THE OCCURRENCE OF IMPORTANT PATHOGENS IN LUMPFISH (*CYCLOPTERUS LUMPUS*) FROM A WILD POPULATION FISHED FOR BROOD FISH

Master thesis in fish health

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Abstract

The use of lumpfish as cleaner fish may be both an effective and environmentally friendly method for louse control in salmon farms. Because of this, demands are high and production growing, so lumpfish is now the third most important aquaculture species in Norway. A major problem is poor survival in pens, mostly due to infection and disease. This project studied the occurrence of; the viral agents infectious pancreatic necrosis virus (IPNV), nervous necrosis virus (NNV), viral haemorrhagic septicaemia virus (VHSV) and lumpfish flavivirus (LFV), opportunistic bacterial infections in the kidney and the parasites *Kudoa islandica* (musculature), *Nucleospora cyclopteri* in kidneys and a yet uncharacterized coccidian in caeca (here: Coccidium 'X'), in the most important broodfish population in Norway. All of which are considered important pathogens, and most of them are already detected in lumpfish. Bacteria were screened for using culturing on agar-plates, while the other agents were detected with molecular methods targeting their RNA or DNA. An additional aim was to examine the relationship between the potentially immunosuppressive microsporidian *N. cyclopteri*, developing inside leukocytes, and co-infections for evidence of facilitation or synergism. Coccidium 'X' occurred in 98.8% of the fish, *N. cyclopteri* was detected in 60% and the myxosporean *K. islandica* in the musculature of 21.2%. Two bacteria isolated may have originated from infections in the lumpfish, *Vibrio* sp. aff. *splendidus* and *Psychrobacter* sp. No viral agents were detected. No associations or correlations were detected between *N. cyclopteri* and the other infections, but the very light infections with the microsporidian and the few coinfections were not well suited for examining this. This finding is positive for the use of this stock as brood fish, particularly regarding the viral agents some of which could be vertically transmitted. Also, the baseline prevalence provided here may aid future studies into the environmental effects such as the potential impact on wild lumpfish from diseases spread from conspecifics stocked with salmon in pens.

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1.0 Introduction

Any organism hosts an array of parasites living on or in its body, such as virus, bacteria, fungi, protists or multicellular parasite groups (e.g. tapeworms, lice). Some of these may cause disease (acting as pathogens). In nature each fish individual carries a subset of the local parasite repertoire, while in aquaculture (e.g. hatcheries) various hygienic measures aims at excluding them. The much higher density in fish-farms compared to wild populations of fish can result in increased infection pressures due to more rapid transmission of agents with horizontal transmission, and increased susceptibility, due to stressful rearing conditions (Vadstein et al., 2008). Therefore, pathogens that are infrequent in wild populations and usually do not cause clinical disease, may cause epizootics with disease and mortality in farmed fish (Raynard et al., 2007). If hosts are continuously available, there may also be a selection for more virulent variants of the pathogens, such as the fastest reproducing types (Pulkkinen et al., 2010).

There may be a feedback from farms to wild stocks with respect to pathogens, that originally came from wild fish. It is well documented that sea lice in farms release larvae that may infect and affect wild salmonids. Such elevated infection pressure from aquaculture may also be true for tapeworms, certain protists and viral infections (Grefsrud et al., 2018). Transportation of live fish across great distances also poses a risk of spreading known and unknown pathogens to new areas (Johansen et al., 2016, Grefsrud et al., 2018). In the case of Norwegian salmonids, there is hardly any reference material or information on pathogens from the time prior to aquaculture. Therefore, it may now be impossible to ascertain the changes that may have occurred, such as increased prevalence, increased virulence and introductions of alien species or variants.

Epizootics may occur when naïve hosts are exposed to pathogens they would not normally meet in their natural habitats, and which they may be more susceptible to (Krkošek, 2017). Moving cleaner fish between different geographic areas for use in aquaculture may results in the spread of pathogens to naïve populations. This is an obvious danger associated with the transports of wild caught wrasse (e.g. Sweden to Norway) (EURL, 2016), but may also apply to hatchery reared wrasse and lumpfish. The widespread use of juvenile lumpfish in farms may represent an altered infection pressure on wild conspecifics. When epizootics occur, such as Viral Haemorrhagic Septicaemia, VHS, in both Scottish wrasse and Icelandic lumpfish, there is a need for background information such as VHS Virus (VHSV) prevalence and genotype repertoire. Obviously, this may not be obtained from the affected environment,

which may then be changed. Such considerations also concern other infectious agents that could infect lumpfish. In addition, information on the baseline prevalence of pathogens is necessary for risk assessments, concerning aspects such as movement of fish and the use and commercial distribution of fish eggs potentially carrying vertically transmittable contagion.

1.1 Lumpfish in aquaculture

In Norwegian seawater salmonid aquaculture, lumpfish (*Cyclopterus lumpus*) is together with several wrasse species, used as a cleaner fish to control sea lice infestations. The use of cleaner fish represents a potentially effective and environmentally friendly supplement to the medical and chemical substances that are also used to handle the salmon lice problem (Torrissen et al., 2013). Young lumpfish may devour large numbers of salmon lice (*Lepeophtheirus salmonis*), and in experiments it has been shown that lumpfish can reduce or keep the number of adult lice on the salmon lower than in control groups, not stocked with this cleaner fish (Imsland et al., 2014a). Lumpfish eat lice directly from the salmon (Imsland et al., 2014a, Imsland et al., 2014b, Imsland et al., 2015). In recent years, lumpfish farming has made this cleaner fish the third most important species in Norwegian aquaculture after Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhunchus mykiss*). The production of lumpfish grew from 3.5 million in 2014 to approximately 33 million in 2017 (Fiskeridirektoratet, 2016, Borch, 2018). Around 80% of the lumpfish used in Norwegian aquaculture originates from brood fish caught on Averøy in Møre and Romsdal county (Hosteland, 2017). Brood fish are still mainly wild-caught lumpfish, but there are initiatives to establish farmed brood stocks (AquaGen, 2016) that may allow breeding of lumpfish (EURL, 2016). By collecting lumpfish from several locations along the coast, a high genetic diversity will be ensured. Allowing selection of the preferred genetic traits based on disease resistance, cleaning behaviour and growth (AquaGen, 2016).

Diseases

Lumpfish survival during a production cycle of salmon is poor and most or all lumpfish die during the production cycle (Borch, 2018). The species is susceptible to a range of parasites, bacteria and viruses, some of which may cause disease and mortality (Alarcón et al., 2016a, Bornø et al., 2016, Alarcón et al., 2016b). The most common bacterial infections diagnosed in captivate lumpsucker are different *Vibrio* spp., *Tenacibaculum* spp., atypical *Aeromonas salmonicida* and *Pasteurella* sp. (Alarcón et al., 2016a, Bornø et al., 2016, Småge et al., 2016, Hjeltnes et al., 2017). A common parasite of lumpfish in Icelandic waters is the microsporidian *Nucleospora cyclopteri* (Freeman et al., 2013) and this parasite has also been

found to infect farmed lumpfish in Canada and Norway (Mullins et al., 1994, Alarcón et al., 2016b). Lumpfish is susceptible to amoebic gill disease (AGD) caused by *Paramoeba perurans*, although less so than salmon (Karlsbakk, 2015, Haugland et al., 2017). Viral infections may also be responsible for disease and mortality, but so far little is known. A recently discovered virus, Lumpfish flavivirus, causes liver pathology and mortalities in farmed lumpfish in Norway (Skoge et al., 2017, Vestvik et al., 2017). Elsewhere, infections with Ranavirus have been detected in lumpfish on several locations in the North Atlantic Ocean (Iceland, Ireland, Faroe Islands, and Scotland) (Price et al., 2017) and VHSV genotype IVd in Icelandic brood fish have also been detected (Stagg et al., 2017).

New infectious agents are also continuously being detected and characterized from lumpfish (Scholz et al., 2017a, Scholz et al., 2017b). Some may also be vertically transmitted and be spread by healthy juveniles ("carriers") developing disease later in life. The extent to which pathogens are spread along the Norwegian coast, and contracted by wild lumpfish, are unknown. Thus, there is a need for background information on the occurrence of infectious agents in wild lumpfish before these wild populations may be subject to any impact from aquaculture.

1.2 Viruses

Infectious pancreatic necrosis virus (IPNV)

IPNV is the type species in the genus Aquabirnavirus (Birnaviridae). They are double stranded RNA, dsRNA, viruses, and there are several serotypes and many genotypes of IPNV. (Lago et al., 2017).

IPNV is very contagious and may infect a wide range of wild fish species (Wallace et al., 2008, Bruno et al., 2013). The virus has been found in over 80 different aquatic species in both freshwater and in saltwater around the world, most often without clinical signs of disease (Bandin and Dopazo, 2011). Different genotypes may differ in virulence and pathogenicity due to differences in the VP2 gene (Manríquez et al., 2017, Lago et al., 2017). Some strains of the virus may cause infectious pancreatic necrosis (IPN), an important disease in salmonid fish. A key finding in fish with IPN is necrosis of the exocrine pancreatic tissue, but also some haemorrhages occur, and the heart and liver are often pale (Bruno et al., 2013). Despite what the name may imply, IPNV is present in other organs than the pancreatic tissue. Liver and kidney are good tissues for detecting IPNV (Kitamura et al., 2000, Wallace et al., 2008).

Infections in salmonids can occur in all age groups, but the mortality seems to be higher when outbreaks occur in freshwater (Bang Jensen and Kristoffersen, 2015). Fry and post smolt are most susceptible to the disease and the mortality can be insignificant or become as high as 90%. (Bruno et al., 2013, Taranger et al., 2014, Hjeltnes et al., 2017). The finding of IPNV in wild symptomless fish suggests that some fish can be asymptomatic carriers of the virus (Wallace et al., 2008).

In Norway IPNV has been isolated from farmed Atlantic salmon, turbot, brown trout (*Salmo trutta*), rainbow trout and Atlantic halibut (*Hippoglossus hippoglossus*) (Mortensen et al., 1990, Melby et al., 1994, Johansen et al., 2016). IPNV has also been reported from Atlantic cod (*Gadus morhua*) in Denmark and the Faeroe Islands (Lorenzen et al., 1995, Martin-Armas et al., 2007).

IPNV has been detected in captive lumpfish at three different locations in Nordland and Trøndelag (Bornø et al., 2016). It has also been shown experimentally that they can be infected with IPNV by co-habitant transmission (Breiland and Johansen, 2015) and both naïve and intraperitoneally injected lumpfish tested positive for IPNV, without resulting an IPN outbreak or mortalities (Breiland and Johansen, 2015). This means that lumpfish potentially can become carriers of IPNV, which suggests that there is a risk for disease transmission between lumpfish and salmon in fish farms (Breiland and Johansen, 2015, Bornø et al., 2016).

Nervous necrosis virus, NNV

The nervous necrosis viruses, NNV, belong to a genus; Betanodavirus (Nodaviridae). Nodaviruses are positive sense single stranded RNA, ssRNA, viruses. The genus contains four species approved by the International Committee on Taxonomy of Viruses, ICTV: Tiger puffer nervous necrosis virus (TPNNV), Striped jack nervous necrosis virus (SJNNV), Barfin flounder nervous necrosis virus (BFNNV) and redspotted grouper nervous necrosis virus (RGNNV) (Shetty et al., 2012).

NNV replicates in the central nervous system and retina. Infection with NNV might cause degeneration of the spinal cord, brain and retina, a disease called Viral Nervous Necrosis (VNN) or Viral Encephalopathy and Retinopathy (VER). Typical clinical signs during VNN include abnormal swimming (spiralling or looping), poor coordination, and also changed skin pigmentation (Korsnes, 2008). The disease can cause high mortality rates in farmed fish, up to 100 % especially in larvae and juveniles (Korsnes et al., 2005a, Shetty et al., 2012). In

Norway VNN outbreaks have occurred in juvenile farmed halibut, cod and turbot (*Scophthalmus maximus*), with mortalities at almost 100% (Korsnes, 2008).

Three major types of NNV are found in Norway, including two of the ICTV approved species. BFNNV types have been detected in Atlantic halibut, Atlantic cod and in three wild wrasse (*Labridae*) species, also frequently used as cleaner fish in aquaculture in Norway (Korsnes et al., 2005a, Patel et al., 2007, Korsnes et al., 2017). RGNNV was recently found in a wild wrasse from near Bergen (Korsnes et al., 2017). A very distinct NNV genotype, turbot nervous necrosis virus (TNNV), occurs in turbot (Korsnes et al., 2005a).

Findings on NNV infections in lumpfish have so far not been published, but at FHF's sea lice conference in January 2018 it was reported that nodavirus had been detected in this host in Norway (Østvik, 2018). Details are not yet available.

Viral haemorrhagic septicaemia virus (VHSV)

Viral haemorrhagic septicaemia virus, VHSV, is a member of the family Rhabdoviridae. They are negative sense ssRNA viruses. There are four main genotypes (I-IV) of VHSV, and they are divided into subtypes (a-e). The genotypes differ in geographical distribution, rather than host species preference (Sandlund et al., 2014).

The virus causes characteristic haemorrhage in the skeletal muscle and intestinal mucosa. Fish also show exophthalmia, pale gills, darker skin colour and lethargy. VHSV has a broad spectrum of clinical signs and can cause mortality up to 100% if the temperature is optimal (Gadd, 2013). Horizontal transmission is either by ingestion of infected material, direct contact or through the water (Sandlund et al., 2014). The gills are a good place for sampling (Sandlund et al., 2014).

Genotypes I, II and III are found in Europe, and genotype IV was until recently only found in North America and the North Pacific Ocean (details below). Overall VHSV has been detected in over 80 different species, both from wild and captive origin (Wallace et al., 2008, Bruno et al., 2013, Sandlund et al., 2014).

Genotype Ib has been detected in the north of Norway (Finnmark) in different wild caught fish species. Genotype III, earlier believed to be a low risk type for salmonids, caused an outbreak in marine reared rainbow trout in 2007 (Sandlund et al., 2014). It is also known to have caused disease outbreaks in farmed turbot in Ireland and Scotland, and in wild caught, captive wrasse in the Shetland Isles (Sandlund et al., 2014).

In 2015 VHSV was detected in wild captive lumpfish in Iceland (Dadadottir, 2015). This proved to represent a novel VHSV genotype IV variant, provisionally referred to as IVd (Cuenca et al., 2017). As a result, screening was undertaken for VHSV in wild lumpfish on Iceland, but the 23 examined fish turned out negative (Fjölnisdottir, 2016). In Norway screening projects examining wild fish species have also included some lumpfish, but VHSV have so far not been detected in Norwegian lumpfish (Brudeseth and Evensen, 2002, Sandlund et al., 2014). Transmission between different species seems highly possible, and if the lumpfish is a carrier of VHSV it could represent a risk to salmonids (Sandlund et al., 2014, Fjölnisdottir, 2016).

Lumpfish flavivirus

In 2015 a new virus belonging to the family Flaviviridae, was discovered in diseased lumpfish in Norway (Skoge et al., 2017). The Flaviviridae is a group of positive sense ssRNA viruses. The virus, first referred to as Lumpfish flavivirus (LFV) and is the first flavivirus discovered in fish (Vestvik et al., 2017). Another name, Cyclopterus Lumpus virus (CLuV) has also been proposed (Skoge et al., 2017).

The virus is found in all tissues in the lumpfish. Signs of infection are best seen as changes like paleness and firmness in the liver (Vestvik et al., 2017). Histology shows clear signs of inflammation in the liver, like comprehensive necrosis and degeneration. Also, an accumulation of lipid droplets in the liver. These lipid droplets can also be observed in the gills and kidney, but not to the same extent (Skoge et al., 2017, Vestvik et al., 2017). The liver stands out as the target organ, even in the absence of visible signs. (Vestvik et al., 2017, Skoge et al., 2017). LFV is clearly linked to disease and mortality, but the mortality varies a lot between cases (Vestvik et al., 2017).

