salmon was demonstrated to be caused by a genetic variant of PRV (Takano, Nawata *et al.* 2016).

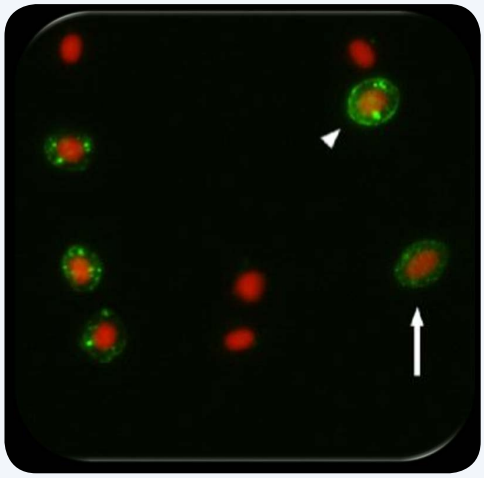

Figure 3. Erythrocytes from PRV-infected Atlantic salmon stained with anti-PRVσ1 antibodies and a secondary antibody emitting green fluorescence. Nuclei are stained red. Five PRV-infected and four uninfected erythrocytes are shown. Photo: Øystein Wessel.

Other pathological observations

Other typical macroscopic signs of a PRV infection include ascites, renomegaly, splenomegaly, hepatomegaly, pale or yellow liver, nutmeg liver, petechial haemorrhage in the liver and visceral fat, pale gills, exophthalmia and jaundice (Di Cicco et al., 2017; Ferguson et al., 2005; Godoy *et al.*, 2016; Kongtorp *et al.*, 2004a and b). However, the link between PRV infection and these pathological findings is unclear.

Ocurrence

HSMI has so far only been reported in farmed fish and not in wild salmonids, although the virus can be found in wild fish as well. In addition to the high prevalence of PRV in farmed and wild Atlantic salmon in Norway, PRV is common in farmed Atlantic salmon and Coho salmon in Chile (Bustos *et al.*, 2011; Kibenge *et al.*, 2013), Scotland, Ireland, Iceland and the Faroe Islands (Biering & Garseth, 2012; Rodger *et al.*, 2014); (Ferguson, Kongtorp *et al.* 2005), in farmed and wild Atlantic salmon in Denmark (Mikkelsen, Arnö *et al.*, 2014), farmed Atlantic salmon (*S. salar)* and wild Chum salmon (*Oncorhynchus keta*), rainbow trout (*Oncochynchus mykiss*), and cutthroat trout (*Oncorhynchus clarkii*) in Canada (Kibenge, Iwamoto et al. 2013), in farmed Chinook salmon (*Oncorhynchus tshawytscha)* and wild Coho salmon (*Oncorhynchus kisutch*) in Alaska, USA (Marty, Morrison *et al.* 2015). Experimentally, PRV has been shown to replicate in Coho salmon, Chinook salmon and Sockeye salmon (*Oncorhynchus nerka*) (Garver, Johnson *et al.* 2016, Garver, Marty *et al.* 2016, Polinski, Bradshaw *et al.* 2016), but has not been reported to induce any heart pathology or immune response in these species. Finally, Bigarre (2016) described mortality in brown trout (*Salmo trutta*) associated with the presence of Piscine orthoreovirus. Very low levels of PRV have been detected in certain marine fish species along the Norwegian coast (Atlantic herring, Capelin *"Mallotus villosus,"* Atlantic horse mackerel *"Trachurus trachurus"* and Great silver smelt *"Argentina silus"*) (Wiik-Nielsen, Lovoll et al. 2012).

In the last couple of years, two PRV genetic variants which appear to be adapted to other salmonid species than Atlantic salmon have been characterized, including a variant causing EIBS in Japanese coho salmon (PRV2) (Takano, Nawata et al. 2016), and a variant causing HSMI-like disease in rainbow trout (Olsen, Hjortaas et al. 2015). The latter PRV-variant was recently reported also to replicate in Atlantic salmon but with fewer signs of pathogenicity compared to rainbow trout (Hauge, Vendramin et al. 2017). This indicates that there are species or host-strain specific PRV variants related to differential disease states.

Trends

HSMI outbreaks occur from south to north along the Norwegian coastline during both summer and winter, indicating that PRV and disease development is rather independent of season and water temperature variation along the Norwegian coast. However, no experimental studies on temperature dependence of PRV infection have been published to confirm this hypothesis.

Although PRV was originally considered a seawater agent and HSMI a disease occurring only after sea transfer of Atlantic salmon, this picture has changed in Norway in recent years. PRV is commonly found infecting young fish in fresh water facilities prior to smoltification (Wiik-Nielsen, Ski et al. 2012), and several HSMI outbreaks have been reported in hatcheries (Hjeltnes, Bornø et al. 2017). Following a questionnaire to Norwegian smolt producers in 2016, HSMI was reported as an important disease problem in several fresh water facilities (Hjeltnes, Bornø et al.

2017). PRV is also common in freshwater in Chile, and outbreaks are also seen there, although bacterial and fungal coinfections confund the picture.

HSMI can also be induced in pre-smolts experimentally, and although HSMI can develop at most stages, a study has indicated that the host transcriptional response to PRV infection differs in pre- and post-smolts, and that the immune responses mounted in pre-smolts may be more effective in eradicating virus (Johansen, Dahle et al. 2016).

Impact

The reported mortalities from HSMI can be up to 20% of the infected population in field outbreaks, but clinical HSMI outbreaks without mortalities also occur, indicating that additional factors are decisive for the outcome. Mortality from HSMI is not commonly observed in experimental trials despite 100% HSMI development. A large variation in HSMI outcome is the typical picture in aquaculture, and still not fully understood. In Norway, HSMI has been a significant disease problem in aquaculture for almost two decades, mostly due to the high number of outbreaks.

Accumulated mortality from clinical HSMI in Chile varies within the production cycle. Mortality in freshwater generally does not exceed 5%. During the growout stage in seawater or estuary, outbreaks occur from two months post-transfer with mortalities ranging from 2 to 10%, with a second peak of clinical cases at about 6 months with mortalities that can reach 30%. This second peak is usually associated with, or followed by, Salmonid Rickettsial Septicaemia (*Piscirickettsia salmonis*).

Clinical HSMI in Atlantic salmon in freshwater in Chile is associated with handling, but also with bacterial (*Flavobacterium psychrophilum*) and fungal co-infections (*Saprolegnia* sp.).

PRV has been shown to persist in the host for more than a year after experimental infection (Garver, Johnson et al. 2016), which is in line with field observations. The long-term effects of PRV are unknown. In 2016, a link between PRV persistence and melanized spots in Atlantic salmon fillets was proposed, and PRV was found in melanized areas of the white muscle tissue (Bjorgen, Wessel et al. 2015). If PRV infection is linked to increasing melanization in Atlantic salmon, which is a quality-reducing factor leading to economic loss, the consequences of PRV infection may be higher than originally anticipated. However, the PRV-melanization link should be further investigated.

Distribution

PRV is ubiquitous in Norwegian salmon aquaculture (Lovoll, Alarcon et al. 2012), and is also found in escaped farmed Atlantic salmon (Madhun, Isachsen et al. 2016) and wild Atlantic salmon (Garseth, Fritsvold et al. 2013) to a lesser extent. The virus has also been detected by RT-qPCR in farmed Atlantic salmon in Ireland (Rodger, McCleary et al. 2014), Scotland, Iceland and the Faroe islands and in wild Atlantic salmon in Denmark (Mikkelsen et al., 2014), but the prevalence is unclear.

In Chile, PRV has been recorded since 2010 (Bustos et al., 2011), being highly ubiquitous in farmed salmonids in both freshwater, seawater or estuary. Later, PRV has also been associated with an HSMI-like condition in Coho Salmon (Godoy, Kibenge et al. 2016) and, less frequently, the virus has also been detected in farmed rainbow trout (*O. mykiss*) (Godoy, unpublished data). It was not until 2016 that the clinical disease in Atlantic salmon (*S. Salar*) was described from Chile (Godoy, Kibenge et al. 2016), although it is highly probable that HSMI was present in the farming systems and was underdiagnosed or disguised by mixed infections. A recent report showed that PRV is enzootic in farmed and wild salmonids on the Canada/US Pacific coast (Siah, Morrison et al. 2015).

Phylogenetic analysis

Due to the variability between isolated sequences, PRV segment S1 has been used for phylogenetic analysis in several studies. Kibenge et al. (2013) sequenced PRV segment S1 from 12 samples including wild cutthroat trout, farmed steelhead trout, wild Chum salmon, from British Columbia and farmed Atlantic salmon from Chile (Kibenge, Iwamoto et al. 2013). Their phylogenetic analyses grouped Norwegian PRV strains into a single genotype of two sub-genotypes (la and l b) with BC strains clustering with sub-genotype la and Chilean strains with sub-genotype Ib. A larger survey performed by Marty et al. (2015)

tested salmonid tissues from Alaska and BC between 1974 and 2013 (Marty, Morrison et al. 2015). Their RT-qPCR tests amplified PRV sequences from salmonid tissues collected from Alaska and BC in the 1980s. Taking advantage of the study of Marty et al. (2015), Siah et al. (2015) performed a phylogenetic analysis using a partial segment of PRV segment S1 (Siah, Morrison et al. 2015). The authors investigated both the occurrence and genetic diversity of PRV sequences isolated from wild and farmed fish collected in these regions. This latter study analyzed 71 sequences isolated from salmonids collected from 21 different locations from Alaska to the Columbia river over a 13 year period (2001-2014). The results revealed ten distinct sequence types with 1.1% maximum nucleotide diversity. The phylogenetic analysis was performed using Garseth et al. (2013) as a reference for consistency (Garseth, Ekrem et al. 2013) and results showed a high genetic homogeneity within western North America as all sequence types were not statistically different and grouped with Norwegian sequence types clustering within Group II (Fig 3). It is noteworthy that the Norwegian sequences and the two Chilean sequences (GenBank accession numbers KC782501 and KC795571) within Group I differ by more than 4% with Group II sequences. This indicates a high variability of PRV segment S1 in the Norwegian area. In contrast, the western North American S1 sequences suggest a low level of PRV diversity in this area. For instance, the archived sequence type isolated from BC salmonids collected in 2001 were identical to samples from Alaska, B.C. and Washington State collected in 2014.

Based on phylogenetic analyses of the available sequences of segment 1 (Seg-S1), it is possible to group strains into genotypes I and II. At the same time, genotype I is subdivided into two sub-genotypes Ia and Ib. Figure 1 shows the relationship between the genetic diversity of PRV and its geographical distribution (Godoy, Kibenge et al. 2016). Takano et al (2016) describe a new virus closely related to PRV based on phylogenetic analyses of segment S1 and the amino acid sequence of the RNA polymerase λ3. The virus is different enough to be designated as Piscine orthoreovirus 2 (PRV-2).

Diagnostics

HSMI diagnosis is predominantly based on histological characterization of the heart combined with determination of PRV-levels by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). In situations of doubt, immunohistochemistry to show localization of PRV in myocytes from the infected hearts can be performed.

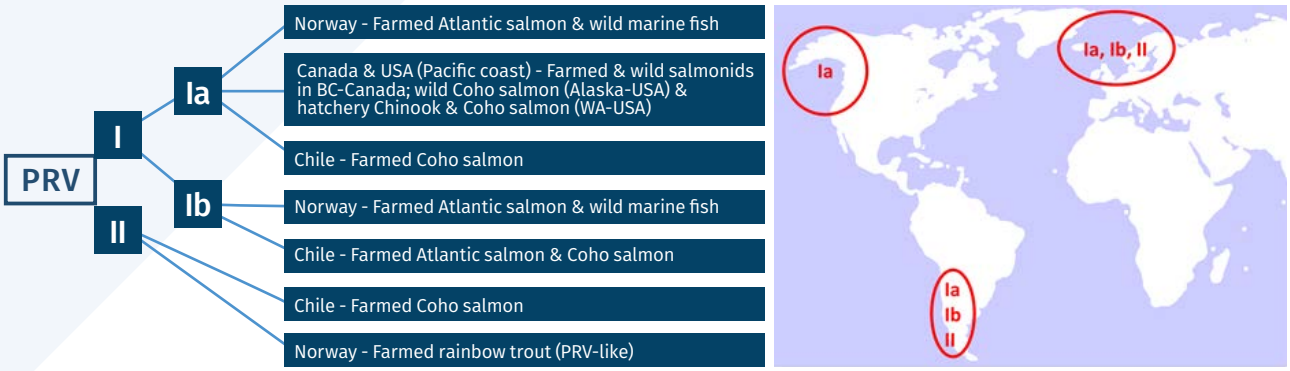

Figure 4. Genetic diversity and global geographical distribution of Piscine orthoreovirus (PRV), based on phylogenetic analyses of segment S1 (Source: Kibenge & Godoy, 2016).