The virus seems to be distributed along the whole coast of Norway and was sporadically detected in most of the different stages of lumpfish production (e.g. hatchery, salmon farms). It has also been detected in lumpfish from Scotland (Vestvik et al., 2017). One experiment has shown that naïve fish can be infected by co-habitant transmission, without producing any pathological sings of LFV infection (Vestvik et al., 2017). Whether the virus can be vertically transmitted is so far unknown. If so, screening of brood fish could minimize the spread (Vestvik et al., 2017).

Ranavirus

Ranavirus belongs to the family *Iridoviriadae*, which are dsDNA viruses. It is known to infect more than 140 species of fish all over the world (Bandin and Dopazo, 2011). In 2014 a Ranavirus closely related to the notifiable Epizootic Haematopoietic Necrosis Virus (EHNV), was detected in lumpfish in the Faeroe Islands (Stagg et al., 2017). Since then the Ranavirus has been detected in lumpfish on Iceland, Norway, Ireland and in Scotland (Stagg et al., 2017). In Ireland, the virus was associated with high mortality in a hatchery, while closely related strains from the Faroes Islands, Norway and Iceland occurred in clinically healthy brood fish (Scholz et al., 2017b). There are still few publications on this genotype of Ranavirus.

1.3 Bacterial infections

The leading cause of mortality amongst lumpfish used as cleaner fish is probably bacterial infections (Nilsen et al., 2014, Hjeltnes et al., 2018). Atypical *Aeromonas salmonicida* and *Pasteurella* sp. were most often detected in 2015-2017 in samples analysed by the Norwegian veterinary institute, NVI (Hjeltnes et al., 2018). General detection of bacteria has been increasing since the introduction of the cleaner fish (Nilsen et al., 2014, Hjeltnes et al., 2018). Other bacteria isolated from lumpfish include *Pseudomonas anguilliseptica*, *Tenacibaculum maritimum*, *Psychrobacter* sp. *Polaribacter* sp. and different *Vibrio* spp. (Nilsen et al., 2014, Småge et al., 2016, Hjeltnes et al., 2018).

Many *Vibrio* spp. are a normal part of the marine bacterial flora, and some of these are good examples on opportunistic infectious agents (Nilsen et al., 2014). *Vibrio* spp. are commonly detected in both deceased and sick fish, but usually in a mixed bacterial flora, so its importance is unclear (Hjeltnes et al., 2018). Even so, some species like, *V. anguillarum* (causing vibriosis) and *V. ordalii* are known to be the cause of disease in fish, including lumpfish (Austin and Austin, 2012, Hjeltnes et al., 2018). In a more general picture are *Vibrio* spp. infections in lumpfish often linked to fin rot, especially the tail- and dorsal fin, but also to acute mortality and decreased appetite (Nilsen et al., 2014).

Vibrio splendidus is frequently detected in cleaner fish (Mortensen et al., 2011, Nilsen et al., 2014, Gulla et al., 2015). Some *V. splendidus* strains can cause mortality in turbot (Thomson et al., 2005, Farto et al., 2006, Sandlund et al., 2010). *Vibrio splendidus* consists of a lot of different genotypes, and is a broad and complex group of closely related *Vibrio* spp. Many of

which are opportunistic and can cause disease and increase mortality of different fish species, e.g. corkwing wrasse (*Symphodus melops*) (Mortensen et al., 2011).

Bacterial outbreaks of e.g. pasturellosis in lumpfish, have in some cases been epizootiologically linked to a common origin (hatchery populations) (Alarcón et al., 2016a).

1.4 Parasites

Coccidians

The coccidians (subclass Coccidia) belong to the Phylum Apicomplexa, a group of intracellular obligate parasitic protists. Coccidia infections are common in fish, usually found in the intestinal epithelium, but some species develop in other cells and organs such as in the swim bladder, liver, spleen, kidney or urinary bladder. (Lom and Dyková, 1992, Freeman et al., 2015).

There are many types of fish coccidians, but not all are well known. The best studied are *Goussia* spp. and *Eimeria* spp. both known to infect both tropical fish species and cold-water fishes (Landsberg and Paperna, 1987, Lom and Dyková, 1992, Hemmer et al., 1998, Munday et al., 2003, Khan, 2009, Bruno et al., 2006).

Coccidians may undergo extensive propagation in the gut epithelial cells, which are destroyed in the process. There are two cycles of merogony, each leading to the production of a high number of new stages. After gamete formation and fertilization, oocysts are produced. These normally sporulate when in the gut epithelial cells, forming four sporocysts each with two sporozoites. However, some species, such as *Eimeria variabilis*, infecting sculpins, release unsporulated oocysts from the fish host (Davies, 1978, Davies, 1990). Sporocyst formation is then completed in the environment (exogenous sporulation). Mature sporocysts with sporozoites are infective to new hosts (Lom and Dyková, 1992). This direct lifecycle may lead to high prevalence and intensities in fish ponds where the exchange of water is low, and therefore the infective stage is retained (Bruno et al., 2006). Coccidiosis is less common in marine fish, which usually are cultured in open pens rather than ponds (Bruno et al., 2006). Heavy coccidian infections in the intestine may lead to enteritis, with necrosis, dystrophy, desquamation and inflammation (Lom and Dyková, 1992).

Heavy coccidian infections have been revealed in the intestine of lumpfish from both Norway and Iceland, in both wild and farmed fish. The coccidian caused lysis of the infected mucosal cells, resulting in extensive pathology. Histology showed areas of exposed submucosa due to

torn layers of epithelial cells (Kristmundsson et al., 2018). The generic assignment of this apparently new coccidian is unknown since sporulation or the oocytes and hence the sporocysts have not been observed. It is therefore plausible that this coccidian has exogenous sporulation.

In this thesis, it will be referred to as Coccidium 'X'.

Kudoa islandica

Kudoa spp. are myxosporeans, a group of obligatory parasitic microscopic, but multicellular animals. The members of the genus *Kudoa* are intracellular parasites that usually infect skeletal muscle fibres (Bruno et al., 2013).

A large number of *Kudoa* spp. are known to infect fish, mostly marine species. The most studied, *K. thyrsites*, shows low host specificity and is known to infect more than 20 different species of fish. Among the hosts are different salmonids including farmed Atlantic salmon in British Colombia, Canada (Whitaker and Kent, 1991, Moran et al., 1997, Moran and Kent, 1999, Bruno et al., 2013).

Kudoa islandica infects the skeletal muscle cells of lumpfish and wolfish (*Anarhichas minor* and *Anarhichas lupus*) (Kristmundsson and Freeman, 2014, Karlsbakk et al., 2014).

The lifecycles of *Kudoa* spp. are not known. All myxosporeans have an alternating host in their lifecycle, usually an annelid, where a different type of spores is produced (actinospores). These actinospores are infective to fish, and the spores produced in the fish are infective to the alternate host only (Yokoyama et al., 2012).

Kudoa spp. form spores in plasmodia intracellular in their hosts muscle fibres. The plasmodia, are surrounded by a membrane, separating the parasite from the sarcoplasm. These structures are called pseudocysts. The pseudocysts of *K. islandica* can be visible to the naked eye, as a white worm-like structures in the muscle (Alarcón et al., 2016b).

Kudoa spp. are not normally associated with disease and mortality in fish and infected individuals appear healthy. Even so, some internal damage, like inflammation, have been observed in *Kudoa thyrsites* infected fish when the intensity of the parasite was extreme (Bruno et al., 2013). Some species like *K. islandica* and *K. thyrsites* cause post mortem myoliquefication, or "soft flesh" (Whitaker and Kent, 1991, Moran et al., 1999). This post

mortem myoliquefication is due to lysis of the host tissue caused by proteolytic enzymes released by the parasite (Whitaker and Kent, 1991, Moran et al., 1999, Bruno et al., 2013).

Lumpfish could potentially have been re-used as cleaner fish, but this is discouraged due to the possibility that they may carry infectious agents between salmon generations and have a poorer appetite for sea-lice when they reach a certain size. Hence normal practice is to dispose-of the lumpfish when the salmon are harvested (Borch, 2018). Another, possibly better, re-use of lumpfish is as a human food resource (Fletcher, 2016, Borch, 2018). This means that *K. islandica* may become a problem for further use of lumpfish, if infections are widespread (Kristmundsson and Freeman, 2014). This parasite has already been detected in farmed lumpfish and wolfish in Norway (Karlsbakk et al., 2014, Alarcón et al., 2016b), but the prevalence is virtually unknown.

Nucleospora cyclopteri

Nucleospora spp. are microsporidians that usually infect the nuclei of hematopoietic cells of fishes. Most other microsporidians infect and proliferate in the cytoplasm of their host cells, which may become hypertrophic or even tumour like.

Microsporidians have two morphologically distinct stages; the proliferation stage, or meront, and the infective stage, or spore stage (Dunn and Smith, 2001). Some have a direct lifecycle and others have an indirect lifecycle using more than one host organism. They also have the ability of infecting both horizontally or vertically, or even both. The microsporidians infecting fish, mostly have a direct lifecycle with horizontal transmission (Dunn and Smith, 2001).

The best known of these parasites is *Nucleospora salmonis*, which may infect several different salmonids in North America, Chile and Central Europe. *N. salmonis* is associated with chronic mortality in several farmed salmonid hosts, due to the disease characterized below (Hedrick et al., 1990, Chilmonczyk et al., 1991). In addition, halibut may be infected by *Nucleospora* sp., and lumpfish with *N. cyclopteri* (Chilmonczyk et al., 1991, Mullins et al., 1994, Nilsen et al., 1995, Alarcón et al., 2016b).

When fish are infected with *Nucleospora* spp. it is common to find internal signs like reno- and splenomegaly and also swelling of the large intestine (Hedrick et al., 1990, Bergh et al., 2001, Freeman et al., 2013). A characteristic histological finding is developmental stages or spores of *Nucleospora* spp. within the nucleus of the host cell. (Hedrick et al., 1990, Chilmonczyk et al., 1991, Alarcón et al., 2016b).

Clinical signs of *N. salmonis* infection include pale gills and occasionally exophthalmia (Hedrick et al., 1990). The microsporidian infection of salmonids induces an abnormal proliferation of hematopoietic tissue, mostly leukocytes, which in turn leads to a leukaemia like condition, and then anaemia (Hedrick et al., 1990). Target cells are in principle haematopoietic cells in the head kidney and spleen, but systemic infections also occur frequently (Hedrick et al., 1990, Chilmonczyk et al., 1991, Foltz et al., 2009).

Nucleospora cyclopteri infections in lumpfish is detectable in different organs, but the kidney is often more affected (Freeman et al., 2013). Infection cause swelling of the kidney, necrosis and degeneration of the hematopoietic tissue in the liver and spleen as well as in the kidney. Oedema in glomeruli and kidney interstitium are also commonly observed (Freeman et al., 2013, Alarcón et al., 2016b). This parasite has been found to infect wild lumpfish in Iceland and farmed lumpfish in Canada and Norway (Freeman and Kristmundsson, 2013, Karlsbakk et al., 2014, Alarcón et al., 2016b).

It has been shown experimentally that the antibody response in fish infected with *N. salmonis* is lower than that in non-infected fish. This is because infection causes impairment of the mitogenic response (triggering mitosis), and the further the infection progresses, the lower is the antibody response (Wongtavatchai et al., 1995), and this clearly implies a suppression of the humoral immune response.

Since *N. salmonis* have suppressive effects on the hosts immune system, it is possible that related species like *N. cyclopteri*, may also infect immune cells and have a similar effect. If so, then lumpfish developing nucleosporosis may be more susceptible to other infections, or co-infections may become more severe. A major problem in the use of lumpfish as cleaner fish is their survival during a production cycle of salmonids. The cause of death is not readily identifiable in dead fish, but Nilsen (Nilsen et al., 2014) found that some 75% of the mortalities (across 6 farms) were due to bacterial infections that would require a normal humoral immune response for resolution. It should therefore be investigated if *N. cyclopteri* infections are associated with these problems. The co-infection of *N. cyclopteri* and *Kudoa islandica* has already been described (Alarcón et al., 2016b).

1.5 Aims

In 2017, the Norwegian Seafood Research Fund (FHF) initiated the project *'Nucleospora cyclopteri* in lumpfish; occurrence, transmission, clinical importance and impact on cleaning behaviour' (project no. FHF901320). This project aim at increasing the current knowledge concerning the microsporidian parasite *Nucleospora cyclopteri* in lumpfish, and the present study is a part of that project, examining the prevalence and density of *N. cyclopteri* in the most important brood fish population in Norway (Hosteland, 2017), and the relationship between this parasite and other infections, 'co-infections' in nature.

In the present study the aim was to use sensitive molecular methods to examine the wild lumpfish population at Averøy, Møre and Romsdal county for selected pathogens. The study aimed at:

i. establishing the prevalence of infectious agents in healthy, mature wild lumpfish caught at Averøy, being:
    - viral agents known to infect lumpfish (IPNV, LFV and VHSV. See sections above)
    - viral agent that likely infects lumpfish (VNN)
    - opportunistic bacterial infections
    - *Nucleospora cyclopteri*
    - a novel coccidian, Coccidium 'X'
    - *Kudoa islandica*
ii. determine the genotypes ("wild types") of any virus detected, and
iii. examine the relationship between *N. cyclopteri* infection and the prevalence and intensity of other infectious agents.

2.0 Materials and methods

2.1 Location

Adult mature lumpfish were caught using nets in the Ramnfjorden – Sveggen area and landed at Skjerneset on the island of Ekkilsøya in Møre and Romsdal, just outside Kristiansund (Figure 1).

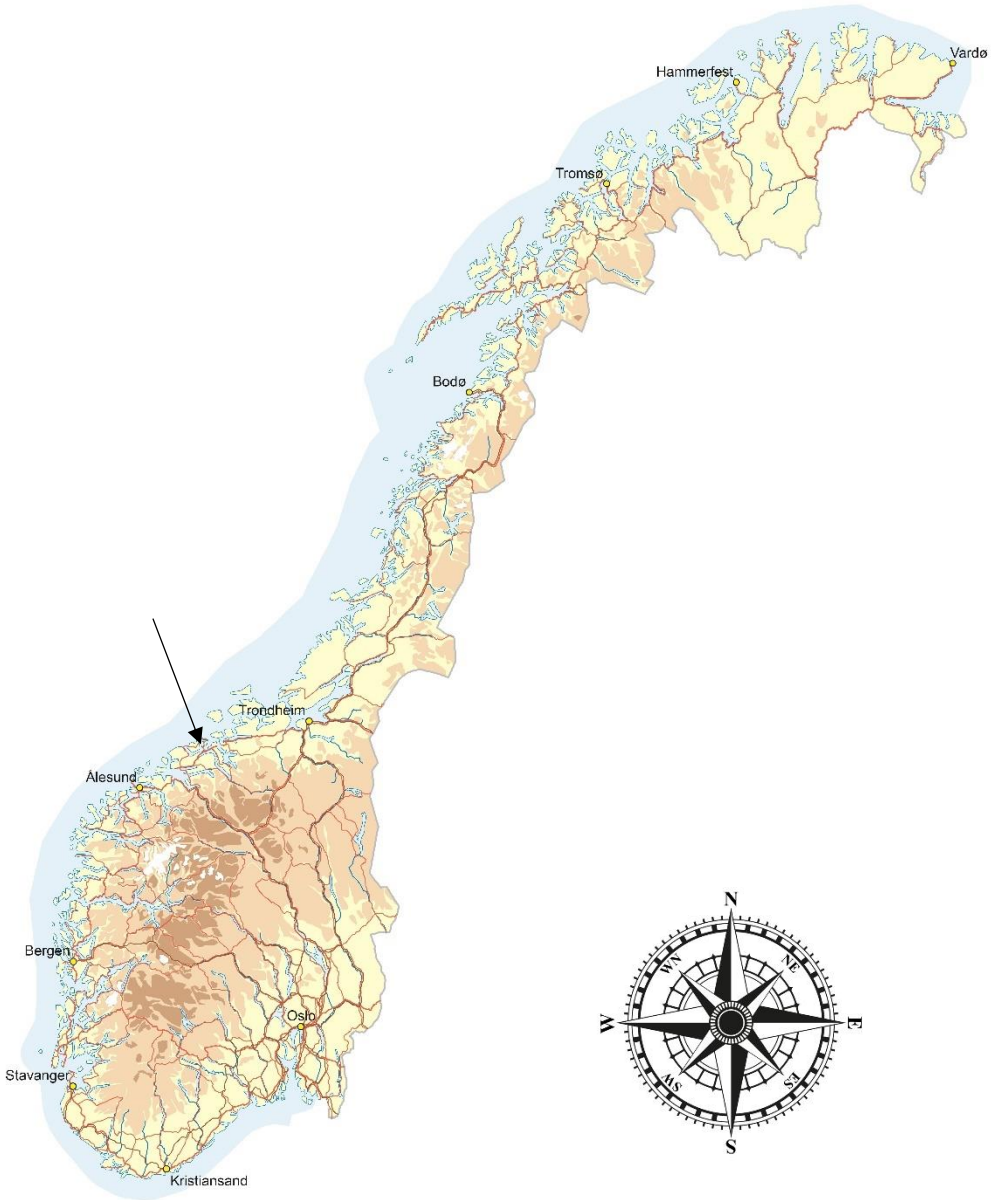

Figure 1: Map. Arrow = Kristiansund

Fish were caught in shallow waters (<10 m), using nets with a mesh size of 268 mm, the 21. June and 1. November 2017. Live fish were kept in tanks in the fishing vessels and transferred to three holding tanks (500 L) at Skjerneset (Figure 2). Some of the fish caught in June were transported live to tanks in a stripping facility were eggs and sperm were recovered from sexually mature fish before the fish were subjected to pathogen examination.