Virus diagnostics

The level of PRV infection is generally monitored by targeting one of the long PRV gene segments, L1, by the RT-qPCR assay. The target tissue used for diagnosis is usually the heart, since the main purpose is to link the virus to HSMI pathology. Since PRV is ubiquitous, its presence in the host is in itself not indicative of disease. However, high levels of virus can be,

and for HSMI diagnostics, PRV is always semi-quantified by providing qPCR cycle threshold (Ct) values along with tissue histology. To avoid false negative answers due to RNA quality issues, tissue housekeeping genes should also be analysed and used for normalization. Ideally, for more accurate description of virus loads, absolute quantification against a standard curve should be performed. PRV levels in infected fish are usually high in blood and in any perfused tissue including the spleen, kidney and heart. Hence, high levels of PRV in heart samples does not necessarily indicate infection of the heart tissue itself or correlate with heart pathology. During an HSMI outbreak, heart and blood PRV Ct values are commonly in the range of 15 to 25. However, experimental studies have shown that the peak amount of virus occurs prior to and in the early phase of HSMI development, and that virus levels in the heart decrease along with inflammatory cell infiltration. For that reason, there is no clear correlation between virus levels and HSMI pathology in samples taken from fish during a field HSMI outbreak, and particularly during the late phase of the outbreak.

So far, there is no information linking PRV genetic markers to virulence, so regular diagnostic investigations in Atlantic salmon do not generally include sequencing or assays designed to detect genetic variants. An exception is when aiming to identify the novel PRV genotypes adapted to other salmonid species, which have less than 90% genomic identity (Olsen, Hjortaas et al. 2015, Takano, Nawata et al. 2016). However, these genotypes are not recognized by the general RT-qPCR assays designed for PRV in Atlantic salmon, and require separate assays and analysis.

Histopathology

The most prominent histopathological finding of HSMI is inflammation in the epi- , endo- and myocardium. An initial epicarditis is followed by a severe infiltration of mononuclear cells and myocardial necrosis in the compactum and spongiosum (Kongtorp and Taksdal 2009, Finstad, Falk et al. 2012, Mikalsen, Haugland et al. 2012). The grade of infiltration in the heart is a basis for scoring the severity of HSMI pathology. The scoring method of choice when assessing the histopathological lesions in HSMI depends on the purpose. A categorical scoring of the heart is sufficient if a yes-no confirmation of HSMI is required for a field outbreak. For more information, the inflammatory changes within each of the cardiac compartments (atrium, epicardium, compactumand spongiosum) can be scored using a semi-quantitative scoring system, in line with the system used for heart lesions in pancreas disease (McLoughlin, Graham et al. 2006). A continuous scoring method using a visual analogue scale can also be used to simplify statistical evaluation of the result, commonly for experimental studies (Finstad, Falk et al. 2012, Mikalsen, Haugland et al. 2012). A more standardized international scoring system for HSMI would be helpful when comparing the HSMI situation based on results from different laboratories.

Transmission

PRV infects through cohabitation in experimental settings (Kongtorp and Taksdal 2009, Palacios, Lovoll et al. 2010), indicating that the virus is shed and transmitted through water. Experimental trials also indicate that the main time of transmission from an infected fish to cohabitants is when the virus reaches peak levels in blood, which is commonly around two-three weeks after experimental infection (Finstad, Dahle et al. 2014). However, in a field setting the transmission time may vary due to lower infection pressure, but this is difficult to assess.

Low levels of viral RNA have been detected in faeces from experimentally infected fish increasing along with blood levels in the first weeks after infection, indicating that faeces may be a PRV shedding route (Hauge, Dahle et al. 2016). The biological routes of PRV entry into new hosts are still unknown, but the virus has been found to enter the blood stream through the intestinal wall within a few hours following injection into the intestine, indicating that intestinal uptake of virus could be a transmission pathway (Hauge, Dahle et al. 2016). Infection by oral intubation was not effective in this experimental setting, and orally injected virus did not appear to reach the gut (Hauge, Dahle et al. 2016). The virus was given in a liquid dilution in this experiment, but if the virus had been present in feed or other solid matrices this could have helped the virus pass the acidic environment in the stomach and infect. Other routes of PRV entry, like gills and skin, may be equally important, but have not been subject to study.

The possibility of vertical transmission has not been extensively studied, and although it is apparently not the main mechanism of transmission, it should be considered until proven otherwise (Wiik-Nielsen et al., 2012a).

HSMI was first described in 1999, but PRV has been found in fish samples from the 1980's, indicating that the virus was around long before the disease was first noticed. Similarly, PRV has been found in North American samples dating back to before Atlantic salmon aquaculture was established on the continent (Marty, Morrison et al. 2015, Siah, Morrison et al. 2015).

So far, there is insufficient data available to predict the spreading routes of PRV. Among the risk factors associated with spread of heart and skeletal muscle inflammation (HSMI) are the proximity to sites with outbreaks and the net connectivity between farming sites (Aldrin et al., 2010). Further studies have shown that the risk of outbreak is associated with a more rapid farming cycle and cohort size within the same site and geographical area (Kristoffersen et al., 2013).

PRV also appears to be common in wild salmonids, but with low pathogenicity, if any (Garseth, Fritsvold et al. 2013). This indicates that the virus may have been able to spread naturally over large distances. Phylogenetic analyses based on selected segments indicate that PRV variants found in Atlantic salmon over several continents, in both farmed and wild fish, are very similar (Siah, Morrison et al. 2015, Godoy, Kibenge et al. 2016). Whether this is due to high selection pressure or recent spreading is not clear. When more whole PRV genome sequences are published, it may be possible to further elucidate the origin of the PRV variant associated with HSMI in farmed salmon.

Control and management

Governmental control

Since 2010, between 100 and 200 Norwegian Atlantic salmon farms have reported HSMI outbreaks annually, reaching a maximum of 181 farms in 2014 (Hjeltnes, Bornø et al. 2017). That year, HSMI was removed from the list of fish diseases notifiable to the Norwegian Food Safety Authority. Since 2015 the number of outbreaks registered by the NVI are therefore no longer complete, and the apparent decrease in reported outbreaks does not represent the general picture in Norwegian aquaculture. The disease situation is most likely unchanged in Norway. There are no governmental reports available from Canada and Chile regarding PRV prevalence and HSMI outbreaks.

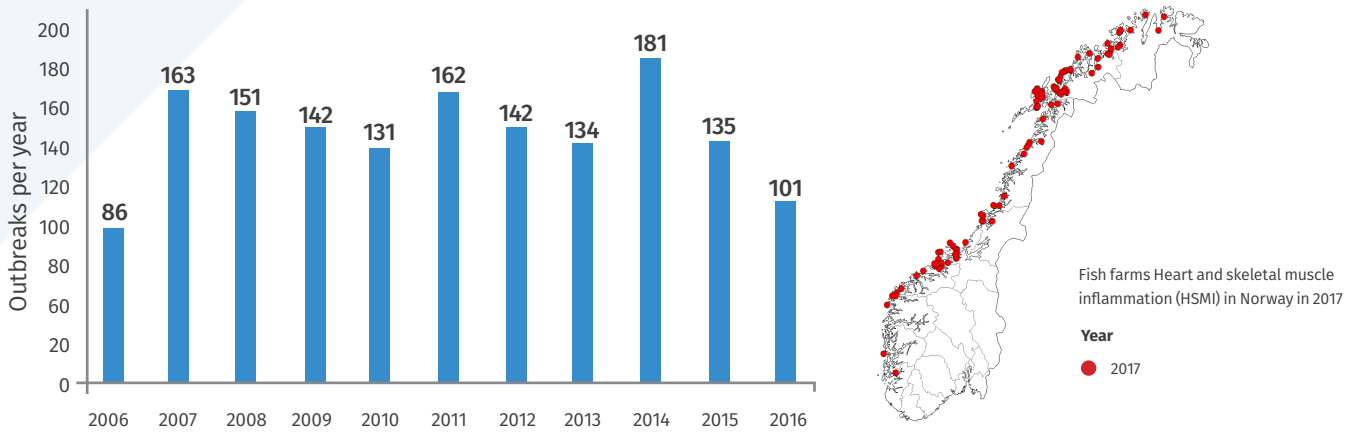

Figure 5. The number and geographical distribution of farms with HSMI outbreaks in Norway. Source: The Norwegian Veterinary Institute.

Management of infected fish

There are currently no interventions in general use in aquaculture against PRV infection and HSMI. Since PRV infection is ubiquitous in farmed salmon and often not associated with disease, the presence of the virus in itself does not initiate any particular procedure, unless PRV is associated with mortality and diagnosis of HSMI.

Since HSMI is a disease induced by inflammation, targeting inflammatory responses has been put forward as a potential intervention. Feed containing a high percentage of marine oils has been associated with reduced HSMI development in experimental studies (Martinez-Rubio, Morais et al. 2012), most likely through anti-inflammatory properties since the level of PRV infection was not affected in these experiments. Several fish feed companies are currently developing HSMI-protective feed.

Field observations from both Norway and Chile indicate that fish suffering from HSMI may be sensitive to stress, and there is a general awareness that infected fish should be handled with care. However, not many studies have directly addressed this topic. One recently published experimental study showed that PRV infected fish were significantly more sensitive to acute hypoxic stress compared to uninfected controls (Lund, Krudtaa Dahle et al. 2017). Stress sensitivity was demonstrated when PRV levels peaked in blood and heart, and heart inflammation was initiated, and the finding was associated with lower hemoglobin levels and reduced heart function (Lund, Krudtaa Dahle et al. 2017).

Vaccination

There are currently no vaccines against HSMI available on the market, or published reports on functional vaccines. However, several companies and ongoing research projects express the aim to develop HSMI vaccines using different strategies ranging from DNA vaccines, recombinant subunit vaccines and whole virus vaccines. A challenge in vaccinating against PRV is the ubiquitous nature of the virus, which indicates that the fish immune system is not able to completely eradicate the virus after infection, and this could be a challenge when aiming to develop effective vaccination. However, PRV infected fish produce PRV-specific antibodies (Teige, Lund et al. 2017), indicating that adaptive immune responses are involved in virus eradication and that vaccination may be a successful approach. There are reasons to believe that results from PRV vaccination trials will be announced in the near future.

Consequences

PRV is an extremely widespread virus, and appears to be very common in farmed Atlantic salmon and Coho salmon, particularly in Northern Europe and Chile. Now, with disease causing PRV genetic variants found in Coho salmon and rainbow trout, the global importance of controlling PRV infections is increasing. Although PRV infection is normally not related to extreme mortality in infected populations, the widespread nature of the virus, the inability of farmed Atlantic salmon to efficiently eradicate it, and the many outbreaks and accumulated high losses in Norwegian aquaculture give many reasons for concern. PRV infection in erythrocytes may lead to additional consequences beyond leading to HSMI, through reducing hemoglobin levels and lowering the tolerance to hypoxic conditions (Lund, Krudtaa Dahle et al. 2017), inducing anemia or liver pathology (Olsen, Hjortaas et al. 2015), or by being involved in melanization of muscle tissue (Bjorgen, Wessel et al. 2015). The subclinical effects of PRV infection may be of more importance than we are aware of today.

Knowledge gaps and challenges

Even though the understanding of PRV and HSMI has increased in recent years, there is still a need for more knowledge on the importance and risks related to the high prevalence of PRV infection in salmonid aquaculture. We also need a more detailed understanding of the factors that control HSMI development and other consequences of the infection. Some obvious knowledge gaps exist.

Why is PRV sometimes pathogenic, sometimes not? It has so far not been possible to pin-point any genetic association to virulence in PRV, and it is possible that factors in the environment are the main triggers of disease. So far, this is still an open question. Although novel experiments have indicated hypoxic stress-mediated mortality in fish with HSMI, hypoxia did not appear to affect the HSMI development itself (Lund, Krudtaa Dahle et al. 2017).

How many PRV variants are out there? The identification of novel PRV variants adapted to specific salmonid species and linked to disease states with different characteristics have opened the door to new ideas of PRV origin, pathogenesis and pathology (Olsen, Hjortaas et al. 2015, Takano, Nawata et al. 2016). These findings have revealed species-specific infection patterns, and raised new questions linked to effects of erythrocyte infection based on the anemia seen in Coho salmon and rainbow trout. Questions related to differences in disease kinetics, tissue specificity, virus transmission and eradication rate can also be raised. Is the explanation linked to genetic factors in the different PRV variants, or to factors in the different salmonid species?

Is there a cell line suitable for culturing PRV? The study of PRV infection mechanisms and the development of whole virus vaccines have been hampered by the lack of a cell culture for PRV replication. Despite many culturing attempts in several labs, PRV has so far only been shown to multiply in primary erythrocytes *ex vivo* (Wessel, Olsen et al. 2015). This challenge is now attempted using novel cell lines and by further dissecting the mechanisms of replication, aiming for knowledge-based construction of a susceptible cell line using novel gene editing techniques.

What is the most effective HSMI intervention? Effective interventions against HSMI are highly warranted, and these may range from vaccines, virus eradication from fresh water facilities by disinfection, HSMI prevention by anti-inflammatory treatment, or breeding of HSMI resistant fish.

Conclusion and discussion

In the years since PRV was identified and associated with HSMI in Norway in 2010, the knowledge on the virus and disease has increased significantly. Most importantly, the PRV-HSMI link has been confirmed, and the global distribution of PRV and the wider consequences of infection is currently unfolding, with novel species-specific PRV variants being identified and linked to disease manifestations that have more or less in common with HSMI. The understanding of PRV pathogenesis and mechanisms behind disease development is evolving, and more tools to study PRV are being produced. Still, the global picture when it comes to PRV infection and HSMI is still under investigation, and there are no effective vaccines or interventions available. The existing knowledge gaps could be closed by continuing focused research on HSMI, along with openly sharing information on the infection and disease status in aquaculture globally. Openness and increased information sharing could bring solutions forward faster.

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Tenacibaculosis

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Introduction

The genus *Tenacibaculum* (Family *Flavobacteriaceae,* Phylum *Bacteroidetes*) represents a group of closely related Gram-negative, yellow pigmented, strictly aerobic, slender, filamentous, rod-shaped bacteria which lack flagella and display characteristic gliding movement. *Tenacibaculum* spp. are common and widespread members of the marine microbiota where they may exist as planktonic cells or in close association with marine organisms (Suzuki et al., 2001; Ferguson et al., 2010) or organic detritus (Kirchman, 2002). *Tenacibaculum* are closely related to the largely freshwater associated genus *Flavobacterium* and both genera perform a vital role in environmental carbon cycling (Kirchman, 2002). Representatives of both genera cause serious disease in farmed fish around the world.

Infections associated with *Tenacibaculum* spp. are commonly referred to as 'tenacibaculosis', a term originally coined to describe the ulcerative disease produced in marine fish by *Tenacibaculum maritimum* (Avendaño-Herrera et al., 2006). The term has replaced various names based on the clinical signs observed, e.g. eroded mouth syndrome and black patch necrosis (Santos et al., 1999). Both the awareness and impact of tenacibaculosis as a problem in salmon farming appear to be increasing. A number of *Tenacibaculum* species and strains associated with this disease have certainly been identified in recent years. Although there exists a great deal of evidence linking in particular *T. maritimum* (Wakabayashi et al., 1986) to disease in farmed marine fish species, the pathogenic role of various other *Tenacibaculum* taxa isolated during diagnostic investigations in salmon farming around the world is not always clear. The clinical picture identified in the field may be difficult to recreate in the laboratory and development of clinical disease may be dependent on a complex balance of host, agent and environmental parameters (Avendaño-Herrera et al., 2006).

Taxonomy/diversity

More than 25 *Tenacibaculum* species have been described (http://www.bacterio.net/tenacibaculum.html) and many more as yet undescribed taxa undoubtedly exist. The described *Tenacibaculum* spp. associated with fish disease include *T. maritimum* (formerly *Flexibacter maritimus*) (Wakabayashi et al., 1986), *T. dicentrarchi,* originally isolated from diseased farmed seabass (*Dicentrarchus labrax*) (Piñeiro-Vidal et al., 2012), *T. discolor* and *T. soleae* isolated from diseased Senegalese sole (*Solea senegalensis*) (Piñeiro-Vidal et al., 2008a; 2008b) and *T. ovolyticum* isolated from Atlantic halibut eggs (*Hippoglossus hippoglossus*) (Hansen et al., 1992). A new species associated with tenacibaculosis in salmon farmed in the north of Norway has been proposed as '*T. finnmarkense*' (Småge et al., 2016), but does not have standing in nomenclature. Recently, *T. dicentrarchi* has been associated with skin ulcers in wrasse (*Labridae*), lumpsucker (*Cyclopterus lumpus*), Atlantic cod (*Gadus morhua*) and Atlantic salmon (*Salmo salar*) in Norway, and a number of as yet undescribed *Tenacibaculum* taxa have also been isolated from diseased fish, including Atlantic salmon and rainbow trout (Habib et al., 2014; Olsen et al., 2017). *T. dicentrarchi* has also been associated with high mortalities in Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) farmed in Chile (Avendaño-Herrera et al., 2016), as well as in Chilean red conger eel (*Genypterus chilensis*) (Irgang et al., 2017). More recently, Apablaza et al. (2017) reported the first isolation of *T. maritimum* from Chilean Atlantic salmon mortalities during a harmful algal bloom caused by *Pseudochattonella* spp.

Tenacibaculum as a pathogen of farmed salmonids

Tenacibaculosis in salmonids has been reported from all salmon-producing areas of the globe, and can be roughly split into two main types, i.e. outbreaks associated with *T. maritimum* and those associated with non-*T. maritimum* species or strains.

Most studies involving *T. maritimum* associated disease have

focused on marine fish species (reviewed by Avendaño-Herrera et al., 2006). This bacterium has, however, for many years also been associated with disease in salmon farming countries with 'milder' climates and moderate sea water temperatures including Tasmania (Schmidtke and Carson, 1995), Spain (Pazos et al., 1996), Ireland (Fringuelli et al., 2012), USA (Chen et al., 1995) and western Canada (Kent, 1988; Frisch et al., 2017). *Tenacibaculum maritimum* has also recently been described for the first time in diseased Atlantic salmon farmed in Chile (Apablaza et al., 2017).

Non-*T. maritimum* species isolated from Atlantic salmon include *T. dicentrarchi* in Chile and Norway (Avendaño-Herrera et al., 2016; Olsen et al., 2017) and '*T. finnmarkense*' in Norway (Småge et al., 2016). Recently several genetic clusters of *Tenacibaculum* spp. consistent with undescribed species have been associated with salmon tenacibaculosis in Norway (Olsen et al., 2017; see below for discussion of the Norwegian situation). The precise aetiological role of these taxa remains to be elucidated. *Tenacibaclum maritimum* has been associated with disease in lumpsucker (Småge et al., 2016) and turbot (Olsen, unpublished data) farmed in Norway, but has not been identified in Norwegian farmed Atlantic salmon (Olsen et al., 2017).

Diagnosis of tenacibaculosis

While tenacibaculosis, particularly when caused by *T. maritimum*, may present as a systemic infection, the majority of cases in Norway and Chile involve topical infections of the integument, mainly affecting head, abdomen, fins and gills as skin ulceration and fin or gill 'rot'. Tenacibaculosis in Atlantic salmon raised on the Pacific coast of Canada is normally restricted to an infection of the jaws and gills, whereas ulceration is also common in Atlantic salmon in Canada.

Fish of all ages farmed in seawater may be susceptible. Tenacibaculosis in salmon caused by different types/species of *Tenacibaculum* may present similar clinical pictures. Mouth rot or bacterial stomatitis (Ostland et al., 1999; Frisch et al., 2017) is one of the most common manifestations of tenacibaculosis in both *T. maritimum* (Ostland et al., 1999) and non-*T. maritimum* associated disease in salmonids. Corneal infections, rupture of the eye (Handlinger et al., 1997; Olsen et al., 2011) and necrotic gills are also reportedly associated with different *Tenacibaculum* bacteria (Chen et al., 1995; Handlinger et al., 1997; Mitchell and Rodger, 2011). The presence of large numbers of *Tenacibaculum* cells may also give affected areas of skin or gills a yellowish hue and macroscopically visible yellow plaques are also reported (Ostland et al., 1999). Histopathological findings common in external lesions of general tenacibaculosis include necrosis in collagen rich tissues like the dermis and the presence of large numbers of long bacterial cells. Inflammatory reactions are usually absent or sparse.

Identification of long, non-motile rods by direct microscopy of smears from ulcers is indicative of *Tenacibaculum* infection. Histopathology is an excellent tool to visualize the infection and increase the detection rate of tenacibaculosis. Immunohistochemistry techniques have been developed to identify the bacteria involved (Olsen et al., 2011; Faílde et al., 2013).

While typical clinical signs may provide grounds for presumptive diagnosis of tenacibaculosis, they do not constitute grounds for definitive diagnosis, as similar clinical signs may result from other causes and ulcerous lesions favor the entry of many different types of bacteria, including *Tenacibaculum* spp.

Several PCR methods have been described for detection of *T. maritimum* (Cepeda et al., 2003; Avendaño-Herrera et al., 2004; Fringuelli et al., 2012), *T. soleae* (López et al., 2010; García-González et al., 2011) and more recently, Avendaño-Herrera et al. (2018) reported a PCR procedure for detection of *T. dicentrarchi* in fish samples.

As tenacibaculosis is not a notifiable disease in Norway, Chile or Canada, the infection is often diagnosed at a local level, based on indicators such as direct microscopy and/or culture of yellow-pigmented colonies of typical morphology. There may, therefore, be a degree of uncertainty relating to the *Tenacibaculum* spp. involved. Local or private diagnosis also means that the incidence of tenacibaculosis is most probably underreported.

Culture and classification

Tenacibaculum species grow poorly or not at all on most

general-purpose agars, even when supplemented with NaCl. They may be cultured by inoculation of ulcer material on low nutrient media containing sea-salts, e.g. FMM (*Flexibacter maritimus* medium) and Marine Agar 2216 (Pazos et al., 1996). The various species of *Tenacibaculum* display a wide range of optimal culture temperatures, which vary from 15°C to 30°C. It appears that *T. maritmum* isolated from salmon and other cold water species may have lower optimal temperatures than *T. maritimum* isolates originating from warmer water fish species (Frisch et al., 2017). Incubation temperatures of between 15 to 18°C are probably suitable for most salmonid related isolates. *Tenacibaculum* are relatively slow growing and cultivation directly from ulcers may be difficult. To increase the likelihood of successful culture, tissue scrapings should be obtained by scalpel and inoculated onto the plate prior to careful spread. Addition of 50 mg/mL kanamycin to culture media has been reported to aid recovery through depression of 'contaminating' bacterial growth (Frisch et al., 2017). *Tenacibaculum* spp. grow with pale to bright yellow colonies consisting of rod-shaped, long hair-like bacterial cells, which may become rounded in older cultures. *T. maritimum* colonies are particularly adherent to the culture agar.

Tenacibaculum spp. are biochemically relatively poorly active and identification to the species level using traditional phenotypic methods may be challenging, particularly given the phenotypic differences reported between Chilean and European isolates of the same species (Piñeiro-Vidal et al., 2012; Avendaño-Herrera et al., 2016; Irgang et al., 2017). With the ever-increasing availability of advanced analytical methods such as PCR, gene sequencing and proteomics, phenotype based studies are becoming less frequently used.

Given the diversity of undescribed *Tenacibaculum* taxa within the environmental flora, development of specific molecular detection analyses may also present challenges. However, tools for identification of particular taxa such as the various PCR-based assays mentioned previously have been developed and allow non-culture based highly sensitive detection of the target bacterium within the range of matrices for which the assays were tested.

Genotyping by multilocus sequence analysis (MLSA) and multilocus sequence typing (MLST) has been developed for *Tenacibaculum* spp., http://pubmlst.org/tenacibaculum (Habib et al., 2014).

Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry, which differentiates bacterial species and strains on the basis of mass-charge of (mainly) ribosomal proteins has also recently been demonstrated as a useful and extremely rapid tool for the identification and differentiation of *Tenacibaculum* species (Fernández-Álvarez et al., 2017).

Reservoirs and transmission

Tenacibaculum spp. are common and widespread in marine waters, suggesting that the main sources of infection are environmental. While *Tenacibaculum* spp., as rich producers of extracellular proteases (van Gelderen et al., 2009) are almost certainly responsible for the majority of the tissue losses observed during outbreaks of tenacibaculosis, the epidemiology may be complex and outbreaks of disease are often associated with environmental challenges, suboptimal management and/or poor resistance in affected fish. Following establishment of an infection, shedding of bacteria from ulcerated fish will undoubtedly increase the risk of spread of infection, but as fish in neighbouring cages may or may not be affected, the degree of infectivity is unclear. *Tenacibaculum maritimum* may be associated with marine organisms, and blooms of jellyfish could conceivably provide both a colonization site (via initial nematocyst related injuries) and a source of *Tenacibaculum* infection (Ferguson et al., 2010; Delannoy et al., 2011). Barker et al. (2009) also suggested the sea lice (*Lepeophtheirus salmonis*) might serve as an organic substrate capable of extending the persistence of *Tenacibaculum* cells in seawater. The mode of transmission and route of infection of tenacibaculosis in salmon in general remain unclear. Consistent reproduction of disease under controlled laboratory conditions is often difficult, although clinical signs consistent with *T. dicentrarchi* infection and mortality have been demonstrated in Atlantic salmon and rainbow trout exposed to 3.78×10^5 CFU/mL *T. dicentrarchi* for 60 minutes (Avendaño-Herrera et al., 2016). Despite the association between *T. dicentrarchi* isolation and observed clinical changes in salmon in Chile, there is no conclusive evidence that this bacterium is a primary or opportunistic pathogen.