2.2 Samples

Samples were collected from 25 female individuals the 21st and 22nd of June and 60 individuals (49 females and 11 males) on the 1st and 2nd of November 2017. 20 of the 25 fish in June were received after they had been stripped for eggs at Skjerneset Fisk's stripping facilities at "Mork" and kept in individual plastic bags on ice in a fridge until examination. The remaining five fish were kept alive in tanks until examination. All 60 individuals samples in November were kept alive in tanks until examination. Fish were killed by a blow to the head either directly after stripping or directly before examination. An overview of the details of the sampled fish is given in Table 14, Appendix II. The fish from June were labelled 101-125, and the ones from November were labelled 201-260.

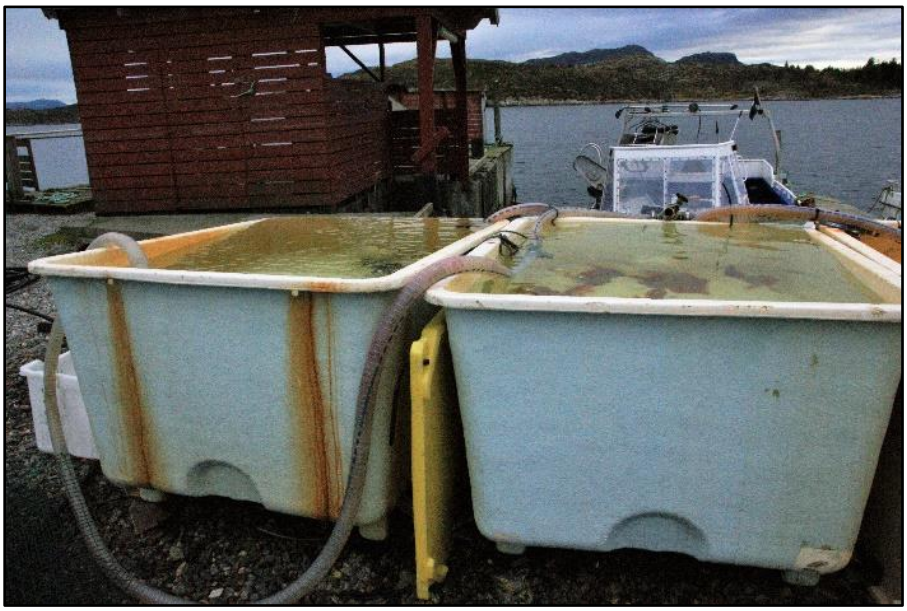

Figure 2: Fish holding tanks. Photo: Kathrine Nilsen

Four types of samples were taken:

i. Gill ('G'), Liver ('L'), anterior kidney/head-kidney ('AK') and medulla oblongata ('CNS') in RNA preservation solution for RNA extraction and quantitative PCR (qPCR)
ii. Muscle ('M') and pyloric caecum ('C') in 96% ethanol, for DNA extraction and PCR testing.
iii. Blood-agar plates with 2% NaCl (BAS) inoculated from the head-kidney.
iv. Formalin fixed tissue samples for histology of L, G, AK, M and C, from the first 10 fish at each sampling, and from individuals with lesions.

Table 1: Targets, assays and tissue samples analysed by them. Karlsbakk, E. designed the 18S fish coccidia primers based on a sequence of the lumpfish Coccidium 'X' kindly provided by Mark A. Freeman.

Targeted agent	Tissues	Targeted gene	Assay name	Reference
Nucleospora cyclopteri	Head-kidney	16S	NC16S	PatoGen
Lumpfish elongation factor	All RNA	Elfa 1α[1]	RKEla	PatoGen
IPNV	Liver	VP2[2]	IPNV	(Watanabe et al., 2006) (as "VP1")
NNV	Medulla oblongata	RNA2	NNV	(Korsnes et al., 2005b)
LFV	Liver	EP[3]	CLuV	(Skoge et al., 2017)
VHSV	Gill	NP[4]	VHSV	(Jonstrup et al., 2013)
Kudoa islandica	Musculature	*18S*	"Kud" (PCR)	(Kristmundsson and Freeman, 2014)
Coccidium 'X'	Pyloric caecum	*18S*	"Cocc" (PCR)	Karlsbakk, E. Unpublished[5]

[1]Elfa= Elongation factor 1α, [2]VP2= Viral protein 2, [3]EP= Envelope protein, [4]NP=Nucleoprotein, [5]The 18S fish coccidia primers were designed based on a sequence of the lumpfish Coccidium 'X' kindly provided by Mark A. Freeman and other fish coccidia sequences from GenBank. They are not specific for Coccidium 'X'

2.3 Dissection

The fish were killed, measured (to the nearest 0.5 cm) and weighed (in grams, g). The skin was examined for wounds and other lesions. Sea lice (*Caligus elongatus*) was also collected (not part of this project). The lumpfish was placed on a sterile sheet, always laying on the right side facing left (Figure 3A). The second left gill arch was then removed to a sterile petri dish, and the gill sample was taken out from the same gill arch. Thereafter, the abdomen was cut open; starting at the anterior ventral side, cutting alongside the sub-operculum, past the pectoral fin to the top of the abdominal cavity, then towards the posterior end and down towards the anal fin. The abdominal cavity was then exposed by folding down the left belly-flap (Figure 3D). The BAS inoculum was collected with a hot inoculating loop by penetrating the head kidney (Figure 3B), before the head kidney sample was taken out. The following tissue samples were dissected out in the most convenient order to avoid cross contamination (e.g. liver → caecum → muscle→brain) (Figure 3C and 3D) and placed in sterile petri dishes. Three small pieces (approximately 5 mm^3) of each tissue were collected for qPCR and PCR and one big for histology, put in the right preservative (see point i, ii and iv above, page 19) and stored in the fridge. The last sample taken was the medulla oblongata. After a frontal incision just above the eyes, a transverse incision was made where the skull ends to expose the brain (Figure 3E). The whole brain was carefully removed, the medulla oblongata separated from the brain and sliced into three pieces before it was put in preservative.

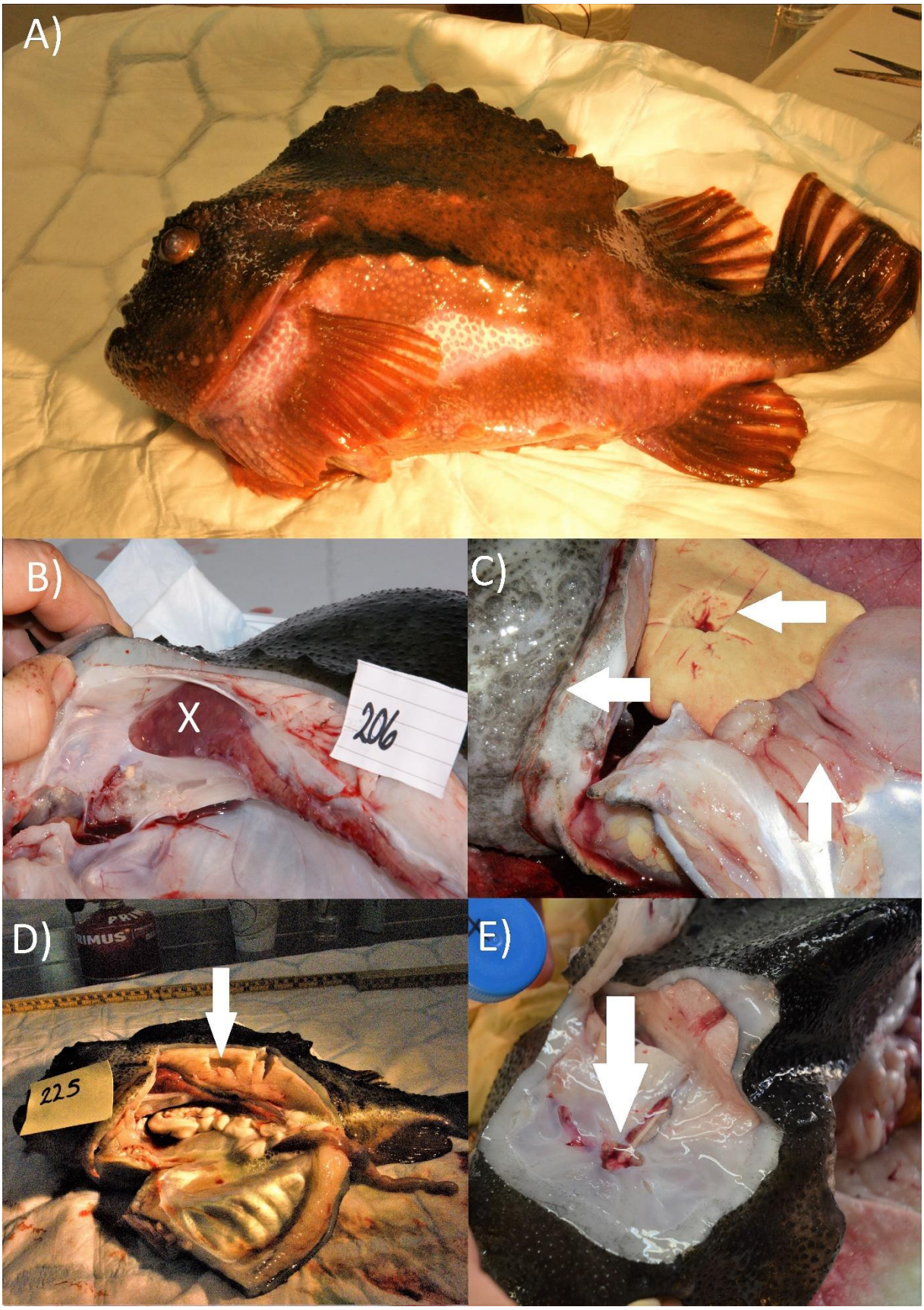

Figure 3: A) Red male lumpfish. B) Head kidney, X = BAS inoculum collection spot C) Organ package, arrows from top to bottom: Liver, operculum (covering the gill) and pyloric caecum. D) . Abdominal cavity exposed, Arrow: muscle sample location. E) Brain and medulla oblongata exposed. All photos: Kathrine Nilsen

Histology

All histological slides were prepared by the Norwegian Veterinary Institute, NVI. The tissue samples were fixed in formalin and the formalin fixed tissues were cast in paraffin. And the slides where coloured with Haematoxylin and Eosin stain (H&E stain) (Alarcón et al., 2016a).

The slides were examined with an axio scope A1 light microscope (Carl Zeiss). Photos were taken through the microscope with the Axiocam 105 color and edited with the software ZEN (Carl Zeiss).

2.4 Bacteriology

The BAS plates were incubated at 16 ˚C, and checked for growth at 3, 7 and 14 days after inoculation. Any bacterial growth found in the track from the inoculation needle was re-plated to ensure clean mono cultures were obtained before further analysis: single bacterial colonies were transferred from the original plate to a new one and incubated again at the same temperature. This was repeated until the growth on the BAS agar looked homogenous. One bacterial colony was taken from the new BAS plate (now containing colonies from only one bacteria species) and frozen in nuclease free water at -20 ˚C for later DNA extraction.

2.5 RNA isolation

For the RNA isolation TriReagent® was used following the instructions by the manufacturer, but with two changes to the protocol:

1. All the centrifugation steps were set to max (14 800 g).
2. The RNA isolation step 2. (washing the RNA-pellet) was repeated once.

A small tissue sample (50-100mg) was put in a 2mL Safelock tube (Eppendorf®) with 1000µl TriReagent and one 5mm stainless steel bead. This was homogenized in a Tissuelyser II instrument (Qiagen) for 3 minutes. For complete dissociation the sample stood in room temperature for 5 minutes. After 5 minutes, 200µl of chloroform was added and the sample was extracted by shaking vigorously for 15-20 seconds. Then the mixture stood at room temperature for 2 minutes. After centrifugation at 14 800 g for 15 minutes at 4˚C, 450µl of the colourless, upper, aqueous phase (containing RNA) was transferred to a clean 1.5mL microtube tube. RNA was precipitated by the addition of 500 µl isopropanol, and thorough mixing. Again, the sample stood at room temperature for 5 minutes before being centrifuged at maximum speed for 10 minutes at 4˚C. This centrifugation step formed a pellet at the bottom of the tube. The supernatant was removed, and the pellet was washed by adding 500

µl of 75% ethanol, vortexing the sample and then centrifuging at maximum speed for 5 minutes at 4˚C. The ethanol was removed, and the wash step was repeated. After removing as much ethanol supernatant as possible, the pellet was air dried on the bench until it turned transparent. The RNA pellet was dissolved in 50 – 100 µl (depending on the pellet size) nuclease free water, and the RNA was vortexed to dissolve it completely. RNA was kept on ice while the amount of nucleic acid was measured using a NanoDrop 1000 Spectrophotometer, this also provided a measure of the RNA purity.

The RNA purity was assessed based the ratio of absorbance at 260 nm and 280 nm. For RNA a 260/280 value greater than 1.8 was considered as an indicator of good RNA purity (Fleige and Pfaffl, 2006).

2.6 Quantitative Reverse Transcriptase PCR, RT-qPCR (here: qPCR)

Lumpfish were examined for selected RNA virus infections using published and mostly well established and tested qPCR assays (Table 2). The exception was the recently published LFV assay (Skoge et al., 2017). When several assays were available (published), an assay was selected to maximise the likelihood for a detection by amplifying a wide range of genotypes. No assay for the Ranavirus has not yet been published.

For the analysis of the RNA samples a quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was run with AgPath-ID™ One-step RT-PCR reagents (Ambion). The concentrations set in the user guide were followed except for the final volume of the reaction, which was set to 10µl reaction rather than 25µl as suggested in the user guide (concentrations and volumes in Table 6, Appendix I).

Table 2: Assays used for qPCR.

Target	Name	Primers and Probes[5] 5' – 3'	Length (Base pairs)	Eff. [1]	Ref.
Elongation factor 1α lumpfish	RK Elfac_F RK Elfac_R RK Elfac_P	GTTGAGACCGGCATCATCAA AGGTTGCAGGGAGCAAAGG CCCACCATGGTCGTC	56 bp	1.883	Courtesy of PatoGen Analyse AS
16S *Nucleospora cyclopteri*	NC16S_F NC16S_R NC16S_P	AGTCCGTAGTCGTAGATGCAATTAAA GCTCCGCCACAATTCAACA TATGGATCAAGCATACTAAG	69 bp	1.947	Courtesy of PatoGen Analyse AS
VP2 [2] IPNV	IPNV_F IPNV_R IPNV_P	ACCCCAGGGTCTCCAGTC GGATGGGAGGTCGATCTCGTA TCTTGGCCCCGTTCATT	67 bp	1.987	(Watanabe et al., 2006) (as "VP1")
RNA2 NNV	NNV_F NNV_R NNV_P	TTCCAGCGATACGCTGTTGA CACCGCCCGTGTTTGC AAATTCAGCCAATGTGCCCC	54 bp	1.999	(Korsnes et al., 2005b)
NP [3] VHSV	VHSV_F VHSV_R VHSV_P	AAACTCGCAGGATGTGTGCGTCC TCTGCGATCTCAGTCAGGATGAA TAGAGGGCCTTGGTGATCTTCTG	77 bp	2.018	(Jonstrup et al., 2013)
EP [4] Lumpfish flavivirus	CluV_F CluV_R CluV_P	GCCGAGACCTATATAACTTGGAGAGA CGACGTTATGGGCTTCTGAAA ACCACCCTCCATTACGTGA	70 bp	-	(Skoge et al., 2017)

[1]Efficiency (see details in appendix II) [2]VP2 = Viral protein 2, [3]NP = Nucleoprotein, [4]EP = Envelope protein, [5]TaqMan MGB probes (Applied Biosystems)

Master mixes were made according to Table 6 (Appendix I) and kept cool during handling. Eight µl of the different master mixes were dispensed first into each well on the 96 well plate, followed by 2 µl of RNA template, positive control RNA or nuclease free water was added as appropriate.

The qPCR was run with the 96 well qPCR system; Quant Studio 3 (Applied Biosystems by Thermo Fischer Scientific). The thermal cycling conditions shown in the AgPath-ID™ One-step RT-PCR reagents user guide for 96-well machines were used with 45 amplification cycles; reverse transcription was to run for ten minutes at 45˚C, then the RT inactivation/Taq polymerase activation for ten minutes at 95 ˚C. The amplification, consisting of two steps; denaturation at 95 ˚C for 15 seconds and annealing/elongation at 60 ˚C for 45 seconds, was repeated 45 times (Table 8, Appendix I).

All the positive control samples used for the targets in Table 3 were mixed together in an Eppendorf tube. For the *N. cyclopteri* positive control (Ct-value = 30), the head-kidney sample from lumpfish 113 was used. A tissue sample from Atlantic salmon was used as positive control for the IPNV assay (Ct-value = 22), provided by Dr Craig Morton at Institute of Marine Research, IMR. Egil Karlsbakk provided the positive controls for the NNV assay; a medulla oblongata sample from cod (Ct-value = 28), and the VHSV assay; a head-kidney sample infected with VHSV type Ib also cod (Ct-value = 28) (Karlsbakk et al., 2008). The LFV positive control (Ct-value = 22) was provided by Pharmaq Analytiq AS.

Lumpfish were considered positive for IPNV, NNV, VHSV and LFV, when amplification was measured in the qPCR. Fish positive for any viral RNA would be further examined, by testing the other tissues for the agent, and then cDNA synthesis and PCR performed for genotyping.

Fish were considered positive for *N. cyclopteri* when the amplification was measured in the qPCR. Samples were considered negative when amplification was not seen. The head-kidney samples positive for *N. cyclopteri*, but with a Ct value over 36 (standard curve Figure 10, Appendix II) were repeated with RNA extracted from a second, back-up tissue sample. Only when both RNA extractions were positive for *N. cyclopteri* RNA in qPCR, were individuals reported as positive.

Ct-value, or threshold cycle number, is a relative quantitative measure of the qPCR target. The value comes from the number of amplification cycles that are run until the base line (manually set to 0.1 for this project) is crossed by the amplification curve (More target = fewer cycles = lower Ct – value) (Fleige and Pfaffl, 2006).

Assay efficiency

Knowing the efficiency of the assay is important because it provides information on sensitivity, dynamic range and the presence of inhibitors in the RNA template. Theoretically assay efficiency should be 100 %, meaning that during each cycle the target amount doubles.

A tenfold dilution series was made of the positive control samples, to test the efficiency of all the qPCR assays (except for the CLuV assay were no material was available). This was run with triplicates for the four least diluted samples, and quadruplicates for the four most diluted samples. Plotting the Ct-values from the dilution series against the logarithmic scale of the

dilution gives a standard curve (Figure 9 – 13, Appendix II), were the slope is used to calculate the efficiency (E).

The assay efficiency (E), was calculated using the equation below (Equation 1).

Equation 1: $E = 10^{(-1/(slope))}$ (Pfaffl, 2004)

Assay efficiency ranged from 88.3% to 102% (Table 2).

2.7 DNA isolation

DNA was isolated from muscle and caecum samples, and from the bacterial colonies using E.Z.N.A® Tissue DNA Kit. The Tissue DNA – spin protocol was followed, together with the Vacuum/spin protocol (QIAvac 24plus from QIAGEN) for using a vacuum manifold instead of centrifugation.

A small tissue sample was put in a microtube together with 200 µl TL (lysis) buffer and 25 µl OB protease. It was mixed by vortexing and incubated with vigorous shaking at 55˚C (with Eppendorf® Thermomixer compact) for approximately 3 hours to lyse the tissue sample. After the tissue was lysed the sample was centrifuged at max speed for 5 minutes and 200 µl of the supernatant was carefully transferred to a clean tube. Then 200 µl BL buffer was added, and the sample was vortexed and incubated at 70˚C for 10 minutes. After incubation, 200 µl of absolute ethanol (96-100%) was mixed into the sample by pipetting up and down 5-10 times. This was transferred to the HiBind® DNA mini column (which was already mounted on the vacuum manifold). The vacuum was then turned on, and the liquid was drawn through the HiBind® DNA mini column. HBC buffer (500µl) was added to the column, and again the vacuum drew the liquid through the mini column. The same was done twice with 700 µl DNA wash buffer. After washing, the mini column was put in a collection tube and centrifuged at maximum speed for two minutes to dry the mini column. The dry column was placed in a clean 1.5 mL microtube, and 100 µl elution buffer, preheated to 70˚C, was added carefully to the centre of the column. After two minutes incubation at room temperature the DNA was eluted by centrifugation of the column at 14 800 g for one minute.

The quantity and purity of the resultant DNA sample was estimated using a NanoDrop 1000 Spectrophotometer. DNA purity was derived from the ratio of absorbance at 260 nm and 280 nm. The samples were considered “pure” if 260/280 absorbance ratio was >1.7 (O'neill et al., 2011).

2.8 PCR

For the PCR of the DNA samples isolated with E.Z.N.A, GoTaq® Green Master Mix was used. The master mix was prepared according to Table 7 (Appendix I) and kept cool during preparation. General guidelines for amplification with PCR in the GoTaq® Green Master Mix user manual was followed.

Table 3: Assays used for PCR and sequencing.

Target	Name	Primers 5'→ 3'	Annealing Temp.	[3]Frag. (base pairs)	Reference
18S *Kudoa islandica*	**Kud80_F** **Kud730_R**	ACTGCGAAGCGCTCAGTA AGGCACACCTCGCAAGTGAC	55 ˚C	750bp	(Kristmundsson and Freeman, 2014)
18S Coccidium 'X'	**Cocc-F1** **Cocc-R1**	GTATTGGCCTACCGTGGCAG CTCTACCATAAACTATGCCGACTAG	56 ˚C	700bp	Karlsbakk, E. Unpublished[4]
16S Bacteria	**27F** **1518R**	AGAGTTTGATC[1]**M**TGGCTCAG AAGGAGGTGATCCANCC[2]**R**CA	56˚C	1500bp	(Giovannoni et al., 1996)

[1]**M** = A or C, [2]**R** = G or A, [3]Frag. = fragment length, [4]The 18S fish coccidia primers were designed based on a sequence of the lumpfish Coccidium 'X' kindly provided by Mark A. Freeman and other fish coccidia sequences from GenBank. They are not specific for Coccidium 'X'

It was pipetted 19 µl of master mix into each well, and 1 µl DNA template, positive control sample or nuclease free water. They were placed directly in Verti 96 well Thermal Cycler (Applied Biosystems) PCR machine pre-heated to 95˚C. PCR was run with the following thermal conditions; First denaturation at 95˚C for two minutes. Then 35 repetitions of the three steps of amplification; Denaturation at 95˚C for 30 seconds, annealing at the assay specific temperature (Table 3). for 30 seconds, and extension at 72˚C for 45 seconds. Followed by a final Extension at 72˚C for five minutes. (Table 9, Appendix I),

All the positive control samples for the assays in Table 3 were provided by Dr Egil Karlsbakk. Positive controls were not included for the 16S bacteria assay. A *Kudoa* sp. infected muscle sample from a Barents Sea eelpout (*Lycodes gracilis*) was used for the *K. islandica* assay. Two positive controls were initially included for 18S Coccidium 'X' assay, and both were from *Eimeria* sp. infected caecum samples. One was a from the fish *Arctozenus rissoi* caught in the Norwegian sea. The second from a *Bathylagus tenuis* caught in the Southern Ocean.

For analysing the amplified PCR product, a 1% agarose gel was run, using 1xTAE (Tris, acetate EDTA pH 7.6) buffer, and 80-90 volts (Recipe for agarose gel can be viewed in Appendix I). Five µl of each PCR product was applied in separate wells in the gel, and three µl SMART ladder (Eurogenetics) as a molecular weight standard was applied in the first or last well.

Fish were considered positive with *Kudoa* sp. and coccidium 'X' when bands were visible in the agarose gel from gel-electrophoresis at the correct length (Table 3), and positive for any marine bacteria when sequenced and a BLAST search performed.

Coccidium 'X' infections were subjectively scored from 0-3 based on band intensity in agarose gel, where 0 = no visible band on the gel, 1 = weak band, 2 = clear band and 3 = prominent or overloaded band visible (Table 13, Appendix II), for a relative measure of quantity (Guenin et al., 2009).

Sequencing

ExoSAP (Promega) or Illustra™ ExoProStar™ 1-step (GE healthcare) PCR clean-up kits were used to digest the left-over primers and nucleotides to prevent interference in the subsequent sequencing reaction. BigDye chemistry was used for the Sanger sequencing method, and the samples were sequenced by the sequencing facilities at UiB. Sequences were assembled using the software Contig Express™.

2.9 Data analysis

Sequence identification

The bacteria partial 16S sequences were initially identified to genus or species-group using the nucleotide BLAST search at the website of National Center for Biotechnology Information, NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and then compared to the type species sequences of the closest relatives. Based on this comparison each sequence was assigned to the species to which it had highest identity to, using aff. for 'affinity to'.

The *Kudoa* sp. 18S sequences were compared to available sequences in GenBank from Iceland, and the still unnamed coccidian (Coccidium 'X') was identified by sequence alignment to a consensus sequence kindly provided by Mark. A. Freeman (University of Keldur, Iceland).

Normalized expression

Normalized expression (NE) was calculated following Simon (2003) and was used as a density measure for *N. cyclopteri*. Using RK Elfac (elongation factor 1α) as the reference, and NC16S (*Nucleospora cyclopteri*) as the target. The density in samples where the target was not detected was set to 0 (Table 12, Appendix II).

Equation 2: $\mathrm{NE} = \frac{(\mathrm{E}_{reference})^{\mathrm{CT}_{reference}}}{(\mathrm{E}_{target})^{\mathrm{CT}_{target}}}$ (Simon, 2003)

Statistical analysis

Pathogen prevalence, the proportion of samples infected (Bush et al., 1997), were compared in two samples using Fisher exact tests (FET).

The density of *N. cyclopteri* was compared using the non-parametric Mann Whitney U-test (MW), since the NE data generally were heteroscedastic and not-normally distributed. Correlations were examined using the non-parametric Spearman rank order correlation coefficients (r_s).

All statistical tests were performed using the software Statistica 13.3 (Tibco Inc.)

3.0 Results

3.1 RNA and DNA purity and quantity

RNA and DNA purity were good with little variation. For RNA the 260/280 ratio generally were ~2.0. For DNA the 260/280 ratio were ~2.0 (Table 10 and 11, Appendix II).

The isolated RNA quantity varied between the different tissues, if they measured >1000 ng/µl they were diluted to <1000 ng/µl. DNA quantity for the muscle samples were all <100 ng/µl. The DNA isolated from the caecum samples measured from 100 ng/µl up to 400 ng/µl.

3.2 Comparison of the two different samplings, June and November

There was no significant difference in size between the females sampled in June and the ones sampled in November (T-test, p = 0.208). No males were available in June and only 11 were caught in November. These were generally smaller than the females.

Table 4: Biometric data of the examined lumpfish. Mean length and weight, sample date and sex.

Sample	**Sex**	**N**	**Length (cm)** **Range** **Mean±SD**	**Weight (g)** **Range** **Mean±SD**
1 – June	Females	25	36.0 – 51.6	1600 – 3840
			44.1 ± 3.5	2603 ± 669
2 - November	Females	49	39.0 – 53.3	2022 – 5196
			45.1 ± 3.1	3254 ± 791
	Males	11	30.6 – 41.8	875 – 2101
			35.1 ± 3.1	1480 ± 374
Total N:		85		

N = number of individuals, SD= Standard deviation

3.3 Infectious agents detected

The infectious agents detected in this study were *N. cyclopteri, K. islandica,* Coccidium 'X', and *Vibrio* sp. aff. *splendidus, Psychrobacter* sp. The prevalence of the selected pathogens is listed in Table 5.

The different positive controls always tested positive.

Table 5: Prevalence of all infectious agents screened for in the studied adult mature lumpfish (N=85) caught off Averøy in 2017.

Pathogen	**Tissue**	**N infected**	**Prevalence**	**$CI_{95\%}$ (min-max)**
Infectious pancreatic necrosis virus (IPNV)	Liver	0	0	0 - 4.4%
Nervous necrosis virus (NNV)	Medulla oblongata	0	0	0 - 4.4%
Viral haemorrhagic septicaemia virus (VHSV)	Gill	0	0	0 - 4.4%
Lumpfish flavivirus (LFV)	Liver	0	0	0 - 4.4%
Bacteria	Head kidney	2	2.4%	0.3 – 8.2%
Vibrio sp. aff *splendidus*	Head kidney	1	1.2%	0.03 – 6.4%
Psychrobacter sp.	Head kidney	1	1.2%	0.03 – 6.4%
Coccidium 'X'	Pyloric caecum	84	98.8%	93.7 – 99.9%
K. islandica	Musculature	18	21.2%	13.3 – 31.1%
Nucleospora cyclopteri	Head kidney	51	60.0%	48.8 - 70.1%

N = number of individuals, $CI_{95\%}$ = confidence interval with 95% certainty, BAS = Blood agar plate with 2%NaCl

Viral infections

The viruses IPNV (liver), VHSV (gill), NNV (medulla oblongata) and LFV (liver) were not detected (Table 5), suggesting a prevalence less than 4.4% with a 95% certainty.

Since the selected viral infectious agents were not detected in the primary tissue, further tissues were analysed, and thus cDNA synthesis and PCR for genotyping were not performed.

Bacterial infections

The growth on the BAS plates was sparse, with only single colonies observed. One colony (fish 104) was green greyish and showed alpha hemolysis. Another (fish 118) was white and showed weak beta hemolysis. The others showed no hemolysis and where yellow, grey, white or transparent (fish 223).