The ability of particular genotypes of *T. maritimum* (Habib et al., 2014) and other *Tenacibaculum* spp. (Olsen et al., 2017) to colonize multiple fish species within restricted geographic areas may indicate that the pathogen/host relationship is regulated by geographical limitations rather than host specificity within certain strains.

Treatment of disease

As *Tenacibaculum* spp. infections are most often external, antibiotic treatment may not be successful and is not generally recommended. Antibiotic treatments are at times considered necessary, however (see regional situation reports below), and the response to treatment seems to be variable. One measure to reduce mortalities once the infection is detected is rapid removal of fish with macroscopic lesions, thereby attenuating further transmission of infection.

Prophylaxis

Currently, vaccination is the only measure to prevent the appearance of diseases of bacterial origin in fish farming. Some success has been achieved in developing vaccines against *T. maritimum* infections in marine fish species (Romalde et al., 2005). Despite the favorable results generated following intraperitoneal application of an adjuvanted vaccine against *T. maritimum* related tenacibaculosis in Atlantic salmon (van Gelderen et al., 2010), no commercial vaccine against tenacibaculosis in salmonids is currently available. The genetic and possibly antigenic variety amongst the bacterial isolates associated with tenacibaculosis, particularly in non-*T. maritimum* associated cases, may complicate the choice of candidate for vaccine development (Irgang et al., 2017, Olsen et al., 2017). Handlinger et al. (1997) highlighted the improvement of fish management in general as an important reason for the decline in tenacibaculosis outbreaks in Australia. Handling and other management routines, such as mechanical anti-lice treatments, should therefore be kept to a minimum. Great care should be taken to avoid compromising the skin barrier of the fish.

The Norwegian situation

Tenacibaculum spp. have been associated with ulcers of farmed Norwegian salmonids since the late 1980's both as co-infections with *Moritella viscosa* (Olsen et al., 2011) or as the dominating bacterium. During the last decade, however, tenacibaculosis (sometimes referred to as 'atypical' winter ulcer) typified by extreme necrosis and tissue loss involving the head/jaw has become increasingly considered a serious threat to the farming industry. The northernmost areas of the country, i.e. Finnmark and Troms, are most severely affected, although outbreaks may occur over the whole coastline. Case histories may vary considerably, but a common Norwegian tenacibaculosis scenario appears to be related to outbreak of acute disease in smolts newly transferred to very cold seawater. The prevalence within affected cages may be extremely high (>80%) and associated with a severe, acute mortality period. In some cases, such outbreaks have been associated with jaw/nose abrasions following sea transfer and/or salmon louse treatment. In some farms, the disease is reported to transmit in an infectious manner, with cage-to-cage spread, while in other farms the disease has caused heavy mortalities in single cages without further apparent spread.

The reasons for the recent emergence of this extreme type of tenacibaculosis as a severe disease in Norway are unclear, but it is thought that current farming practices may be contributing to the situation. The Norwegian aquaculture industry is extremely dynamic and continuously driven towards increasing efficiency. Stocking practices have changed in relatively recent times from single annual transfers of smolts to sea in the spring, to continual sea-transfer of 'developmentally manipulated' smolts. Even in Finnmark, the most northerly region of the Norwegian mainland, smolts are now transferred to sea throughout the year. The introduction of mechanical de-licing methodologies including treatments based on warm-water, physical brushing and/or water-jets, as well as broad use of H2O2 in chemical treatments, may also play an important role.

While *T. maritimum* has recently been isolated from farmed lumpsucker (Småge et al., 2016) and turbot (Olsen, unpublished data) in Norway, this bacterium has not yet been described from Norwegian farmed salmon. A number of taxonomic studies have been performed on *Tenacibaculum* isolated from Atlantic salmon in Norway (Olsen et al., 2011; Habib et al., 2014; Småge et al., 2016; Olsen et al., 2017) and all indicate

that various species and strains may be involved. Four main clades of related bacteria have been identified by MLSA (Olsen et al., 2017) of a collection of isolates collected over two decades from seven species of fish. Salmon isolates were represented in all four clades. While members of one clade showed a high degree of similarity to *T. dicentrarchi*, the three remaining clades probably represent undescribed species. It is not clear at this time whether *T. finnmarkense* sp. nov., isolated from a tenacibaculosis case in northern Norway and proposed by Småge et al. (2016) represents one of the three 'novel' clades identified by Olsen et al. (2017). The overall lack of clonality and host specificity among the majority of Norwegian *Tenacibaculum* isolates examined, together with a certain tendency towards regional separation (Olsen et al., 2017), may indicate that *Tenacibaculum* infections primarily occur as local epidemics involving one or more strains. The lack of intra-outbreak clonality also indicates that at least in these cases, fish to fish transmission may not represent the primary transmission route.

The Irish situation

In Ireland, tenacibaculosis in Atlantic salmon is associated with *T. maritimum* infection and is usually present as superficial infections, most likely opportunistic in nature. The disease is commonly observed in post-transfer smolts with poor fin condition, and in skin and gill associated lesions in ongrowing salmon. It is commonly associated with jellyfish damage.

Tenacibaculum maritimum has been detected on the gills of Atlantic salmon smolts at very high prevalence in the late summer and autumn, without any evidence of tenacibaculosis.

The Canadian situation (British Columbia, BC)

Tenacibaculosis, an emergent disease in Canada, may cause significant outbreak events in post-seawater entry Atlantic salmon smolts that can result in significant mortalities and is a bacterial disease of concern in BC.

Tenacibaculum maritimum associated tenacibaculosis is referred to in BC as 'yellow mouth' due to the visible yellow plaques on the mouth of affected fish. Yellow mouth has been reported in BC and Washington State since the beginning of Atlantic salmon fish farming in the late 1980s (Kent, 1988). The disease remains important and can lead to significant revenue losses. Recent observations reported a bimodal spike of mortalities with a first spike one to two weeks post entry to seawater and a second peak when fish are about 400 grams. It also reported that environmental conditions, particularly high salinity and temperature, may significantly contribute to the severity of outbreaks. The current mitigation strategy for tenacibaculosis is treatment with an antibiotic such as florfenicol or potentiated sulfonamides during the first two months after seawater entry. While tenacibaculosis is one of the few remaining bacterial infections in salmon farming treated regularly with antibiotics in BC, several treatments may be required to mitigate and stop the disease which can continue for several months. The costs involved in both treatment and production losses are estimated in the millions of dollars.

The Chilean situation

While Bernardet (1998) suspected *T. maritimum* to be responsible for epizootics in Chilean Atlantic salmon farming in the 1990s, and 35 diagnoses relating to *T. maritimum* were reported between 2013-2015 by the National Fisheries and Aquaculture Service (SERNAPESCA), infection involving this bacterium and Atlantic salmon in Chile was confirmed only in 2016 (Apablaza et al., 2017). Unfortunately, this research did not include reproduction of Koch's postulates, so the virulence properties of the Chilean isolate of *T. maritimum* Ch-2402 cannot be confirmed. *Tenacibaculum dicentrarchi* Ch-2402, was also isolated from the same farming site.

Avendaño-Herrera et al. (2016) described the isolation, identification and characterization of six isolates of *T. dicentrarchi* obtained from two outbreaks in salmon occurring at a farming site in Puerto Montt, Chile, in October 2010 and 2014. The bacterium has, therefore, been present in the aquatic environment and associated with salmon farming in Chile at least since 2010. In the first outbreak in 2010 involving 25-30 g fish, *T. dicentrarchi* infection resulted in mortality rates of 50-60%, with only 40% of the dead fish showing clinical signs of the disease. As has been reported in some cases in Norway, only some cages of fish may be affected. The reason for this selective situation is not known, since the Atlantic salmon specimens have the same origin, they are subject to the same

environmental farming conditions and the handling is not different among cages.

Mixed infection with *Piscirickettsia salmonis* are relatively common. This situation again raises the question as to whether *T. dicentrarchi* is a primary or secondary, opportunistic pathogen.

More recently, seven draft genomes of *Tenacibaculum* strains, including the *T. dicentrarchi* (USC 3509T) and *T. finnmarkense* (HFJT) type strains, as well as five field isolates from Chile and Norway selected on the basis of available MLST data (Olsen et al., 2017) Bridel et al. (2018) led to the correct affiliation of strain AYD7486TD, which actually belongs to the species *T. finnmarkense* rather than to *T. dicentrarchi* as previously claimed (Grothusen et al., 2016). Importantly, this result demonstrates that *T. finnmarkense* is also present in Chilean fish farms, suggesting that *T. dicentrarchi* strains form a cohesive group whereas *"T. finnmarkense"* strains are split into two subclusters.

Knowledge gaps

It is clear that tenacibaculosis in farmed Atlantic salmon, despite many commonalities, is not a single disease/infection. The situation in Australia and west-coast Canada appears to be entirely dominated by *T. maritimum* associated infections, the Chilean situation by both *T. dicentrarchi* (Avendaño-Herrera et al., 2016) and *T. maritimum* (Apablaza et al., 2017) while the Norwegian situation may be associated with a number of *Tenacibaculum* species or strains including speciated (Småge et al., 2016; Olsen et al., 2017) and unspeciated (Olsen et al., 2017) taxa. While *T. maritimum* has been identified with tenacibaculosis in Ireland, the putatively pathogenic nature of the *Tenacibaculum* microbiota of Scotland and the Faroe Islands (both important producers of Atlantic salmon) is less clear.

It is evident, therefore that tenacibaculosis may involve different *Tenacibaculum* species and it is possible that different communities of bacteria may contribute to the clinical signs observed. Although the body of work is increasing, it is clear that we lack a good understanding of the population structure of the pathogenic Tenacibaculum species, the pathogenesis of tenacibaculosis and basic knowledge on whether prophylaxis through vaccination is a viable alternative particularly for non-*T. maritimum tenacibaculosis* in salmon.

Already several whole genome sequencing (https://www.ncbi.nlm.nih.gov/genome/?term=tenacibaculum) projects are completed or underway and it is hoped that further genomic investigations will help identify and characterize relevant strains/species involved. Such information will help us understand the pathogenesis of disease, and the virulence and antibiotic resistance mechanisms involved. It will also provide an improved basis for development of sensitive and specific diagnostic methodologies and therefore better management of the disease at the farm level.

The traditional diagnostic methods for *Tenacibaculum* spp. are inexpensive, simple to use and offer reliable results, but are time-consuming. Thus, developments of molecular techniques for the rapid presumptive and/or confirmatory diagnosis of the various taxa are essential.

Little is known of the antigenic and genetic heterogeneity within *Tenacibaculum* populations, particularly non-*T. maritimum* isolates. Such knowledge will be essential for the development of effective biological products such as vaccines.

Up to now, no studies have been carried out to identify the primary reservoirs of infection, or the role of farming water in the epidemiology of these infections.

Although the available evidence indicates environmental factors to be of crucial importance in the pathogenic potential of various *Tenacibaculum* species, manifestation of tenacibaculosis could be deeply influenced by other factors. Therefore, thorough epidemiological studies are required to precisely determine risk factors such that this knowledge may be used to control and prevent future outbreaks.

Finally, while antibiotic treatment should be considered a last resort, research is needed to evaluate the efficacy of various antibiotic treatments. Likewise, alternatives to chemotherapeutics, such as probiotics and antimicrobial peptides should be investigated.

Future perspectives

As salmon farms become ever larger and farming more technically demanding, there are few reasons to believe *Tenacibaculum* problems will disappear in the near future, unless management routines now known to damage skin health are improved upon or discontinued.

Climate change and expansion of salmon farming into new geographical areas may also lead to new *Tenacibaculum* related disease problems. The recent isolation of *T. maritimum* from lumpsucker in Norway and salmon in Chile show that this bacterium is already present in areas of relatively cold water. Even slight increases in water temperature may result in establishment of *T. maritimum* associated disease in salmon farming in more northern areas, e.g. Norway. There are constant expansion pressures in salmon farming and increasing sea temperatures may allow farming of salmon in polar areas. This will very likely result in problems associated with the colder water types of *Tenacibaculum*. There are many good reasons therefore to maintain awareness and a research focus on *Tenacibaculum*.

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Bacterial Kidney Disease

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Introduction

Bacterial kidney disease (BKD) is a worldwide disease in wild and farmed salmonid fish causing losses at all stages of the salmonid life cycle in both fresh and saltwater. A chronic, granulomatous inflammation reminiscent of mycobacterial tuberculosis is caused by the Gram positive etiologic agent *Renibacterium salmoninarum* (Rs). The lack of satisfactory vaccines and chemotherapeutics make avoidance strategies important. These strategies vary according to the epidemiological situation in the different countries as described in the following sections from Chile, British Columbia and Norway. Fairly recent dissemination of Rs due to movement of covertly infected fish and the industrial scale of salmon aquaculture in new areas makes BKD an important emerging disease. BKD is a constant, unpredictable threat to salmonid fish health. Improved control of BKD is thus needed. The genome sequencing of Rs was a landmark that opened for new research approaches (Wiens et al., 2008). However, experimental studies necessary for improved vaccines are still very demanding due to the chronic disease development and the slow growth of Rs. More information on the history of BKD and research efforts can be found in Fryer & Sanders (1981) and Wiens (2011).

Taxonomy – diversity

R. salmoninarum is the only member of its genus. The closest known ancestors are *Arthrobacter* spp. which are soil bacteria with genomes of about 5 million base pairs. This is reduced to about 3 million base pairs in Rs reflecting its parasitic lifestyle. The size of Rs is only 0.3–1.5 µm by 0.1–1.0 µm. It is an aerobic, non-motile, non-spore forming, slow growing and facultative intracellular bacterium with a high Guanidine-Cytosine (G+C) content of genome of 56.3% (Sanders & Fryer, 1980; Wiens et al., 2008). Rs from many different sources show an unusually high phenotypic and antigenic homogeneity (Fiedler & Draxl, 1986; Getchell, Rohovec, & Fryer, 1985; Grayson, Cooper, Atienzar, Knowles, & Gilpin, 1999). There is also a very close phylogenetic relatedness, but analysis with both genome-wide single-nucleotide polymorphism and multilocus VNTR identified two disparate Rs groups (Brynildsrud et al., 2014; Matejusova et al., 2013). One large Rs group is found globally from a wide range of salmonid hosts, includes the 1974 ATCC type Leaburg strain from Oregon and could reflect spread of Rs by trade with fish. Another, smaller Rs group found only in Scotland and Norway from genus *Salmo*, includes the 1960 River Dee strain and could reflect an original European Rs clade. In coding genes, most variation is seen in surface associated virulence factors called major soluble antigen (msa)/p57 and p22 (Brynildsrud, Gulla, Feil, Norstebo, & Rhodes, 2016; Wiens, Pascho, & Winton, 2002).

Renibacterium salmoninarum as a pathogen

Impact

BKD results in significant mortalities of both farmed and wild salmonids in fresh- and seawater worldwide, especially in Chinook salmon and Coho salmon farming, while Atlantic salmon and rainbow trout appear more resistant. BKD mortality rates can come close to 80% in stocks of Pacific Salmon and 40% in stocks of Atlantic salmon (Evenden, Grayson, Gilpin, & Munn, 1993). However, the main economic impact is probably due to the chronic disease resulting in poor growth, seen as many runts, and increased susceptibility to additional diseases. Such losses are best visualized by analysis of accumulated mortality and productive indexes. Recently, epidemiologists have proposed case definitions to help estimation of BKD losses (Boerlage, Stryhn, Sanchez, & Hammell, 2017).

Clinical disease and gross pathology

Clinical and external disease signs are non-specific and include lethargic swimming, exophthalmia, dark skin, pale gills, fin base bleeding and abdominal distension. Occasionally skin vesicles, abscesses and ulcers can be found. Further, skin rashes without any internal pathology have been reported in rainbow trout broodfish (Ferguson, 2006). In contrast, autopsy often raises suspicion of BKD due to the presence of severe nodular, whitish lesions that may enlarge the kidney considerably. At times

miliary (millet-like) small lesions can be found in most organs (Figures 1 and 2). Further, blood tinted ascites and petechiae in the muscle under the peritoneum also reflect the underlying hematogenous dissemination. The ascites are usually quite yellow indicating an exudate and during winter, a chronic peritonitis in Atlantic salmon may lead to severe adhesions between organs (Smith 1964). When the lesions become large, caseous necrotic conglomerates, they compromise organ functions. The anemia and immunosuppression in BKD may reflect the extensive destruction of lympho- and hemato-poietic tissues. The frequent involvement of the heart may lead to circulatory disturbances. Occasionally, cave-like muscle lesions can compromise the meat quality of Coho Salmon. In dead fry and small parr, internal lesions can be indistinct, soft and pale and thus easily confused with autolysis, but the tissues are necrotic and being digested by Rs.

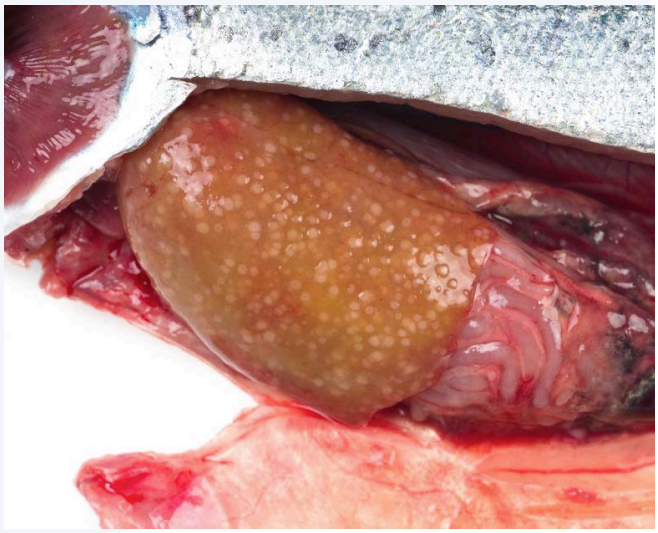

Figure 1. Atlantic Salmon (*Salmo salar*) with clinical signs of BKD. Multiple white nodulations can be observed in the liver, a manifestation macroscopically indistinguishable from miliary tuberculosis. Photo: Elanco Animal Health.

Histopathology of BKD

The visible lesions vary from necrosis with many bacteria (Figures 3 and 4) to well organized granulomas with few or no visible bacteria, but many epithelloid macrophages and lymphoid cells (Figures 5 and 6). Occasionally, macrophages coalesce to form multinucleate giant cells, but not as often as in fungal infections. Pseudomembranes composed of a thin layer of fibrin and collagen, phagocytes and bacteria may cover the serosa of organs (Elliott, 2017), especially at low temperature (Smith, 1964). The bacteria occur both extracellularly as well as intracellularly in phagocytic, epithelial, endothelial, neutrophil, reticular and sinusoidal cells of any tissue, although most prominently in the kidney and spleen (Bruno, 1986; Elliott, 2017; Ferguson, 2006; Flaño, López-Fierro, Razquin, Kaattari, & Villena, 1996). By transmission electron microscopy (TEM), electron-dense glomerular subendothelial deposits resembling immune complexes have been observed, but the nature and impact of this pathology remains to be explored (Sami, Fischer-Scherl, Hoffmann, & Pfeil-Putzien, 1992; Young & Chapman, 1978).

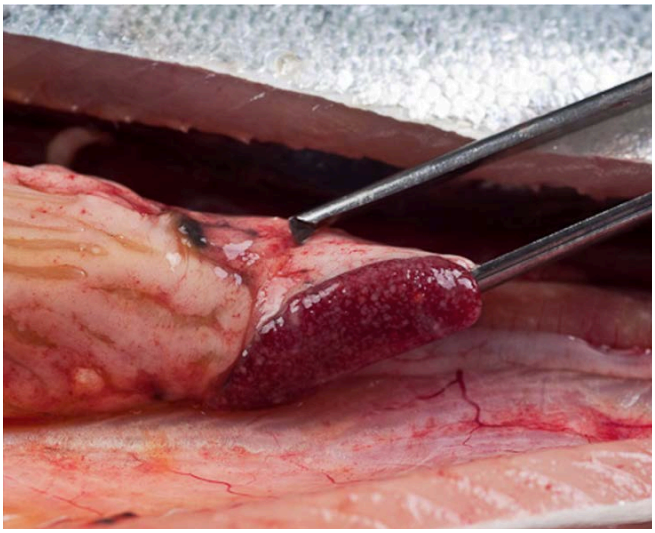

Figure 2. Atlantic Salmon (*Salmo salar*) affected by clinical signs of BKD. Multiple white nodulations can be observed in the spleen (photo: Marcos Godoy).

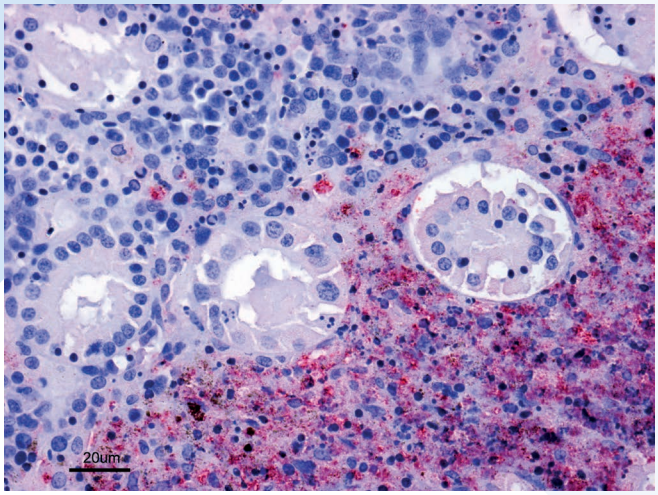

Figure 3. Immunohistochemistry of a necrotic kidney lesion in Atlantic salmon using a monoclonal antibody (4D3) against p57 (Wiens, Chien, Winton, & Kaattari, 1999) coloring the bacteria red. The high specificity of the Mab makes IHC useful to identify Rs quickly when pathology raise suspicion of BKD in Norway. The lack of granulomatous inflammation will make the texture of this necrotic lesion soft (Photo: Ole Bendik Dale).

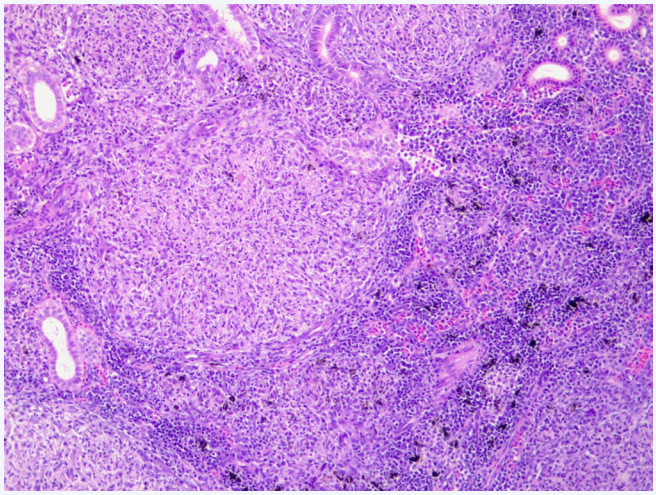

Figure 5. Atlantic Salmon (*Salmo salar*) kidney (H&E, medium magnification). The presence of multiple granulomas in the renal interstitium can be observed. The texture of granulomas is hard compared to the soft necrotic lesions (Photo: Marcos Godoy).

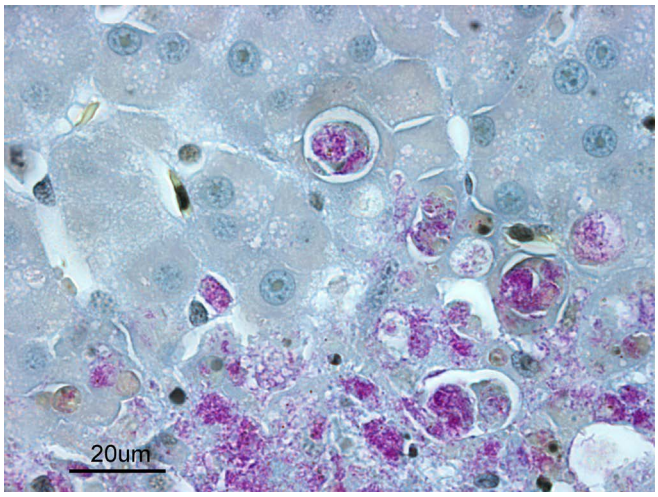

Figure 4. Atlantic salmon necrotic liver lesion with purple stained intracellular bacteria from using a modified PAS stain: Lillies Allochrome that demonstrate polysaccharides of the cell wall. Intracellular, Gram and PAS positive, but not acid fast bacteria in salmonids are very likely Rs (Photo: Ole Bendik Dale).

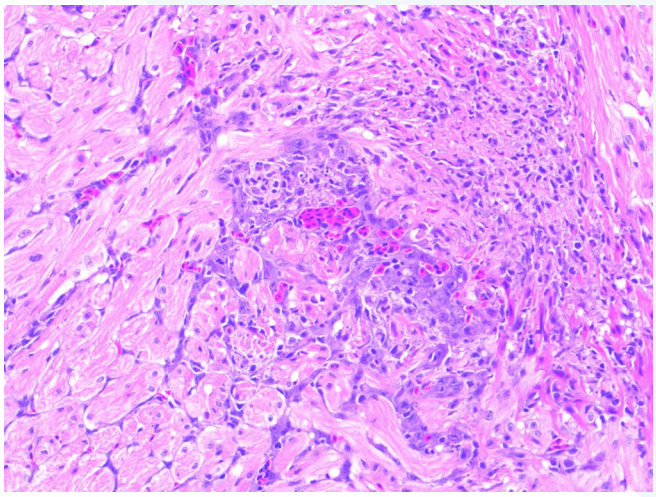

Figure 6. Atlantic Salmon (*Salmo salar*) heart (H&E, medium magnification) with granulomatous inflammation in the myocardium (Photo: Marcos Godoy).