Bacterial colonies were obtained from 10 fish (12%). PCR with general bacterial 16S rRNA gene primers yielded product from six of these according to the agarose gel. BLAST search with partial 16S sequences revealed four of these six as likely contaminations, since their relatives in GenBank were soil bacteria or human symbionts (2 x *Arthrobacter* sp., *Bacillus* sp. and *Staphylococcus* sp.). The last two were a *Vibrio* sp. (fish 104) partial 16S sequence (1442 nucleotides, nt) which showed 99.9% identity with EU091337 (*Vibrio splendidus*), and a *Psychrobacter* sp. (fish 223) sequence (1416 nt) which showed 99.9% identity with MH178035 (*Psychrobacter sanguinus*). These were therefore identified to genus and group as *Vibrio* sp. aff. *splendidus* and *Psychrobacter* sp. aff. *sanguinus* (sequences in Appendix II).

Vibrio sp. aff. *splendidus* came from the green-greyish colony that showed strong alpha hemolysis in BAS, and it came from fish 104. The *Psychrobacter* sp. had transparent colourless colonies without hemolysis and originated from 223. The prevalence of bacterial infections was hence considered 2.4% (Table 10).

Coccidium 'X'

A coccidian infection in lumpfish discovered by Kristmundsson et al. (2018), here referred to as Coccidium 'X', was detected in 98.8% of the lumpfish. A single fish was uninfected (Table 5). The PCR product was sequenced from nine fish, selected based on season and band score. All sequences were identical and showed 100% identity to the reference sequence (MF992167) of Kristmundsson et al. (2018).

In histology, various developmental stages typical for coccidian infections, were seen in high densities (Figure 4 and Figure 5).

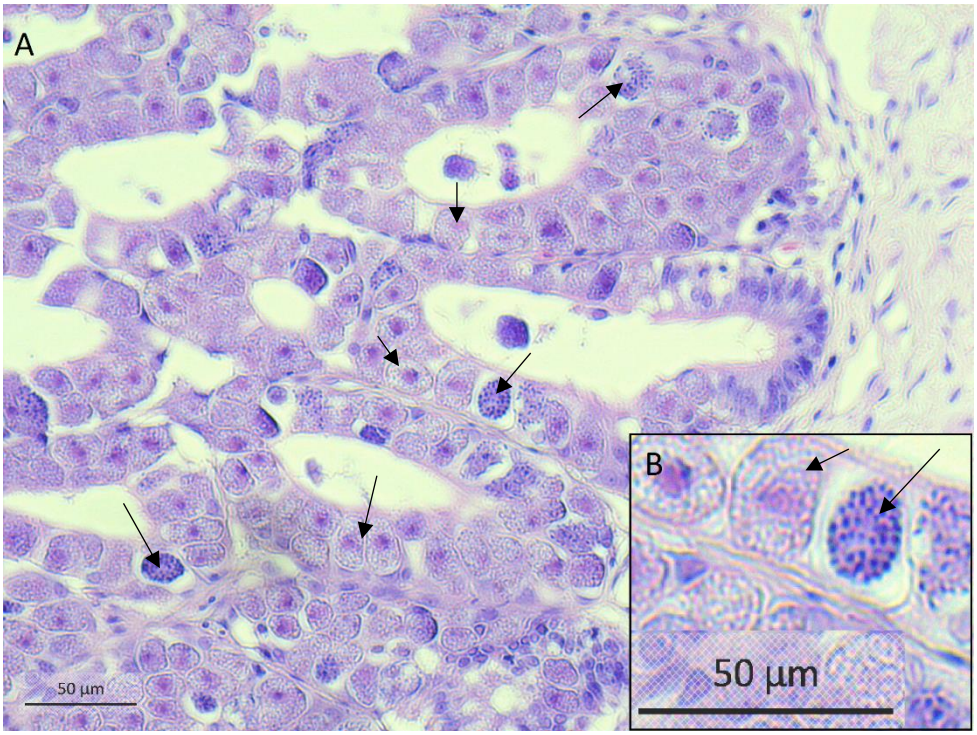

Figure 4: Coccidian ('Coccidium X') infected caecum of lumpfish, showing the parasite inside intestinal epithelial cells. A) Several infected cells containing densely stained microgamonts, and paler granular macrogamonts and macrogametes which are difficult to distinguish. B) Close up of micro- and macrogamont Left arrow: macrogamont or macrogamete with unstained granules. Right arrow: densely stained macrogamont producing sperm. Photo: Kathrine Nilsen

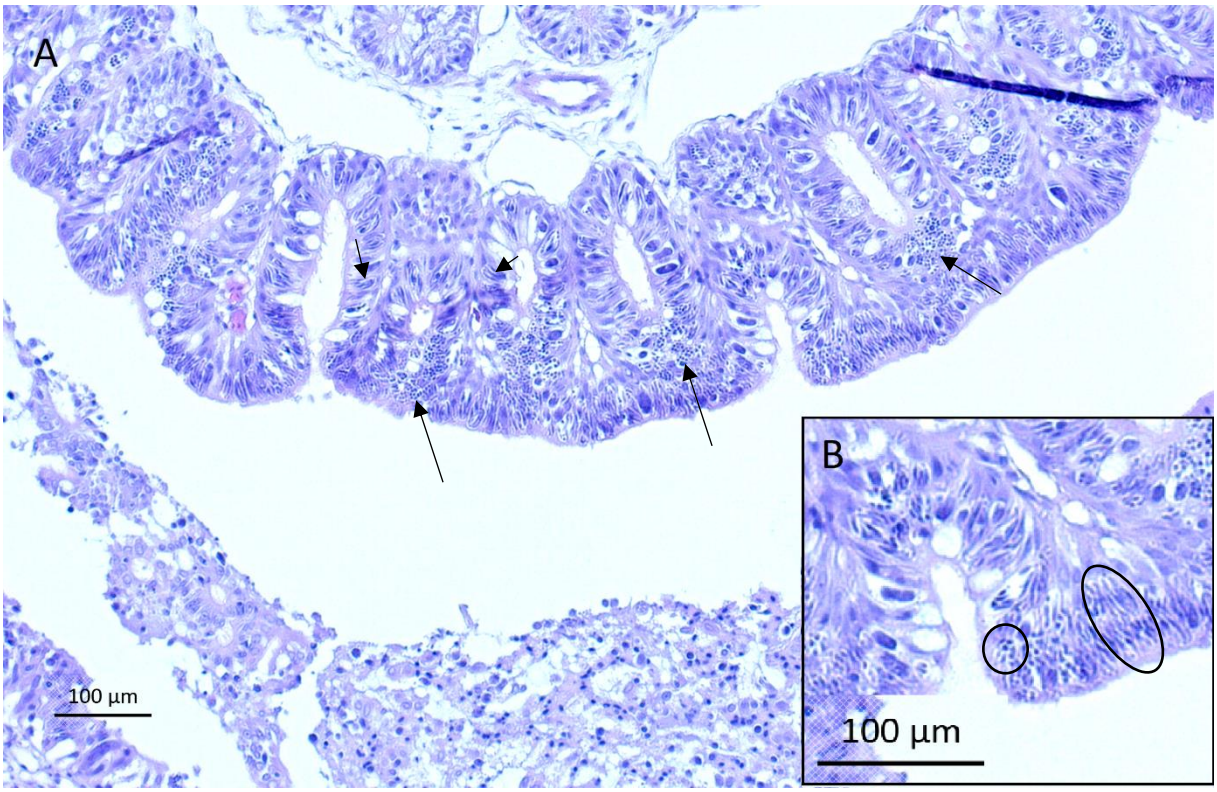

Figure 5: Coccidian ('Coccidium X') infected caecum of lumpfish, showing the parasite inside intestinal epithelial cells. A) A high proportion of the cells harbour merogonial (proliferation) stages, with large numbers of bacilliform merozoites formed inside the cells. B) Higher magnification of merozoites in transverse- (left circle) and longitudinal (right circle) section. Photo: Kathrine Nilsen

Band intensity in the agarose gels varied. A band score based on the intensity of the bands did not correlate with fish length in any samples (June r_s=0.051, p=0.810; November females: r_s=0.050, p=0.731, males: r_s= -0.302, p=0.366).

Kudoa islandica

In 85 muscle samples run with PCR, 20 were positive, but at gel-electrophoresis two bands showed deviating migration, suggesting the two products had higher molecular weight (longer fragment). These two were sequenced and proved to represent *Parvicapsula asymmetrica* 18S with 99% identity (AY584191). Eight products with the expected DNA fragment length according to the DNA standard were sequenced and all were 100% identical to *K. islandica* (KJ451388). All 18 products with this precise band migration were therefore considered to represent *Kudoa islandica* giving a prevalence of 21.2% (Table 5).

Histological sections from infected fish showed muscle fibres with pseudocysts packed with spores (Figure 6).

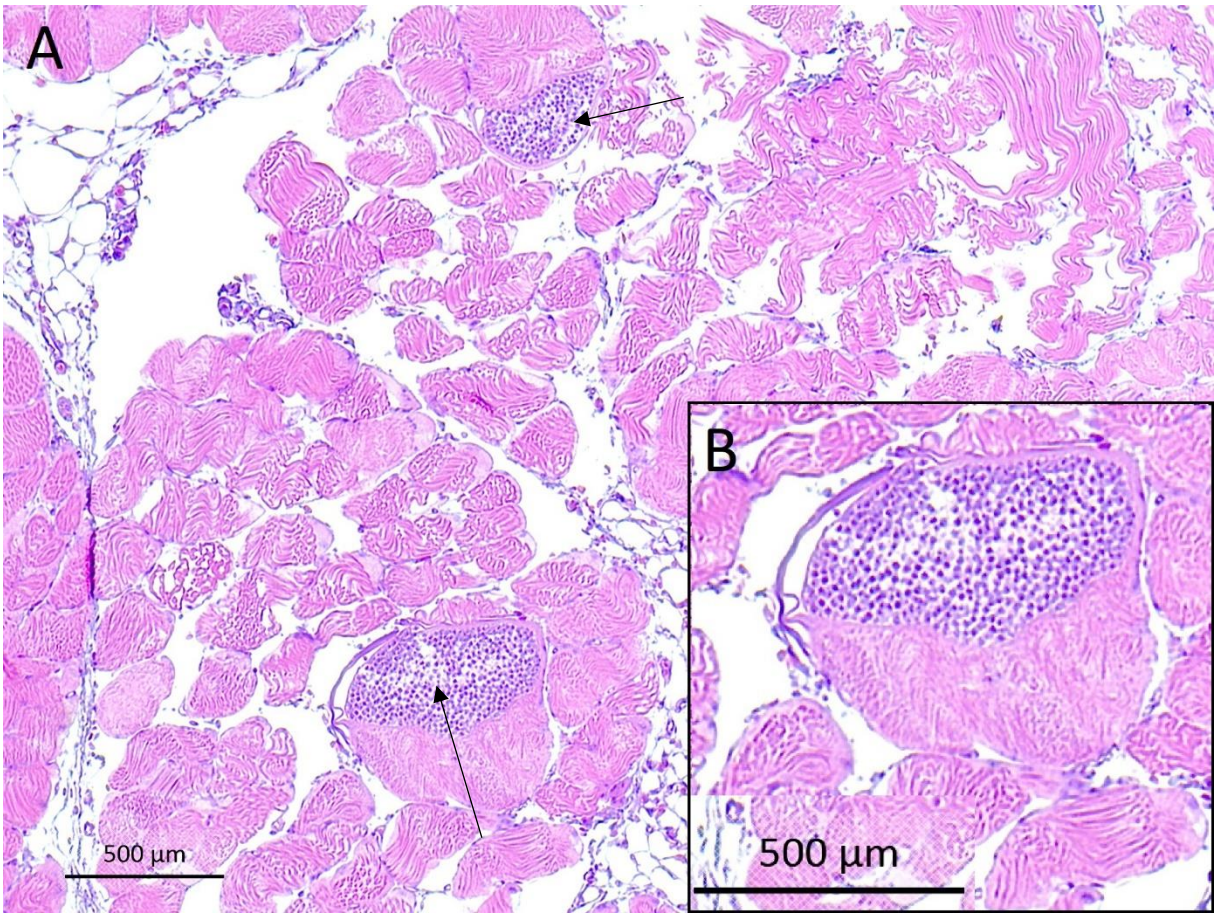

Figure 6: *Kudoa islandica*.infection in the skeletal musculature of lumpfish.A) Two pseudocysts (arrows) inside muscle fibres. B) Magnified pseudocyst (plasmodium) containing spores. Photo: Kathrine Nilsen

Prevalence of *K. islandica* was higher in fish sampled in June than the ones sampled in November (FET, p=0.041).

There was no indication that prevalence differed between males and females (November, FET, p = 0.66). Fish length did not differ between *K. islandica* infected and uninfected individuals (June, T-test, p = 0.979; November, T-test, females: p = 0.371, males: p = 0.146).

Nucleospora cyclopteri

Analysis of head-kidney samples for *N. cyclopteri* infection resulted in a prevalence of 60% (Table 10). The density (Figure 8) of the microsporidian in the kidney showed large variation between the fishes. 16 fish showed moderate changes in the kidney. These changes were mainly observed in the head-kidney, in the form of modest enlargement, but also presence of whitish nodules (Figure 7). In two cases with single nodules, one nodule did not contain *N. cyclopteri* (fish 247; Figure 7B) and the other showed a similar Ct/NE value as the surrounding kidney tissue. In total, five out of the uninfected individuals showed either one singular nodule, or very modest swelling (only male individuals). Five fish with patchy pattern of white nodules (Figure 7A), modest swelling and a moderately paler kidney, had a Ct-value between 5-15. The last six fish (Ct-value between 15-35) showed modest swelling or paleness in the kidney (Figure 8).

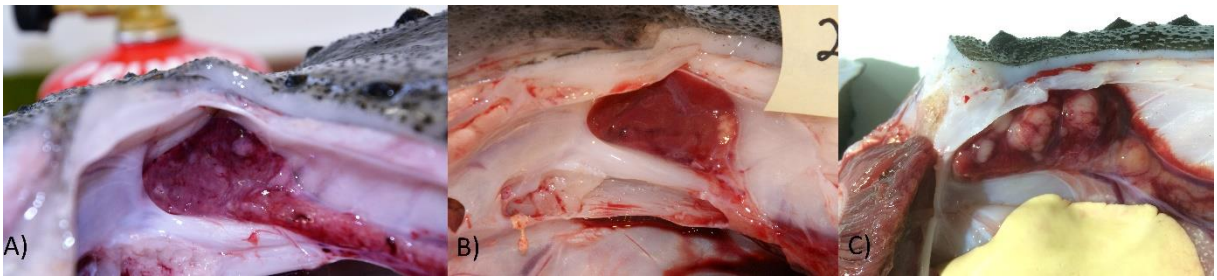

Figure 7: Head kidney lesions seen in lumpfish, with and without *Nucleospora cyclopteri* infection A) Numerous small white nodules in kidney of ~normal size (fish 113), fish infected (Ct=10.5). B) White nodule posterior in an otherwise normal head kidney (fish 247), fish not infected. C) Example of nucleosporosis, swollen head kidney with several large protruding white nodules (fish not from present material, Ct=7.2). Photo A) and B): Kathrine Nilsen. Photo C): Leon Stranden from Marine Harvest Vanylven.

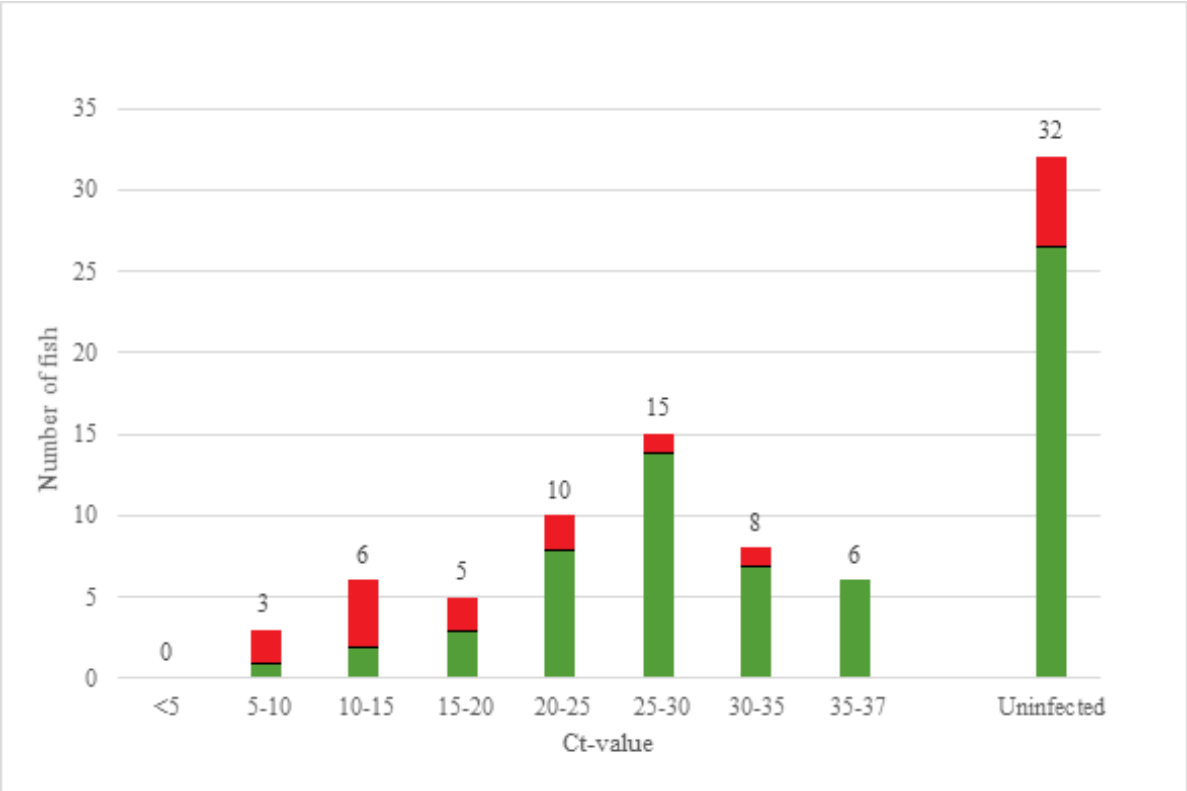

Figure 8: Illustration of the *N. cyclopteri* density observed in the examined lumpfish, shown as normalized Ct-values (Ct calculated on the basis of NE for a set reference (Elongation factor 1α) Ct=10 (Equation 2)). The red area represents the number of fish with visible changes in the kidney.

Fish length showed no relationship with *N. cyclopteri* NE (females, $r_s = 0.139$, $p = 0.237$, males, $r_s = -0.165$, $p = 0.628$).

No difference in *N. cyclopteri* density or prevalence was found between the fish sampled in June vs. the fish sampled in November (density: MW, $U = 711.0$, $p = 0.710$. Prevalence: FET, $p = 0.467$).

Relationship between *Nucleospora cyclopteri* and other infectious agents

No viral infections were detected, so coinfections with *N. cyclopteri* were only seen with bacteria isolated from the head-kidney, *Kudoa islandica* in the musculature and Coccidium 'X' in the caeca.

Bacterial growth from the head-kidney had no relation to *N. cyclopteri* density.

There was no evidence that fish with *Kudoa* infection differed in *N. cyclopteri* density from uninfected fish (MW, $U_{18,67} = 545.5$, $p = 0.54$).

A negative correlation was seen between *N. cyclopteri* density and the Coccidium 'X' band score for females sampled in November (r_s = -0.380, p = 0.007), but not in from June (r_s = 0.250, p = 0.229), or the males from November (r_s = 0.253, p = 0.454).

4.0 Discussion

Using wild-caught lumpfish as brood fish, is presently the only way of breeding lumpfish. However, there are risks associated with the use of wild fish. The fish can be carriers of vertically transmissible disease agents, virulent types that could cause epizootics among the progeny, or opportunistic pathogens, that cause disease first when the fish is exposed to stress and their immune system is supressed. There is also a risk with regards to vertically transmitted diseases and genetic changes from avirulent types to more virulent ones (Karlsbakk et al., 2008a).

It is important to study the presence of potential pathogens early in the development of a relatively new aquaculture species like lumpfish, since the pathogen repertoire can change both quantitatively (e.g. elevated prevalence) and qualitatively (e.g. genetic change) over time (Karlsbakk et al., 2008a). It is known already that the captive lumpfish is suffering from high mortality of both known and unknown reasons, bacterial infections and new viruses, which have been, and are being discovered (Nilsen et al., 2014, Stagg et al., 2017, Skoge et al., 2017). Wild lumpfish populations may over time be affected, perhaps particularly exposed are juveniles in the kelp forests along the coast.

Mainly due to increased transmission, but also from imperfect vaccines and other medical measures (e.g. antibiotics), agents persisting in aquaculture population over long time can possibly develop increased virulence and become more dangerous (Davies and Davies, 2010, Kennedy et al., 2016). However, in order to detect such genetic change, the naturally occurring genotypes should be known (Karlsbakk et al., 2008a). A more complex situation could arise for disease agents that may be transmitted between different fish species, such as IPNV might be transmitted between lumpfish and salmon (Breiland and Johansen, 2015).

Lumpfish bacteria and viruses could also be moved from their original area of distribution in or outside of Norway, to new areas of the Norwegian coastline where fish potentially may be naïve to them and much more susceptible. Screening projects such as the present study are therefore important, since information on both the background prevalence of infectious agents can be obtained prior to potential change from introduction to aquaculture, and also on which wild type infectious agents are present. Another aspect is the acquisition and storage of

samples (Biobank), that can be used later to study the occurrence of presently unknown infectious agents.

4.1 Evaluation of materials and methods

The examination of lumpfish from only one location, as opposed to from several different parts of the country, was in this case a conscious choice. As mentioned in the introduction, the roe produced in Averøy, is used by a very high proportion (80%) of the Norwegian aquaculture farms producing lumpfish for use as cleaner fish (Hosteland, 2017). Hence it is particularly important to examine lumpfish brood fish from Averøy for potentially vertically transmitted agents.

A sample size of 85 fish is not high for a screening project, sometimes with over 1 000 fishes like Sandlund et al. (2014) had when screening for VHSV along the Norwegian coast. The original goal was to sample at least 100 fish. However, since a particular fishery was organized for both sampling periods, all fish that became available were examined. The costs associated with this fishery were high, about 1000 NOK per fish, and this cost was a factor prohibiting further sampling. It was therefore chosen to examine the available fish for a wide range of important infectious agents, instead of examining a higher number of fish for fewer. Also, other samples taken from the same fish were used for a range of other purposes besides the present study. As indicated above, collecting a range of good samples from each fish, also allows for later use such as screening for new viruses that are detected.

All except the 20 last individuals sampled in June, were sampled directly post mortem. These 20 fish were however kept cool in a fridge on ice from the time of death until sampling, which for the last fish sampled means about 6-7 h post mortem. This could have impacted the quality of the RNA in these samples, because RNA is known to have a rapid degeneration post mortem (Seear and Sweeney, 2008). However, the RNA quality was good. There was no difference in the Ct-value from the reference gene (elongation factor 1α) assay between these 20 fish, and all the other fish were sampled directly post mortem. Therefore, RNA quality likely did not affect the results in the present study.

The bacterial sampling was limited to inoculation from any wounds onto marine agar (MA), and from the head kidney onto BAS. No wounds of interest were detected on any of the fish, all external lesions observed appeared to originate from the nets used for capturing these lumpfish. Therefore, samples were not inoculated on MA, and only the inoculum from head kidney onto BAS provided results. The BAS is a moderately selective medium that supports

the growth of most opportunistic fish infecting bacteria, such as different *Vibrio* spp. (e.g. *V. splendidus*) and *Aliivibrio* spp., and also important lumpfish pathogens such as *Aeromonas salmonicida, Pasteurella* sp. and *Pseudomonas aguilliseptica* (Austin and Austin, 2012). *Aeromonas salmonicida* was not targeted using BAS, since qPCR could be a better method to detect carriers of the bacterium, and other agar media are better suited for its isolation.

DNA was not obtained from four single bacterial colonies from four of the fish. The reason for this is obscure, because the bacterial colonies were frozen from fresh plates.

qPCR is a highly sensitive method, making it possible to detect target RNA from e.g. viral agents, at extremely low concentrations. In addition, qPCR is rapid and has good reproducibility and it is also a good tool for analytic and quantitative measures (Pfaffl, 2004). The qPCR and PCR assays used here were selected; in order to detect a wide range of genotypes (IPNV, VHSV, NNV, Coccidium 'X' and bacterial 16S), being the only available (LFV) or being a published assay previously used for the specific agent (*Kudoa islandica*). The efficiency calculated for the qPCR assays indicated their reliability. Unfortunately, the efficiency for the CLuV assay was not calculated. Since there was no infected tissue detected, and since the positive control run in four separate wells showed minimal variation, this was not preformed.

For the IPNV, VHSV and NNV assays were chosen by their ability to detect many different genotypes of the agent, checked *in-silico* in alignments of available relevant sequences from the GenBank. The primers used for the IPNV match different genotypes from different wild marine fish species in the North Atlantic, but also IPNV from farmed salmon, which was used as a positive control sample. The suitability of the VHSV assay used is uncertain. It should amplify all the known marine variants in the northeast Atlantic as well as the IV genotypes, but the sequences of the genotype IVd variant detected in lumpfish brood fish from Iceland is still not available.

There are no qPCR assays available for *K. islandica* and Coccidian 'X', and this was not considered important. Both parasites occur intracellularly and are present in high numbers of host cells. Hence, they should be detected with conventional PCR. The primers used were not species specific, and sequencing revealed that the primers for *K. islandica* also could amplify *Parvicapsula asymmetrica*. This is a related myxosporean, but the primer mismatch towards *P. asymmetrica* makes it unlikely that this species would be amplified in the presence of

Kudoa islandica. In addition, *K. islandica* could be differentiated from *P. asymmetrica* by the different molecular weight observed in the agarose gels. The differentiation was also confirmed through sequencing of a selection of samples with fragment lengths indicative of *K. islandica*.

Some agents were not screened for because suitable qPCR assays were not available within the time frame of this study (*A. salmonicida*) and in the case of Ranavirus even sequence information to design an assay is not yet available.

4.2 Evaluation of results

RNA and DNA quality

The RNA and DNA quality were measured using the Nanodrop spectrophotometer. This may reveal the purity of the sample, but not RNA or DNA degeneration and fragmentation.

RNA breaks down to shorter sequences, which is a natural process of the decay occurring post mortem (Seear and Sweeney, 2008). A higher degree of fragmentation can have an impact on the Ct value in qPCR with primers that produce longer amplicons (>400bp), but it has an insignificant effect on shorter amplicons (70-250bp) (Fleige and Pfaffl, 2006). Therefore, for the qPCR assays used in the present project this is probably irrelevant, both because all the assays designed yield relatively short products, and because the sampling was performed immediately post mortem securing minimal breakdown of RNA (Fleige and Pfaffl, 2006, Seear and Sweeney, 2008).

DNA is highly stable and does not break down quickly post mortem (Allentoft et al., 2012). Also, the targeted microparasites will probably live many hours after the death of the host. According to O'Neill et al. (2011), a pure DNA sample should have 260/280 absorbance ratio between 1.7 – 2.0. The 260/280 absorbance ratio of the isolated DNA in this study had on average approximately 2.0. Higher values than this may indicate the presence of RNA (O'neill et al., 2011). Even though the samples had absorbance ratios in the high end of the scale according to O'Neill et al. (2011), they were considered good because the PCR gave amplified products. Any presence of RNA or appeared to not inhibit the PCR reaction and it is therefore regarded as insignificant for the results.

The assays used in this thesis were considered good, because the efficiency, E, was as close to 100% as possible, but also all the positive control samples used in this study were positive

every time, with only small variations in Ct. Although it was not possible to validate the sensitivity and dynamic range of the LFV assay the positive control sample used in this study were positive four times with little variation, suggesting that the assay used is a reliable assay.

Infectious agents

In a previous project screening captive lumpfish from several different locations in Norway, IPNV was detected in one facility in Nordland, and two in Nord-Trøndelag (Bornø et al., 2016). The IPNV detection was without clinical signs and was not linked to disease or mortality at either location. It has also been experimentally shown that IPNV can infect lumpfish in co-habitant situations (Breiland and Johansen, 2015). Similarities between genotypes found in wild marine fish, and genotypes found in aquaculture suggests that there may be disease interactions between wild and farmed fish (Bain et al., 2008). IPNV may be vertically transmitted (Bovo et al., 2005), making it a potential problem in lumpfish aquaculture facilities. The origin of the virus in the lumpfish from Nordland and Trøndelag is unknown. Besides in salmonids, the virus has caused epizootics in other marine fish such as cod and halibut (Lorenzen et al., 1995, Biering, 1999). It is believed that the virus is widespread in Norwegian wild halibut (Bergh et al., 2001), but this has never been studied.

The present results represent a rare case in the sense that data on virus occurrence in an important wild stock have been gathered before epizootics have occurred in aquaculture. The prevalence in healthy adults appears to be low. More data is needed from both captive and wild lumpfish of all ages.

NNV infections have been detected in many wild marine fish species in Norwegian waters, amongst them, cod, other gadoids, flatfish, mackerel (*Scomber scombrus*) and wrasse (Korsnes et al., 2005a, Korsnes et al., 2017). So far, nothing is published on NNV in lumpfish, but at the sea lice conference in January this year (2018) it was claimed to have been detected in captive lumpfish (Østvik, 2018).

There has not been any NNV screening projects on wild lumpfish in the past. An important finding is co-habitant transmission between turbot and cod, shown under experimental conditions (Korsnes et al., 2012). This could indicate a risk of transmission between other unrelated hosts, such as wrasse and lumpfish together in salmon pens. Since NNV infection may have been detected in captive lumpfish, and NNV may show vertical transmission (Bovo et al., 2005), there is also a risk that VNN outbreaks eventually may occur in lumpfish aquaculture. The 85 individuals examined for NNV with qPCR in this project were negative,

so the prevalence may not be high. In wild wrasse from Sweden and Norway the prevalence was 6.7% in 466 individual fish (Korsnes et al., 2017). While in wild healthy cod from Norway it was found to be 10-15% (Karlsbakk et al., 2008b). This requires further investigation, since NNV can cause disease and high mortality.

In 2015 VHSV infections were detected in wild-caught captive lumpfish intended to be used as brood fish in Iceland (Dadadottir, 2015). After this detection a study examining the prevalence of wild lumpfish was performed on Iceland, but the 23 fish examined were negative (Fjölnisdottir, 2016). Wild lumpfish from Norwegian waters (N=72) have been examined for VHSV infections in past screening projects, without detecting the virus (Brudeseth and Evensen, 2002, Sandlund et al., 2014).

The present study also did not detect VHSV in Norwegian lumpfish, so a total of 157 lumpfish has now been found negative for the virus. A very low prevalence is typical for this virus e.g. Sandlund et al. (2014) examined 1927 fishes from 39 different species caught in Norwegian waters and detected the virus in only twelve samples (0.6%).

In 2015 a massive screening of captive lumpfish was performed at the behest of lumpfish breeders who had concerns with the health of their lumpfish. The fish showed clear signs of infection, with liver damage and increased mortality, and no known infectious agents were detected. This turned out to be a new virus tentatively named Lumpfish flavivirus. It was detected in facilities along the whole coast of Norway, and in some lumpfish produced in Scotland (Vestvik et al., 2017).

The prevalence of LFV in brood fish in Norway is low, but the virus is present, according to Vestvik et al. (2017). Actual prevalence was not reported, but the present results (85 LFV negative lumpfish) supports a low prevalence.

The virus appears to be distributed along the whole coast of Norway, but the result from this project suggests that the virus might not originate from the wild populations at Averøy. This may or may not be true. Although it was not possible to validate the sensitivity and dynamic range of the LFV assay the positive control sample used in this study were positive four times with little variation, suggesting that the assay used is a reliable assay.