Clinical chemistry - hematology

In experimental BKD, circulating erythrocytes decrease by 59-66% while a transitory increase in neutrophils, monocytes and thrombocytes is seen. The anemia is microcytic and erythrocyte sedimentation is increased (Bruno & Munro, 1986). Blood chemistry changes include decreased cholesterol and sodium, electrophoretically faster migrating serum proteins, and increased serum bilirubin, blood urea nitrogen and potassium concentrations (Wiens, 2011).

Pathogenesis and virulence factors

Rs infection may result in different clinical outcomes: immediate BKD; persistent infection without clinical signs (healthy carrier); late BKD developing from a persistent infection. In an infected population there is probably a continuous spectrum of conditions in between these categories, and even within the same individual fish, considerable lesion heterogeneity and intermediate states may co-exist.

Although the pathogenesis is largely unknown, some features can be suggested. Vertical transmission seems important for the perseverance of Rs over generations of fish (Evelyn, Ketcheson, & Prosperi-Porta, 1984). It may not be accomplished in many fish, but sufficiently to start dissemination by horizontal transmission, e.g. by the fecal-oral route (Balfry, Albright, & Evelyn, 1996). However, the exact mode of invasion is unknown. After invasion, opsonisation (Rose & Levine, 1992) may result in rapid uptake of Rs by phagocytes (Bruno, 1986) where Rs may survive by escape from the phagocytic vacuoles (Gutenberger, Duimstra, Rohovec, & Fryer, 1997). Thus, the very cells that should destroy invaders can become "Trojan horses" disseminating Rs to various tissues. The ensuing granulomatous inflammation can be counterproductive as it fails to contain the infection and instead, destructive conglomerates of necrosis and granulomas are formed. However, in some cases Rs can be few or absent in the granulomas and a healthy carrier state, or at best sterilizing immunity is achieved. However, Rs may become dormant, perhaps due to hypoxic conditions in the lesions, and disease reactivation may take place later if immunosuppressive events occur. On a population basis, relapse in maturating brood fish may ensure another round of vertical transmission.

The molecular traits of Rs have been the subject of numerous studies as reviewed in detail elsewhere (Wiens, 2011). Some remarkable features are mentioned here. Rs has its own "wake up call" or resuscitation-promoting factors (Wiens et al., 2008) that may be important to go from a dormant state to start replicating. The bacterial wall appears very resilient and when trypsinized, 60% of the dry weight comes from a special polysaccharide (Sorum, Robertsen, & Kenne, 1998). A capsule 50-100 nm thick has been described (D. Dubreuil, Lallier, & Jacques, 1990). Further, hemolytic activity (Grayson, Gilpin, Evenden, & Munn, 2001), iron-acquisition ability (Grayson, Bruno, Evenden, Gilpin, & Munn, 1995) and a zinc-metalloprotease (Grayson, Evenden, Gilpin, Martin, & Munn, 1995) have been described. Unique to Rs is the major soluble antigen (msa), also known as p57, that agglutinates salmonid spermatocytes and leukocytes, and accumulates in the fish tissues (Daly & Stevenson, 1990; Wiens & Kaattari, 1991). It is possibly related to fimbria seen on TEM (J. D. Dubreuil, Jacques, Graham, & Lallier, 1990). The msa/ p57 protein has two sequence repeats, one related to known adhesion-repulsion molecules, while the other seems unique to Rs (Chien et al., 1992; Wiens et al., 1999). The msa/p57 proteins seem able to suppress bactericidal phagocytic activity (Siegel & Congleton, 1997), the respiratory burst of phagocytes (Densmore, Smith, & Holladay, 1998) and antibody response which a smaller soluble protein, p22, also does (Rockey, Turaga, Wiens, Cook, & Kaattari, 1991) (Fredriksen, Endresen, & Wergeland, 1997). Virulent Rs strains auto-agglutinate in contrast to a non-virulent aberrant isolate with a p57 unable to associate with the bacterial surface (Bruno, 1988; Senson & Stevenson, 1999). How much p57 an Rs isolate produces (Rhodes, Coady, & Deinhard, 2004) could partly explain variation in virulence (O. B. Dale, Gutenberger, & Rohovec, 1997). In sum, Rs seem to be a master of perseverance through robustness and host manipulation.

Host resistance to Rs infection

Experiments show that host resistance to BKD is heritable (Withler & Evelyn, 1990; Beacham & Evelyn, 1992; Hard et al., 2006) and natural epidemics may increase BKD resistance (Purcell et al., 2014). Innate immunity is probably essential for host resistance and gene expression studies point to some potential mechanisms (Booy, Haddow, Ohlund, Hardie, & Olafson, 2005; Rhodes, Wallis, & Demlow, 2009).

Disease diagnosis and detection of infection

As both disease diagnostics and Rs identification can be challenging there is a multitude of bewildering studies done over the years. The good news is that a recent validation study is a revelation, but the bad news is that no perfect detection test for Rs exists (Elliott, Applegate, Murray, Purcell, & McKibben, 2013). The fundamental obstacle is the ability of Rs to go into a low-level, dormant, localized infection anywhere in the fish. A tiny tissue sample from an infected, but healthy carrier fish may actually not contain any Rs, and then all tests will inevitably produce false negative diagnostic results. However, meaningful surveillance programs for populations can still be designed for various purposes. In disease diagnostics, the situation is easier and autopsy and particularly histopathology are useful. The latter can be combined with immunohistochemistry to identify Rs in histological lesions, but more often Rs is detected in fresh material from lesions by immunofluorescence, ELISA, PCR or isolated by culture. These most relevant methods and some challenges of surveillance are described in the following sections. A source of updates on diagnostic procedures is also available from American Fisheries Society - Fish Health Section in the blue book accessible at: http://afs-fhs.org/bluebook/bluebook-index.php.

Culture

Culture is the gold standard for identifying Rs, and isolates are valuable for research and epidemiology. Rs is fastidious, but culture is usually successful when procedures are optimized. Rs is strictly aerobic and media must be supplemented with L-cysteine. For primary isolation, the 20% serum containing KDM2 is recommended and should be freshly made as growth support is gradually lost over a three month period (Evelyn, 1977). Adding 1.5% v/v Rs-conditioned, spent medium will supply growth factors (Evelyn, Prosperi-Porta, & Ketcheson, 1990) that could contain the resuscitation-promoting factors (Wiens et al., 2008). Adding antibiotics to make a selective medium, SKDM (Austin, Embley, & Goodfellow, 1983) will reduce contamination problems and enable isolation of Rs from the environment, but selectivity is only relative (Olsen, Hopp, Binde, & Gronstol, 1992). As fungal contamination and drying out of plates can become problematic during prolonged incubation, sealing all plates with parafilm is helpful. For secondary culture and studies where serum is unwanted, KDM-C with charcoal instead of serum is useful (Daly & Stevenson, 1985). The optimum growth temperature is 15-18°C and incubation times are usually about 2 weeks in clinical cases, but up to 19 weeks have been reported in subclinical cases (Benediktsdottir, Helgason, & Gudmundsdottir, 1991). The colonies are whitish, circular and usually of varied sizes and with creamy texture (Figure 7) (Evelyn, 1977). Atypical thin film like growth have been reported when culturing from subclinical cases (Hirvela-Koski, Pohjanvirta, Koski, & Sukura, 2006). There are few phenotypic tests useful to identify Rs, but the failure to grow on ordinary medium, production of catalase, but not cytochrome oxidase are easy to check. Usually immunological identification by e.g. IFAT with a specific antibody is used (Figure 8). Several things can increase the chance of primary isolation. Most important is simply to increase the amount of tissue for inoculation, but also to wash (centrifuge) and avoid tissue inhibitory factors (Daly & Stevenson, 1988; Elliott et al., 2013). However, ordinary plate-spreading on several plates will also dilute, and using a few seconds extra to move the inoculation loop widely around in the kidney underneath the capsule will also increase the chance recovering Rs as they are not uniformly distributed in an healthy carrier fish (Austin & Rayment, 1985).

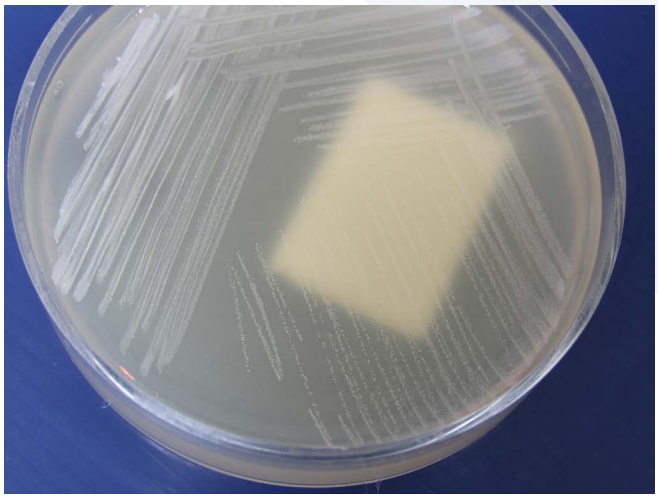

Figure 7. Development of colonies that are characteristic of *R. salmoninarum* in SKDM agar obtained from the seeding of kidney with an acute case of Renibacteriosis (Photo: Elanco Animal Health).

Large scale culture is often done in broth versions of the media with vigorous shaking to aerate.

Very dense growth can also be achieved in a biphasic medium, most conveniently in large tissue culture flasks with agar at the bottom and a thin overlay of 0.1% w/v peptone in physiological saline. Young, actively growing cultures should be harvested for freezing at -80°C. Colonies on old plates with may look unchanged, but sometimes few bacteria are cultivable.

Immunodiagnostics

When immunofluorescence antibody techniques (IFAT) were introduced (Figure 8) (Bullock & Stuckey, 1975), this was a great help in speeding up diagnostics. By IFAT one could immediately identify Rs in lesion material with many Gram positive bacteria, and also find typical Rs cells when Gram staining gave no conclusive results (Figure 9). However, there are limitations as fluorescing Rs-like objects that are impossible to identify by other methods are sometimes found. This has led to speculations about dead Rs cells, viable but non-culturable Rs or cross-reacting agents (Austin & Rayment, 1985; Cvitanich, 2004). Other immunodiagnostic formats were developed like staphylococcal coagglutination that does not require specialized equipment (Kimura & Yoshimizu, 1981). To help identify suspect Rs in histopathological lesions immunohistochemistry (IHC) (Evensen, Dale, & Nilsen, 1994) based on a monoclonal antibody reacting with p57 (Wiens & Kaattari, 1991) has been most useful (Figure 3). Further, a quantitative IFAT for ovarian fluid is very sensitive, but far more labor intensive than the ELISA format which often will be sensitive enough (Elliott et al., 2013). The ELISA targets soluble Rs antigens that may accumulate in tissues. The validation study of Elliot and coworkers (2013) showed that sensitivity of most immunodiagnostics was fairly similar to PCR, but that culture and IFAT of ovarian fluid performed better, probably as larger sample volumes increased the chance of including bacteria in the samples. To ensure specificity of the immunological methods, affinity purified polyclonal antibodies or carefully chosen monoclonal antibodies should be used (Wiens, 2011).

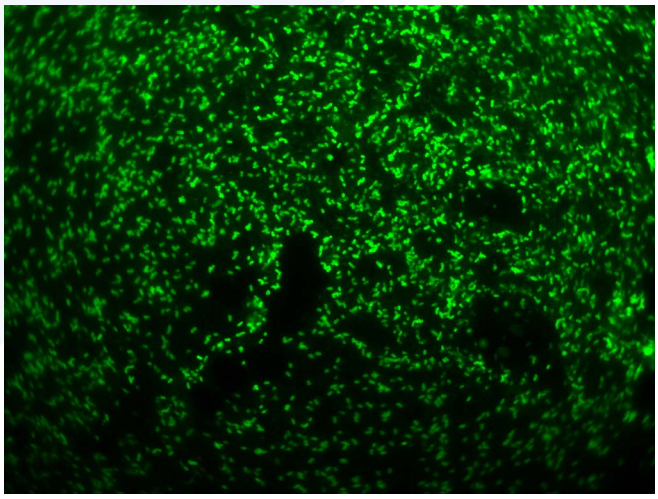

Figure 8. IFAT for BKD (100x) in a smear made from a positive bacteriological culture of *R. salmoninarum*. In the sample, it is possible to observe the presence of abundant quantity of fluorescent green bacilli and diplobacilli (*R. salmoninarum*) grouped irregularly and distributed throughout the whole area focused (Photo: Marcos Godoy).

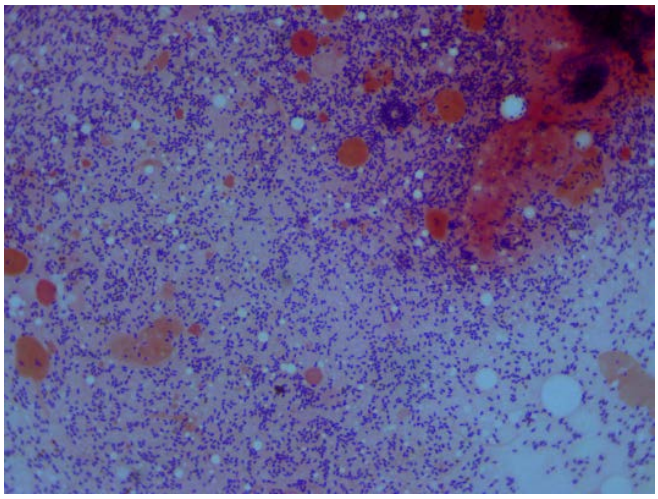

Figure 9. Gram Staining (100x), in Atlantic Salmon (*Salmo salar*) kidney smear with signs of Renibacteriosis. In the sample, it is possible to observe the presence of abundant quantity of Gram Positive (+) bacilli and diplobacilli (*R. salmoninarum*) grouped irregularly and distributed throughout the whole area focused. Smears from lesions in other organ than kidney may avoid melanin granules that sometimes make a Gram stain difficult to interpret (Photo: Marcos Godoy).

PCR

Many variants of nucleic acid based detection techniques have been developed as reviewed by Wiens (2011), but validated quantitative PCRs (Elliott et al., 2013) that target the unique msa/p57 gene are most relevant for practical purposes today

(Chase, Elliott, & Pascho, 2006; Powell, Overturf, Hogge, & Johnson, 2005; Rhodes, Durkin, Nance, & Rice, 2006). The qPCR protocols include lethal sampling, but a recent study shows that skin mucus can be a good, non-lethal sample for detecting Rs and reflects the kidney infection level (Elliott et al., 2015). Careful optimization of primer design, control of PCR product contamination, inhibition, design of positive control etc are as important to optimize PCR for Rs as any other target. When optimized the sensitivity of PCR appears limited foremost by the small sample size when comparing with culture and IFAT of ovarian fluid that examines a larger sample volume. Nevertheless, since qPCR performs as well as immunodiagnostics and can be automated, it is increasingly being used for screening purposes. For any test, including PCR, it should be kept in mind that negative results do not mean freedom from Rs infection, only that any infection is below the detection limit of the assay.

Serology and cellular immunity tests in surveillance

Serology has been much used in surveillance of listed diseases in warm-blooded vertebrates. The reason is that infection usually results in an immune response that can be detected over a wide time span in healthy animals readily available for testing. However, although it is possible to detect an antibody response to Rs (Evelyn, 1971; Jansson & Ljungberg, 1998), serology is not used as the often low or undetectable titers are difficult to interpret. Practical test formats for cellular immunity that are so important in tuberculosis surveillance could be more relevant, but have not been sufficiently researched in fish.

Reservoirs and transmission

Reservoirs

With Australia, New Zealand and Ireland as the most notable exceptions, BKD has been reported from where there are wild or farmed salmonids of the genera *Oncorhynchus*, *Salmo* and *Salvelinus* plus Grayling (*Thymallus thymallus*) and Danube salmon (*Hucho hucho*). Further, ayu (*Plecoglossus altivelis*), whitefish (*Coregonus lavaretus*), sablefish (*Anoploma fimbria*) and Pacific herring (*Clupea harengus pallasi*) have been shown susceptible (Wiens, 2011). Isolation of Rs have also been reported from kidneys of sea lamprey *(Petromyzon marinus)* (Eissa, Elsayed, McDonald, & Faisal, 2006). As Rs is a fastidious organism and takes some effort to isolate there could very well be a larger host reservoir than presently known.

Transmission

Rs can infect horizontally as well as vertically from the female parent to progeny. The vertical transmission was demonstrated by Allison (1958) and Bullock, Stuckey, & Mulcahy (1978) when historically disease-free farming sites received eggs infected by this pathogen and originated clinically infected progeny. Rs has been detected both outside and inside the egg where it will survive surface disinfection as shown by Evelyn et al. (1984). Thus wide dissemination of Rs both by trade with infected eggs and natural migration is possible and in keeping with recent phyologenetic studies (Brynildsrud et al., 2014; Matejusova et al., 2013). Horizontal transmission appears important in disease outbreaks and occurs both in fresh water (Mitchum & Sherman, 1981) and seawater (Murray et al., 1992). Exact mechanisms are unknown, but ingestion of fish carcasses (Wood & Wallis, 1955) and feces containing Rs (Balfry et al., 1996) infect through the gastrointestinal tract. Viable Rs in fresh- and seawater as well as sediments (Austin & Rayment, 1985; McKibben & Pascho, 1999) makes several modes of entry possible and eye and skin lesions without obvious internal pathology have been reported (Ferguson, 2006; Hoffmann, Popp, & van de Graaff, 1984).

Control and management of the disease

Bacterial kidney disease (BKD) is one of the bacterial diseases of fish which is most difficult to control (Elliot *et al,* 1989). No single measure seems able to control BKD, but combining several measures and adjusting to the local circumstances can minimize losses.

Reducing the infection pressure and increasing the disease resistance

In the countries of the British Isles and Scandinavia, BKD is listed as an unwanted disease subject to control measures despite that OIE has chosen to unlist BKD. With the exception of Northern Ireland all of these areas have experienced BKD outbreaks and possibly only isolated broodstocks are free from Rs infection. However, the control efforts are continued

as they seem to make outbreaks with significant losses rare. The cornerstone of these efforts is to eliminate or minimize the infection in the broodstocks. Any control program for BKD should start with production of eggs free from Rs. Sanitary control of broodstock (screening) and the sanitary standards applied to spawning and incubation, such as the delimitation of areas, egg disinfection (external elimination of bacteria) and individual incubation are some relevant measures. Avoidance of Rs in the early lifestages is especially important in hatcheries using recirculation systems as efficient Rs disinfection is not easily achieved. Protection against Rs infection in young fish may also help maximize the effect of vaccines as already infected fish may respond poorly. These specific efforts against BKD are best exploited with good overall health standards covering feed, environment and biosecurity during the whole production cycle. Strict year-class separation using "all-in all-out" principles with fallowing in between are critical to avoid BKD problems during intensive farming. Importantly, these general measures for good health also help against most other diseases.

For declaration of freedom of Rs infection, the shortcomings of all the test methods becomes critical (Elliott et al., 2013). To protect valuable BKD free populations, it is paramount that fish to be moved in are truly Rs infection free. Certification of freedom from Rs infection should not rest on a few screening tests performed over a short time span. None of the pathogen detection tests resolve the sampling conundrum created by both low infection levels and low prevalence persisting for long periods, especially in the more resistant species. A surveillance program for several years combining systematic disease diagnostics and screening with the best tests available may show if a population is truly free from Rs infection.

Immune prophylaxis

Vaccination against BKD has recently been reviewed (Elliott, Wiens, Hammell, & Rhodes, 2014): Although some immunization and challenge studies show adaptive immune responses in the form of antibodies (Evelyn, 1971; Jansson & Ljungberg, 1998; Sorum, Leivsdottir, & Robertsen, 1998) and cell mediated response (Jansson et al., 2003), protective immunity seems difficult to induce. Various vaccine platforms have been investigated including traditional whole bacteria or bacterins killed by heat or formalin treatments. When looking for an avirulent Rs as a vaccine candidate, *Arthrobacter davidanielii* with a carbohydrate surface similar to that of Rs was discovered and found to protect Atlantic salmon against BKD better than a live attenuated Rs strain (Burnley, Stryhn, Burnley, & Hammell, 2010; Griffiths, Melville, & Salonius, 1998; Salonius, Siderakis, MacKinnon, & Griffiths, 2005). However, this live vaccine of *Arthrobacter* sp. shows limited (Rhodes, Rathbone, Corbett, Harrell, & Strom, 2004) or almost no efficacy in Coho Salmon (Alcorn, Murray, Pascho, & Varney, 2005). Nonetheless, Renogen® a live non-virulent lyophilized culture of *Arthrobacter* was the first registered vaccine for BKD in Canada and Chile (SAG N° 0734-B).

Chemotherapy

Treatment of clinical BKD with antibiotics is possible and erythromycin by feed for about one month is efficacious (Peters & Moffitt, 1996). Further, erythromycin injections of broodfish can reduce vertical transmission of Rs (Brown, Albright, & Evelyn, 1990). However, the chance of remission is considerable as Rs is often not eliminated and could also easily be re-introduced (Austin & Rayment, 1985). It could be difficult to achieve therapeutic levels of erythromycin for necessary length of time intracellularly and in necrotic foci within granulomas that may contain dormant Rs. As Rs also can become resistant to erythromycin (Bell, Traxler, & Dworschak, 1988; Rhodes et al., 2008), antibiotic treatment is a poor option to manage BKD, although antibiotic use may be of value for treating particularly valuable, endangered broodstock.

The Norwegian BKD situation

In 1980, the first five cases of BKD were found in Norway. Three cases were in commercial stocks, while two cases were in feral stocks reared for mitigation purposes. As no imports to any of these farms are known, feral brood fish used were judged to be the probable source of infection. This notion may need to be revised as recent phylogenetic studies indicate that we have both an original European Rs group and a second Rs group spread globally through trade in Norway (Brynildsrud et al., 2014; Matejusova et al., 2013). BKD has been found in wild salmonid fish from 17 different rivers in Norway, but not inland south-east of the mountain range along the north-western

part of Norway which appears free from BKD. The National Veterinary Institute has diagnosed 369 outbreaks of BKD in farms along the coast from 1980 to 2006 (O.B. Dale et al., 2007) (Figure. 10). In the salmon farming industry the number of cases peaked in 1990 when 60 seawater farms had disease outbreaks. Since then a steady decrease of outbreaks has been achieved, and in 2006 no BKD cases were found. Following this, the yearly prevalence has varied between 0 and 3 cases.

BKD is most often seen as a chronic disease with highly unpredictable losses. It has been noted that farm populations with both BKD and infectious salmon anemia (ISA) may suffer especially high losses. As the epidemic appeared limited in geographical spread and there were no satisfactory treatments or vaccines, an avoidance strategy was chosen to control BKD in Norway. The most essential step was to keep the broodstocks free from the infection. In Norway we fortunately still had BKD free populations after the epidemic in the 1980ies. The selection of BKD free brood stocks was done quite simply by screening at slaughter of sister groups of potential brood stocks. Several thousand fish in each population were examined for visible kidney lesions which then were tested by histopathology and IHC (Evensen et al., 1994) or ELISA, both based on the highly specific p57 Mab 4D3 (Wiens & Kaattari, 1991) to verify BKD. We thus did not select individual brood fish based on testing. In our experience, no test can reveal all covert carriers in an infected population, including PCR. Moreover in some BKD cases skin wounds could be the dominant pathology with little or no kidney involvement and together with histopathological observations, it was clear that a covert carrier could have sequestered Rs in tiny granulomas elsewhere than in the kidney. Thus, we aimed at maximizing the chance of finding at least one verifiably infected fish in each tested stock and discard all infected stocks for breeding purposes. In hindsight, the approach was very successful to select BKD free broodstocks. However, there is a risk of horizontal infection during the production cycle of these broodstocks. Thus intensified disease diagnostics including testing for BKD is done especially in the months before sexual maturation. If BKD or Rs infection is found, the whole stock is slaughtered if still immature sexually. If BKD is overlooked and is found first when stripping eggs, the loss is maximal as neither eggs nor fish have any market value. Needless to say the economic incentive to find BKD early is strong. So far, only a few commercial brood stocks have had to be culled since selection of BKD free stocks became a prime concern for the egg producers around 1990.