Bacterial infections are frequently detected in lumpfish, and they are often the cause of disease and mortality (Nilsen et al., 2014, Bornø et al., 2016). Commonly found in lumpfish are atypical *Aeromonas salmonicida* ssp., causing atypical furunculosis, *Pasteurella* sp.,

causing pasteurellosis, and *Vibrio anguillarum*, causing vibriosis (Nilsen et al., 2014, Karlsbakk et al., 2014, Hjeltnes et al., 2018). Other bacteria, namely; *V. ordalii, V. splendidus, Pseudomonas anguilliseptica* and *Tenacibaculum maritimum,* have also been detected in association with disease, but not as frequently (Nilsen et al., 2014, Gulla et al., 2015, Småge et al., 2016, Hjeltnes et al., 2018).

Vibrio sp. aff. *splendidus* and *Psychrobacter* sp. were the only two bacteria isolated from the head kidney of the lumpfish in this study, that likely had a marine origin and originated from the fish.

There are no previous studies on bacterial infections in healthy wild lumpfish. Bacterial growth from the head kidney could reflect a septicaemia in fish but could also simply be due to bacterial entry from wounds, and antigen trapping in the kidneys. The latter is the most likely explanation in the present case, since both bacteria are common in seawater and fish mucus (McCarthy et al., 2013, Gulla et al., 2015). Both bacterial types have previously been isolated from lumpfish (Gulla et al., 2015, Småge et al., 2016). Many of the bacteria known to infect lumpfish in hatcheries or when stocked in salmon farms are opportunistic and may not normally cause disease in healthy lumpfish. Therefore, the apparent absence of *A. salmonicida, V. anguillarum* or *Pasteurella* sp. could be expected; the present results suggest that these infections are rare in wild adult mature lumpfish.

Kristmundsson et al. (2018) described a case with a new species of pathogenic coccidian infecting lumpfish, both from wild adult fish from Iceland and from farmed diseased juvenile lumpfish in Norway. The parasite infects gut mucosa, causing epithelial destruction. That particular coccidian was not identified due to the lacking observation of mature oocysts and sporocysts. The identification of fish coccidians to a genera and species level relies mostly on sporocyst morphology (Lom and Dyková, 1992). The coccidian here conveniently referred to as Coccidium 'X', most likely represent a new species which sporulates outside the fish host. However, 18S rDNA sequences were sequenced, allowing for its identification.

From the pyloric caecum samples taken in this project, 84 out of 85 fish tested positive for Coccidium 'X'. Based on rDNA sequencing of samples from 9 fish, the parasite was identified as being 100% identical to the coccidian of Kristmundsson et al. (2018).

The very high prevalence observed indicates that it is common in wild populations and maybe not pathogenic under normal conditions, such as in healthy adults. However, lesions in the gut

epithelium could represent portals of entry for other infectious agents, such as opportunistic bacteria. Its importance as a primary pathogen must be clarified in controlled challenge experiments. This project is the first project screening wild lumpfish for this coccidian.

Kudoa islandica infections have been detected in musculature of lumpfish, both wild caught and farmed lumpfish in Iceland and in Norway (Kristmundsson and Freeman, 2014, Alarcón et al., 2016b). Five out of ten Norwegian farmed lumpfish were infected (Alarcón et al., 2016b), while all five wild Icelandic lumpfish had the infection (Kristmundsson and Freeman, 2014). The occurrence of this parasite in a farmed population in pens (Alarcón et al., 2016b) or reared in water from moderate depths (Karlsbakk et al., 2014, Kristmundsson and Freeman, 2014), suggests that the so far unknown infective stages of this myxosporean occur in coastal shallow waters.

As far as is known this myxosporean is not normally pathogenic to lumpfish, only slight inflammation has been observed, then in association of ruptured pseudocysts (Kristmundsson and Freeman, 2014). The main problem this parasite represents is in the use of lumpfish as human food due to myoliquefication of the musculature. With a *K. islandica* prevalence of 21%, a relatively large proportion of the lumpfish fillets are at risk of being discarded. However, the infections may not always be apparent, and post-mortem myoliquefication seems particularly associated with heavy infections (Moran et al., 1999, Kristmundsson and Freeman, 2014). Lumpfish can likely become infected with this parasite when living in salmon pens, from infective spores in the water. Therefore, this parasite represents a potential problem in the re-cycling of lumpfish (fillets) from pens as human food.

A slightly higher prevalence was seen in the June samples compared to the November samples. This could be due to seasonal changes in infection, as noted among other *Kudoa* spp. (Ishimaru et al., 2014). Another possibility is that the spring and autumn spawners does not belong to the same stock. The present study represents the first study of the occurrence of this parasite in a wild lumpfish population in Norway.

The microsporidian parasite *Nucleospora cyclopteri* was first detected in Norway in wild lumpfish, with macroscopical signs of nucleosporosis such as an enormously swollen head-kidney (Karlsbakk et al., 2014). Infections have later been found in captive lumpfish in Norway (Alarcón et al., 2016b, Hansen et al., 2017, Hjeltnes et al., 2018), also associated with disease and mortalities (Alarcón et al., 2016b). A study of adult wild lumpfish on Iceland has detected this microsporidian at 12 of 43 different sites around Iceland, often associated with

visible kidney changes (e.g. swollen, pale). Such changes were noted in 23% of the fish (Freeman et al., 2013). The actual prevalence of infection was not examined, neither was it known for wild lumpfish in Norway prior to the results from the present study.

N. cyclopteri was detected in head-kidney samples from 51 out of the 85 examined fish. A small amount of the fish (16 individuals) showed kidney changes somewhat similar to the ones Freeman et al. (2013) and Karlsbakk et al. (2014) describes (e.g. swollen, pale kidney, several white nodules), but not to the same degree. Two fish showed a singular white nodule, but this was unrelated to det *N. cyclopteri* infection since no difference in Ct-value of the nodule and the surrounding tissue was detected, one of these fish was even free from *N. cyclopteri* infection. Meaning that the nodule alone is not a good indication of nucleosporosis. Another uncertain sign is the modest swelling, which was observed in five uninfected individuals. This could mean that the swelling is cause by other factors such as bacterial infections, although not in this case. Considering that all the fish registered with solely modest swelling and negative Ct-value were males, there is a possibility that this was normal kidney morphology, and not the swelling it was believed during the time of sampling. Still, all the three kidney changes combined seems to be a good indication of nucleosporosis.

It is suspected that this microsporidian infection, affecting lymphocytes like leukocytes, could have immunosuppressive effects on the host. The related *N. salmonis* infecting salmonids causes a leukaemia-like condition associated with a reduced antibody response and impaired mitogen-induced lymphoproliferation, suggesting a suppression of the immune system (Wongtavatchai et al., 1995). If the parasite has a similar effect on lumpfish, then this could also represent a predisposing factor to the widespread bacterial problems and poor survival seen among fish stocked as cleaner fish in pens.

One aim here was to test this possibility by examining the association and correlation of *N. cyclopteri* in infections and densities with concurrent infections. Since no viral and virtually no bacterial infections were revealed, only the parasitic co-infections could be considered. This however, did not disclose any association or synergism between *N. cyclopteri* and the other parasites detected. The only pattern seen was less coccidian infection in the fish with the highest *N. cyclopteri* density in November, i.e. the opposite effect, negative correlation. All the lumpfish in this project appeared healthy. The relationship between this microsporidian and its host, or occurrence of infections are probably best studied under experimental conditions, by challenge experiments. No challenge model is yet available, but *N. salmonis*

have been transmitted by injection, feeding of infected tissue and by co-habitation (Hedrick et al., 1990, Baxa-Antonio et al., 1992, Gresoviac et al., 2007).

Parasites usually live together with their host in some sort of equilibrium, where the host is infected, but with minimal negative effects. Microsporidians can exist in their host at low concentrations under normal conditions but could possibly proliferate and cause disease when the host is weakened by experiencing stress, like rapid environmental changes or by other infections (Alarcón et al., 2016b). This might be the reason for the mortalities reportedly caused by this parasite among farmed juveniles or captive lumpfish in pens (Mullins et al., 1994, Alarcón et al., 2016b).

The related *N. salmonis* has likely been introduced into Chilean aquaculture several times (Gresoviac et al., 2000). Salmonids does not occur naturally in Chile, so the most likely source are the salmonid eggs imported to Chile each year, from Europe or North America. The *N. salmonis* genotypes identified in Chile are identical to genotypes of American Pacific and North Atlantic origin (Gresoviac et al., 2000). This suggests that *Nucleospora* spp. could potentially be vertically transmitted. Vertical transmission is relatively common among microsporidia (Stentiford et al., 2013). So far, there is no clear evidence for this in lumpfish, although there are indications (e.g. frequent detections in roe and milt) that it could occur (Lein et al., 2017). If so, then the observed prevalence of 60% suggests that this risk could be high. Indeed, some lumpfish breeding companies today do screen the ovarian fluid or milt of brood fish lumpfish for this parasite, so that egg-batches from positive fish can be discarded.

5.0 Conclusion

In the present study, wild-caught lumpfish from the most important brood fish-fishery in Norway were screened for a selection of important infectious agents, most of which already have been detected in farmed lumpfish and have caused disease. The prevalence of some eukaryotic parasites were high, while infections with bacteria were rare and viral infections were not detected. This finding is positive for the use of this stock as brood fish, but a weakness with the survey was the limited number of fish studied, particularly with respect to some important but rare viral infections. However, background prevalence's of several infectious agents have been established, and this may be important for our ability to later detect potential prevalence changes in wild lumpfish due to an impact from aquaculture. The material may also later be examined for additional, at present unknown infectious agents. The association and correlation of the possibly immune-affecting microsporidian *N. cyclopteri* with other agents (coinfections) could not be examined satisfactorily on the present material, due to the very light *N. cyclopteri* infections and few coinfections. These aspects should be examined experimentally.

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Appendix I: Recipes and thermal cycling conditions

Recipe for 100 ml RNA preservation solution

4 ml of 0.5 M EDTA (18.61g disodium dihydrate per 100 ml pH to 8.0 with NaOH while stirring).

2.5 ml 1 M Sodium citrate (2.9.g Sodium citrate trisodium salt dihydrate per 10ml.)

60 g Ammonium sulphate

93.5 ml H_2O stir on hot plate.

Adjust pH to 5.2 using conc. H_2SO_4

Recipe for 100 ml 1% Agarose gel

100 ml TAE, Tris, acetate EDTA pH 7.6, buffer

1 g SeaKem® LE agarose powder

Dissolve the powder in the TAE buffer by heating in microwave. Store in 60˚C to keep it in a liquid state.

Table 6: Master mix for AgPath-ID™ One-step RT-qPCR reagents, for 10 µl reaction.

Component	*Start conc.*	*Volume for 10 µl reac.*	*Final conc.*
2XRT-PCR Buffer	2X	5 µl	1X
Upstream primer	10 µM	0.40 µl	400 nM
Downstream primer	10 µM	0.40 µl	400 nM
Probe	3 µM	0.12 µl	120 nM
Enzyme mix	25X	0.40 µl	-
RNA template	-	2 µl	-
Nuclease free water (H2O)	-	1.68 µl	-

Table 7: Master Mix (MM) volume and concentration used in PCR

Component	*Start conc.*	*Volume for 20 µl reac.*	*Final conc.*
GoTaq Green Master Mix	2X	10 µl	1x
Upstream primer	10µM	1 µl	0.5 µM
Downstream primer	10µM	1 µl	0.5 µM
DNA template	-	1 µl	<250 ng
Nuclease free water (H_2O)	-	7 µl	-

Table 8: Thermal cycling conditions for AgPath-ID™ One-Step RT-PCR Reagents

Stage	*Temperature (˚C)*	*Time (minutes:seconds)*	*Cycles*
Reverse transcription	45	10:00	1
Initial denaturation	95	10:00	1
Amplification	95	00:15	45
	60	00:45	

Table 9: Thermal conditions for GoTaq® Green Master Mix:

Stage	*Temperature (˚C)*	*Time (minutes:seconds)*	*Cycles*
Denaturation	95	2:00	1
Denaturation	95	0:30	35
Annealing	(table 4)	0:30	
Extension	72	0:45	
Extension	72	5:00	1
Refrigeration	4	∞	1

Appendix II: Results

Sequence of *Vibrio* sp. AFF. *Splendidus*, from *Cylopterus lumpus* number 104, partial 16S rRNA gene sequence:

CGACAACATTGAATCTTCGGAGGATTTGTTGGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGC
CTTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAATGCCTACGGGCCAAAGAGGGGGATCT
TCGGACCTCTCGCGTCAAGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACG
ATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAG
CAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTT
GTAAAGTACTTTCAGTTGTGAGGAAGGGGGTAACGTTAATAGCGTTATCTCTTGACGTTAGCAACAGAAGAAG
CACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAA
AGCGCATGCAGGTGGTTCATTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAACTGCATTTGAAACTGG
TGAACTAGAGTGCTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATA
CCAGTGGCGAAGGCGGCCCCCTGGACAGACACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGGCCTTGAGCCGTGGCTTTCGGAGCTA
ACGCGTTAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAAGCCA
GCGGAGACGCAGGTGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCGAGTAATGTCGGGAACTCCAGGGA
GACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACA
CACGTGCTACAATGGCGCATACAGAGGGCAGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGTGCGTCGTA
GTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGT
GAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGCTGCAAAAGAAGTGGGTAGTTT
AACCTTTCGGGGAGGACGCTCACCACTTTGTGGTTCATGACTGGGGTGAAGTCGTAAC

Sequence of *Psychrobacterium* sp. AFF *sanguinis*, from *Cyclopterus lumpus* number 223, partial 16S rRNA gene sequence:

CAAGGTCGAGCGGTAACAGGAGAAGCTTGCTTCTCGCTGACGAGCGGCGGACGGGTGAGTAATACTTAGGAAT
CTGCCCAGTAGTGGGGGATAGCACGGGGAAACTCGTATTAATACCGCATACACCCTACGGGGAAAAGGGGGC
GCTTGCGCTCTCGCTATTGGATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGAC
GATCTGTAGCTGGTCTGAGAGGATGATCAGCCACACCGGGACTGAGACACGGCCCGGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGT
TGTAAAGCACTTTAAGCAGTGAAGAAGACTCTATGGTTAATACCCATAGACGATGACATTAGCTGCAGAATAA
GCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTA
AAGCGAGCGTAGGTGGCTTAATAAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATCTGATACTG
TTGGGCTAGAGTAGGTGAGAGGGAGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAAT
ACCGATGGCGAAGGCAGCCTCCTGGCATCATACTGACACTGAGGTTCGAAAGCGTGGGTAGCAAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTAGTCGTTGGGGAACTTGATTCCTTAGTGACGCAGCTA
ACGCAATAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATATCTAGAATCCTG
CAGAGATGCGGGAGTGCCTTCGGGAATTAGAATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGAT
GTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTTTCCTTAGTTACCAGCGGTTAGGCCGGGGACTCTAAGGATA
CTGCCAGTGACAAACTGGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACA
CGTGCTACAATGGTAGGTACAGAGGGCTGCTACACAGCGATGTGATGCGAATCTCAAAAAGCCTATCGTAGTC
CAGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAA
TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGATTGCACCAGAAGTGGGTAGCCTAACT
TTTAGAGGGCCGCTCACCACGGTGTGGTC

Table 10: RNA purity, 260/280 value derived from Nanodrop spectrophotometer, Samples from kidney tissue, liver tissue, medulla oblongata (nerve tissue) and gill tissue.