To keep avoiding BKD after transfer from the breeding facilities a good overall disease control is required. BKD is thus a listed disease in Norway. Domesticated salmon populations are through their entire life-span subject to a systematic disease surveillance that will reveal BKD. It is not allowed to transfer smolts with BKD to sea-water. If BKD is found in seawater farms, these farms are fallowed in a way that has balanced economy and risk of further spread. In practice, these measures and the very important, general biosafety standards that were introduced in the farming industry to stop ISA, have appeared effective to control BKD in Norway. However, recent outbreaks show that Rs is present and more intensive practices in the industry may increase the significance of BKD and the phylogenetic studies create concern about spread of BKD by trade. Also, feral salmon stocks still represent a reservoir of infection for BKD in Norway. Fortunately, we have never encountered any BKD epidemic in the feral fish, and the BKD prevalence in captured, wild brood fish has been very low as shown by an earlier screening. However, in mitigation hatcheries there have been some severe BKD outbreaks. If infected fish from such hatcheries are released into the waterways, the BKD situation may deteriorate. To avoid this, screening individual, wild brood fish and checking for overt BKD before releasing offspring is important. Release of fish should be limited to the same watershed as the brood fish originated from. Thus,

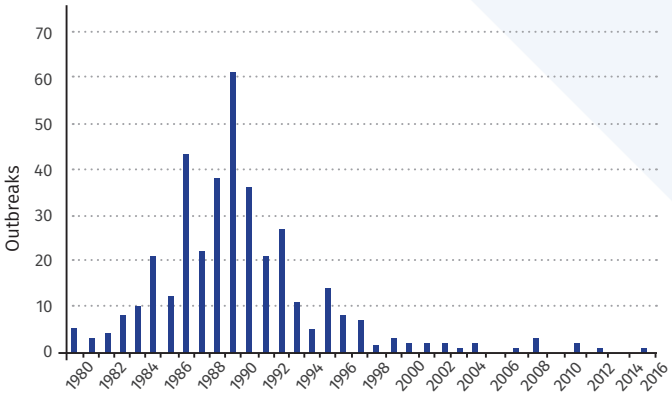

Figure 10. BKD-cases between 1980-2016 diagnosed by Norwegian Veterinary Institute. Strict biosecurity measures in the farming industry were introduced in 1989-90.

if low levels of infection are overlooked, Rs will not be directly disseminated to other watersheds that could be free from Rs.

The high natural resistance of our main aquaculture species, Atlantic salmon and rainbow trout is probably important for the success in controlling BKD. By combining general biosecurity, systematic disease diagnostics, screening for Rs in the broodstocks and not introducing fish from less controlled areas we hope to maintain our relative control of BKD. The most immediate knowledge gap is to understand the dissemination leading to recent, unexpected outbreaks, possibly by applying the new phylogenetic tracing methods.

The British Columbia BKD situation

BKD is prevalent in fish of both the Pacific and Atlantic Canadian coasts and therefore, it has been considered as a disease of concern in Canadian aquaculture facilities. In the Pacific coastal waters, BKD was first reported in 1937 and by the late 1940's identified in wild Sockeye Salmon (*Oncorhynchus nerka*), Chinook Salmon (*O. tshawytscha*), and Coho Salmon (*O. kisutch*) from state fish hatcheries in California, Oregon, and Washington State (Earp, Ellis, & Ordal, 1953; Rucker, 1951).

During the formative years in the BC industry, it was demonstrated that vertical transmission of Rs could be avoided by testing individual spawning fish and discarding any broodstock with detectable levels.

In BC, intensive Rs screening and culling as well as treatment of eggs with iodophore are used to control BKD in farmed salmon. For farmed fish (not wild), coelomic fluid from all female spawners is screened and eggs from positive fish are destroyed to avoid any vertical transmission. Screening is performed using ELISA, IFAT or qPCR based assays and the method is company preference. Some facilities use only ELISA or qPCR whereas others combine ELISA with qPCR tests to assure no false positives or negatives. Proof that these measures are effective is the absence of BKD outbreaks in freshwater parr and smolt for some time.

In some cases, broodstock may be treated with antibiotic prior to spawning to reduce the risk of pathogen presence. To control BKD, administration of antibiotic drugs (oxytetracycline and/or erythromycin) either orally or by injection has been used in BC since the 1980s (Hicks, 1986). However, treatments using antibiotics have been demonstrated to be incomplete, probably due to the intracellular features of Rs (Elliott, Pascho, & Bullock, 1989). To fill the gap of the inefficacy of chemotherapeutants compounds to completely block vertical Rs transmission, a vaccine based on attenuated *Arthrobacter* sp, is available to help mitigate BKD in aquaculture facilities (Salonius et al., 2005). Although this vaccine showed protection in Atlantic salmon, a cohabitation challenge study concluded that none of the tested vaccines provided full protection in Chinook Salmon (Alcorn et al., 2005).

Over the past decades, an overall decline of BKD prevalence has been observed in hatchery-raised salmonids. Many believe that this resulted from both intensive screening of broodstock as well as improved biosafety measures implemented by the companies. It was also hypothesized that the reoccurrence of warm surface seawater of the west coast of North America could be playing a role in this decline by exceeding the thermal tolerance of the bacteria. Support for this notion comes from a recent study showing high water temperature around 15°C suppresses Rs shedding in challenged Chinook Salmon (Purcell, McKibben, Pearman-Gillman, Elliott, & Winton, 2016).

Despite the measures to ensure good hygiene and prevent vertical transmission, Rs is still prevalent in wild migratory salmon. The industry response is to optimize husbandry conditions and apply stringent biosafety controls. As a result, BKD has not been a threat to production in decades.

The Chilean BKD situation

In Chile, this pathology is widespread throughout the territory where salmonid species are farmed. Cases have been reported from hatcheries located in the Metropolitan Region to farming sites that are located in the XII Region.

In 2016, BKD accounted for the second highest percentage frequency of antibiotic treatment amongst freshwater diseases (41.8%) (Figure. 11).

For this same year, the most used antimicrobial products for the treatment of Rs in fresh water were oxytetracycline (83.4%),

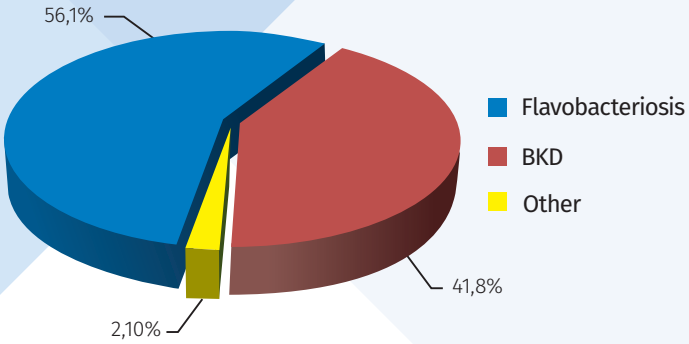

Figure 11. Percentage distribution of the diseases treated with antimicrobial products in fresh water in 2016 (Source: Report on the Use of Antimicrobial Products by the National Salmon Farming Industry in 2016, Sernapesca).

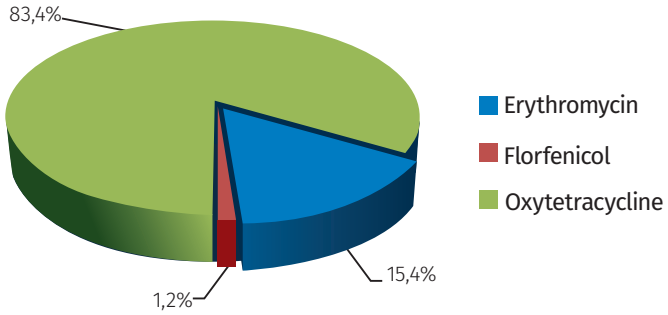

Figure 12. Percentage distribution of antimicrobial products used in the treatment of Renibacteriosis in fresh water in 2016 (Source: Report on the Use of Antimicrobial Products by the National Salmon Farming Industry in 2016, Sernapesca).

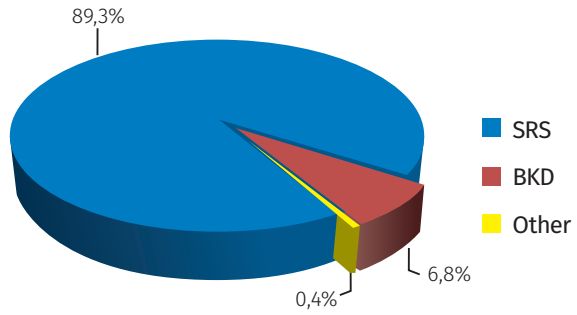

Figure 13. Percentage distribution of antimicrobial products used in seawater per disease in 2016, according to diagnosis (Source: Report on the Use of Antimicrobial Products by the National Salmon Farming Industry in 2016, Sernapesca).

erythromycin (15.4%) and, in a lower percentage, florfenicol (1.2%) (Figure. 12).

In seawater in 2016, BKD again accounted for the second highest percentage frequency of antibiotic treatments, second after SRS (6.8%) (Figure. 13).

For the treatment of Rs in seawater, the most used antimicrobial product was oxytetracycline (58.0%), followed by florfenicol (41.8%) and a very small percentage of erythromycin (0.2%) (Fig. 14).

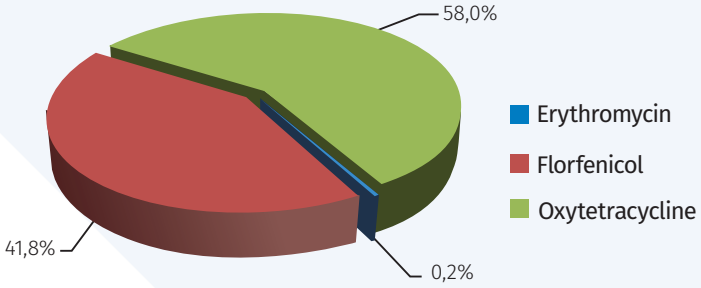

Figure 14. Percentage distribution of antimicrobial products used in the treatment of BKD in seawater in 2016 (Source: Report on the Use of Antimicrobial Products by the National Salmon Farming Industry in 2016, Sernapesca).

Importance of BKD in Chile

Mortality data associated with various infectious causes in the production of Coho Salmon, Atlantic Salmon and Trout in Chile are shown in Table 1 and 2 for 2015 and 2016 respectively. For Coho Salmon in 2015, Rs was the second most infectious cause of mortality (10%). The following year (2016), even though it remained as the second cause of infectious mortality for this species, the percentage of mortality associated to Rs was 22% (Aquabench, unpublished data, cited with permission 2018).

For Atlantic slamon, the situation is quite similar. While in 2015, Rs was the third cause of mortality (3%), the following year, and after registering a considerable increase in the cases produced by this agent, it became the second cause of infectious mortality for this species (9%), an effect also observed in the other salmonid species farmed in Chile.

Historically, Rs has not been the main infectious agent causing mortality in farmed rainbow trout. When analyzing mortality data associated with infectious causes in in this species in 2015, it is interesting to note that the increase in mortality observed in other salmonid species farmed in Chile is also seen in Trout, going from being the cause of 0 (0.1)% of mortality to being directly 1% the following year (2016) (Aquabench, unpublished data, cited with permission 2018).

Table 1. Mortalities associated with infectious diseases in 2015. (Source: Aquabench, unpublished data, cited with permission 2018).

Cause	Coho	Atlantic S.	Trout	Total
Amoeba	2%	1%	0%	1%
BKD	10%	3%	0%	3%
Exophiala	0%	0%	0%	0%
Flavobacteriosis	0%	0%	2%	0%
Furunculosis	0%	0%	0%	0%
Saprolegnia	0%	2%	0%	1%
Ictericia	26%	0%	0%	4%
IPN	0%	4%	0%	3%
SRS	59%	80%	96%	80%
Vibrio	0%	0%	0%	0%
Other Infectious	2%	9%	2%	7%
Total Infectious	100%	100%	100%	100%

Table 2. Mortalities associated with infectious causes in 2016. (Source: Aquabench, unpublished data, cited with permission 2018).

Cause	Coho	Atlantic S.	Trout	Total
Amoeba	0%	3%	0%	2%
BKD	22%	9%	1%	7%
Exophiala	0%	0%	0%	0%
Flavobacteriosis	0%	0%	2%	1%
Furunculosis	0%	1%	0%	1%
Fungus	2%	5%	0%	3%
Ictericia	56%	0%	0%	3%
IPN	0%	1%	2%	1%
SRS	19%	79%	93%	80%
Vibrio	0%	0%	3%	1%
Other Infectious	1%	1%	0%	1%
Total	100%	100%	100%	100

Future perspectives – knowledge gaps

BKD constitutes one of the main sanitary challenges of salmonid farming in the world and particularly in Chile, due to its chronic nature, efficient mechanisms of vertical and horizontal transmission, and the wide range of host salmonid species, affecting fish farms both in the fresh water stage as well as the grow-out stage in the sea. To help minimize losses now, and preferably eradicate BKD in the future, there is a need to work with both applied and basic research. From a short term perspective it could be helpful to use epidemiologic studies to assess the effect of "best practice(s) for BKD management" as suggested by our present knowledge. Further knowledge of how Rs spreads and persists in farming systems and wild fish using the new phylogenetic methods could also be part of such a study.

An immediate, basic research objective would be to find out how the dormant state of Rs is regulated and thus possibly affected by interventions. Dormancy is probably an important mechanism for Rs to survive and wait out hostile host responses and antibiotic treatments and is also a major diagnostic challenge in healthy carriers. Long term research into the host-agent-environment interactions through the application of all the "-omics" disciplines could bring forward better vaccines, resistance breeding and completely novel approaches to control or preferably eradicate BKD. Although the daunting complexity on a molecular level will take time to understand fully, important discoveries could come quickly.

Finally, the establishment of standardized challenge models for the evaluation in controlled conditions of biological and pharmaceutical products, genetic improvement oriented to the resistance of diseases and evaluation of the immune response through molecular or immunological techniques constitute elements that will surely contribute significantly to the control and prevention of this disease.

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