Fish nr.	Kidney tissue	Liver tissue	Nerve tissue	Gill
101	1,79	2,02	1,82	1,97
102	2	1,99	1,86	2
103	1,98	1,97	1,88	1,97
104	1,97	2,03	1,76	1,95
105	1,93	1,99	1,72	1,9
106	1,92	1,95	1,81	1,95
107	1,93	1,93	1,69	1,93
108	2	1,98	1,81	1,97
109	2,02	1,97	1,86	1,97
110	1,8	1,86	1,81	1,89
111	2,01	2,02	1,88	1,95
112	1,96	1,97	2,01	1,93
113	1,98	1,94	1,78	1,95
114	1,97	1,99	1,75	1,84
115	2,02	1,98	1,74	1,96
116	1,95	1,99	1,94	1,9
117	1,94	1,97	1,72	1,96
118	1,98	2	1,83	1,96
119	1,92	1,96	1,94	1,92
120	1,87	1,99	1,95	1,93
121	1,86	2,03	1,96	1,96
122	1,98	2,03	1,97	1,92
123	2,01	2	1,94	1,95
124	1,96	2	1,96	1,97
125	2,03	1,96	1,91	1,71
201	1,95	2,03	1,95	2,02
202	1,94	2,01	1,89	2
203	1,97	1,8	1,83	2,02
204	2	1,99	1,87	2,01
205	1,98	2,02	1,87	1,99

206	1,98	2	1,87	2,04
207	1,98	2,01	1,95	2,03
208	1,98	2,01	1,88	2,01
209	1,98	1,84	1,91	2,01
210	1,91	2,03	2	1,93
211	2	2,02	1,95	2,02
212	2,01	1,99	1,88	2,01
213	1,96	1,97	1,97	2,02
214	1,97	2,02	1,9	2,02
215	1,98	1,97	1,9	2,02
216	1,96	2,01	1,88	1,97
217	2,02	2,01	1,94	2
218	2	1,95	1,89	2,01
219	1,97	1,97	1,86	2,03
220	2,01	2	1,91	2,02
221	2	2	1,92	2,01
222	1,97	1,99	1,83	2
223	2	2,03	1,85	1,96
224	1,98	1,94	1,88	2
225	2	1,96	1,84	2,01
226	1,99	1,99	1,9	1,99
227	1,95	1,91	1,84	2,02
228	1,97	1,98	1,86	1,98
229	1,95	1,94	1,9	2,02
230	1,96	1,96	1,77	2,02
231	2,03	1,99	1,98	1,95
232	2,03	1,99	1,96	1,99
233	1,88	1,95	2,07	1,94
234	2,02	1,96	2,04	1,99
235	2,02	2,03	1,96	1,93
236	1,98	1,99	1,96	2
237	1,89	2	1,95	1,9
238	2,02	1,96	1,9	1,93

239	1,96	1,94	1,93	1,75
240	2,02	2,01	2,05	1,99
241	1,96	2,02	2,05	1,99
242	2	1,98	2,08	1,98
243	2,01	2	2,07	1,89
244	2	1,96	2,05	2,02
245	1,95	1,93	2,03	2,01
246	2,02	1,99	2	1,96
247	1,99	2,01	1,97	1,97
248	2	2	1,97	2
249	1,98	2,03	1,98	1,9
250	2,02	1,96	1,9	1,99
251	2,01	1,98	1,87	1,97
252	2,03	1,97	1,97	1,9
253	2,02	2	1,98	1,99
254	2	1,92	1,87	1,91
255	1,96	2	2,02	1,98
256	1,96	2,03	1,94	1,97
257	2,05	1,97	1,91	1,94
258	2,02	1,99	1,95	1,92
259	1,99	2,01	1,89	1,96
260	1,98	2,03	2,01	1,93

Table 11: DNA purity. 260/280 value measured with Nanodrop spectrophotometer. Samples from muscle and caecum tissue.

Fish number	Muscle tissue	Caecum tissue
101	2,08	1,96
102	2,14	1,99
103	2,09	2,02
104	2,11	2,04
105	2,13	2,06
106	2,17	2,06
107	2,11	2,02
108	2,15	2,03
109	2,11	1,96
110	2,1	2,05
111	2,15	2,01
112	2,1	2
113	2,1	2
114	2,18	2,18
115	2,08	1,99
116	2,13	2,02
117	2,13	1,99
118	2,14	2,01
119	2,03	2,1
120	2,07	2,01
121	2,01	2,05
122	2,04	2,04
123	2,16	2,07
124	2,06	2,03
125	1,99	2,02
201	1,99	2,01
202	2,04	2,01
203	2,03	1,97
204	2,01	1,95
205	1,96	1,97

206	1,96	1,99
207	2,1	1,95
208	1,97	1,97
209	2,03	1,97
210	1,94	1,99
211	1,9	1,95
212	1,98	1,97
213	2,09	2,02
214	2,1	1,98
215	2,05	2,02
216	2,02	2
217	1,97	1,98
218	2,04	2,03
219	2,09	2,05
220	2,08	1,99
221	2,16	1,98
222	2,17	1,96
223	2,1	2,03
224	2,09	2
225	2,1	2,07
226	1,98	2,01
227	1,98	2,02
228	2,01	2
229	1,98	2,06
230	2,08	2
231	2,07	2
232	2,04	1,99
233	2,09	2
234	2,06	2,05
235	2,09	1,95
236	2,04	2
237	2,04	2,01
238	2,07	2,05

239	2,04	1,96
240	2,11	1,94
241	2,05	2
242	2,19	2,02
243	2,04	2,01
244	2,02	2,02
245	2,1	2,06
246	2,04	2,01
247	2,02	2,02
248	2,08	2
249	2,07	2,04
250	1,98	2,02
251	2,08	2,05
252	2,04	2,05
253	2,01	2,04
254	1,79	2,02
255	2,12	2
256	2,06	2,02
257	2,04	2
258	2,1	1,98
259	2,02	1,97
260	2,09	2,05

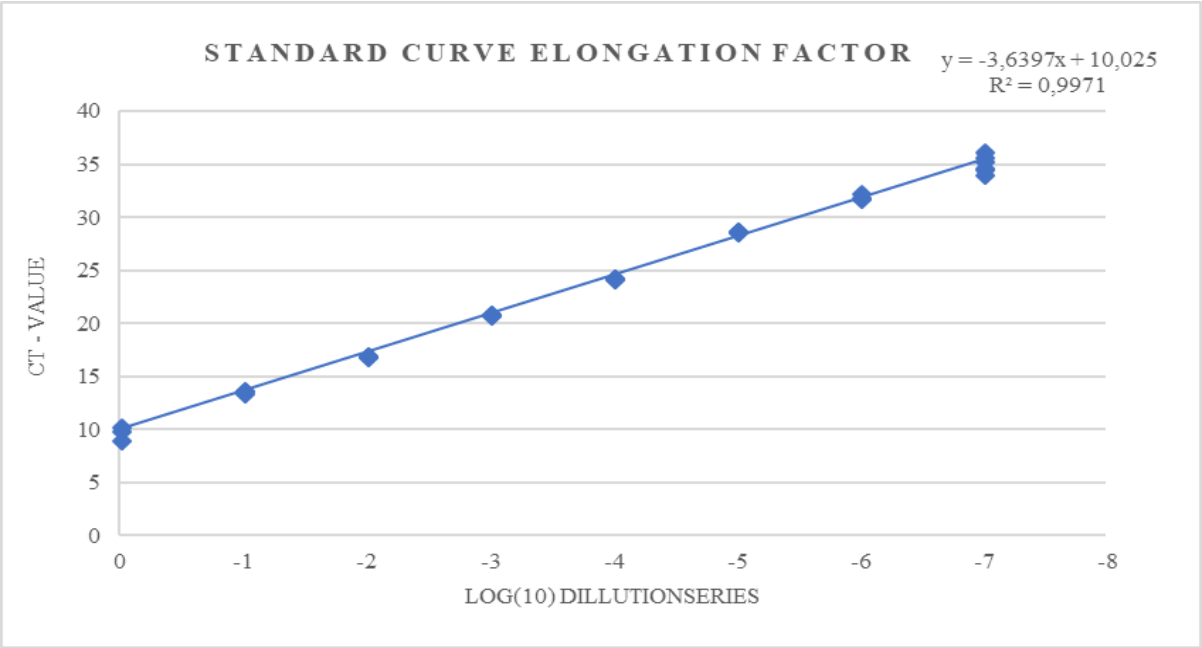

Figure 9: Standard curve Elongation factor α1.

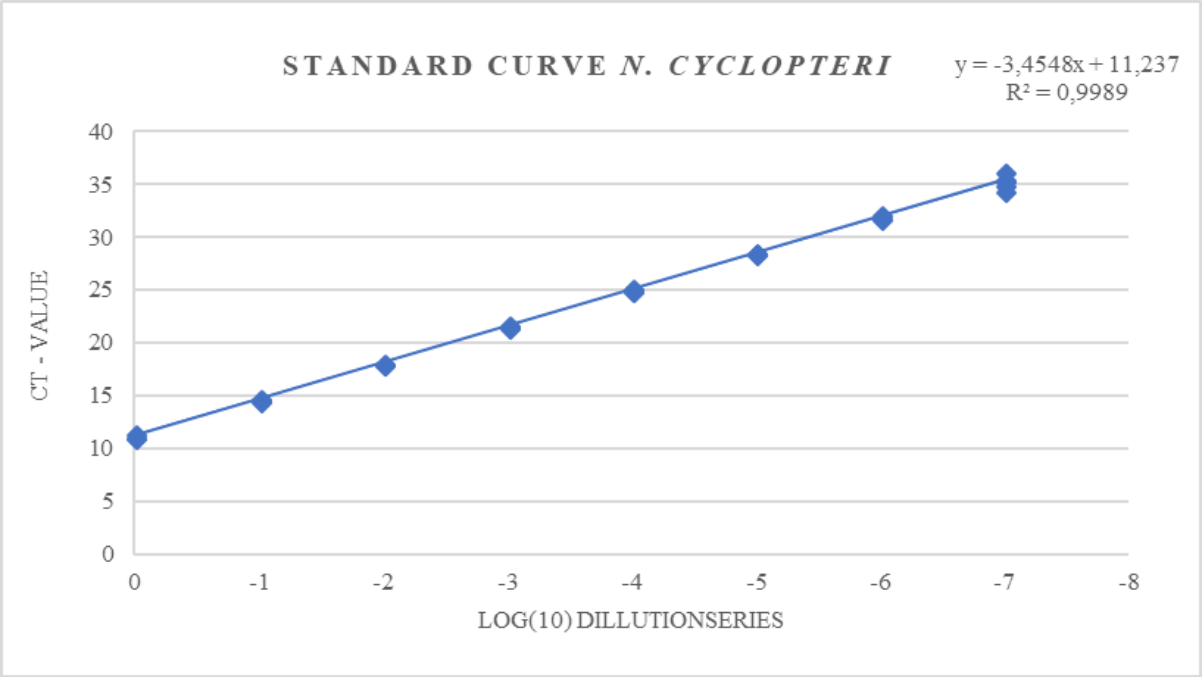

Figure 10: Standard curve *Nucleospora cyclopteri* assay. Ct value = 35.4 was the detection limit from the dilution series.

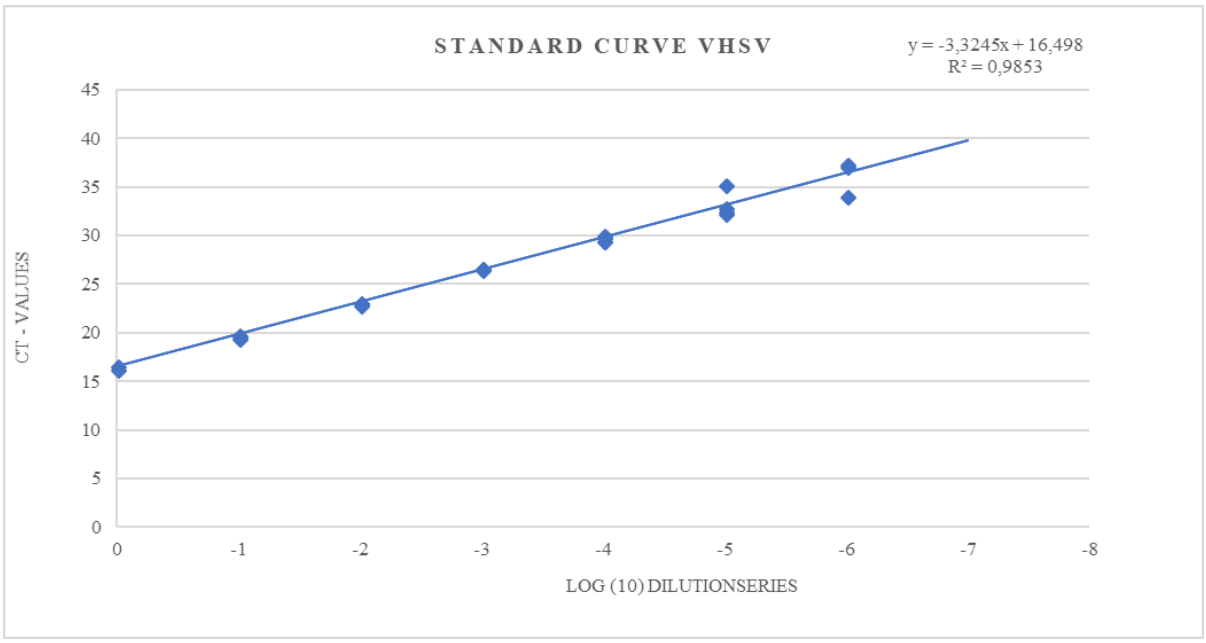

Figure 11: Standard curve for Viral Haemorrhagic Septicaemia Virus, VHSV assay used for qPCR.

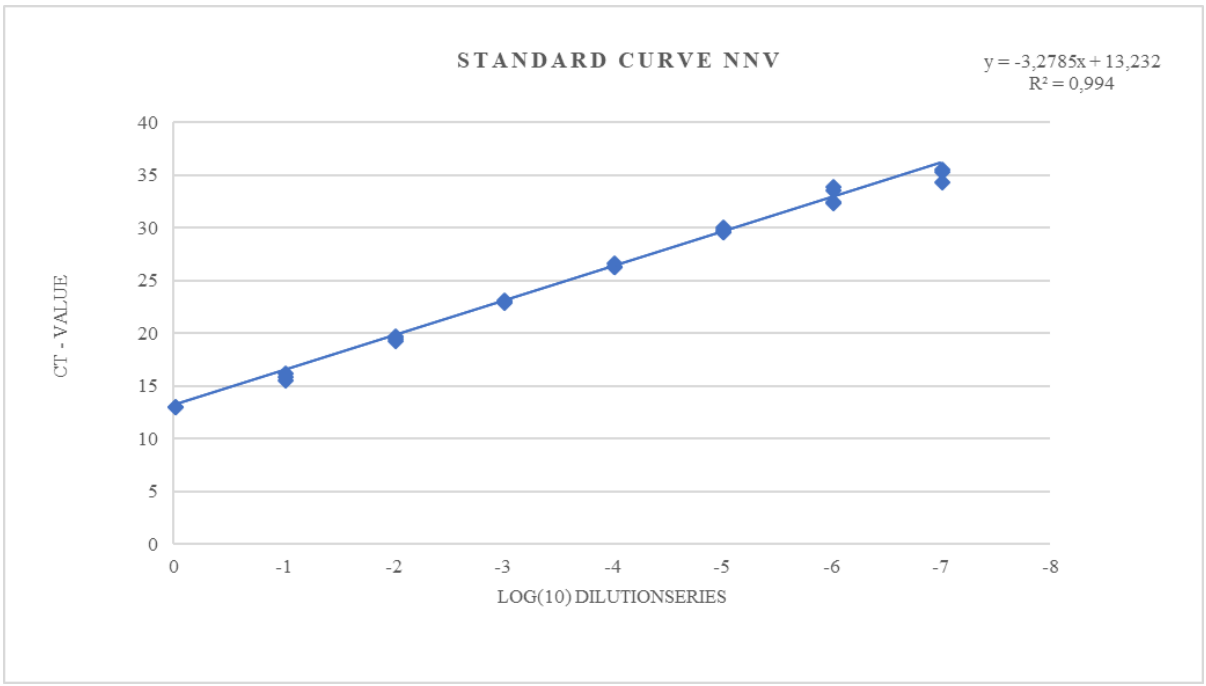

Figure 12: Standard curve for Neverous Necrosis virus, NNV assay used for qPCR

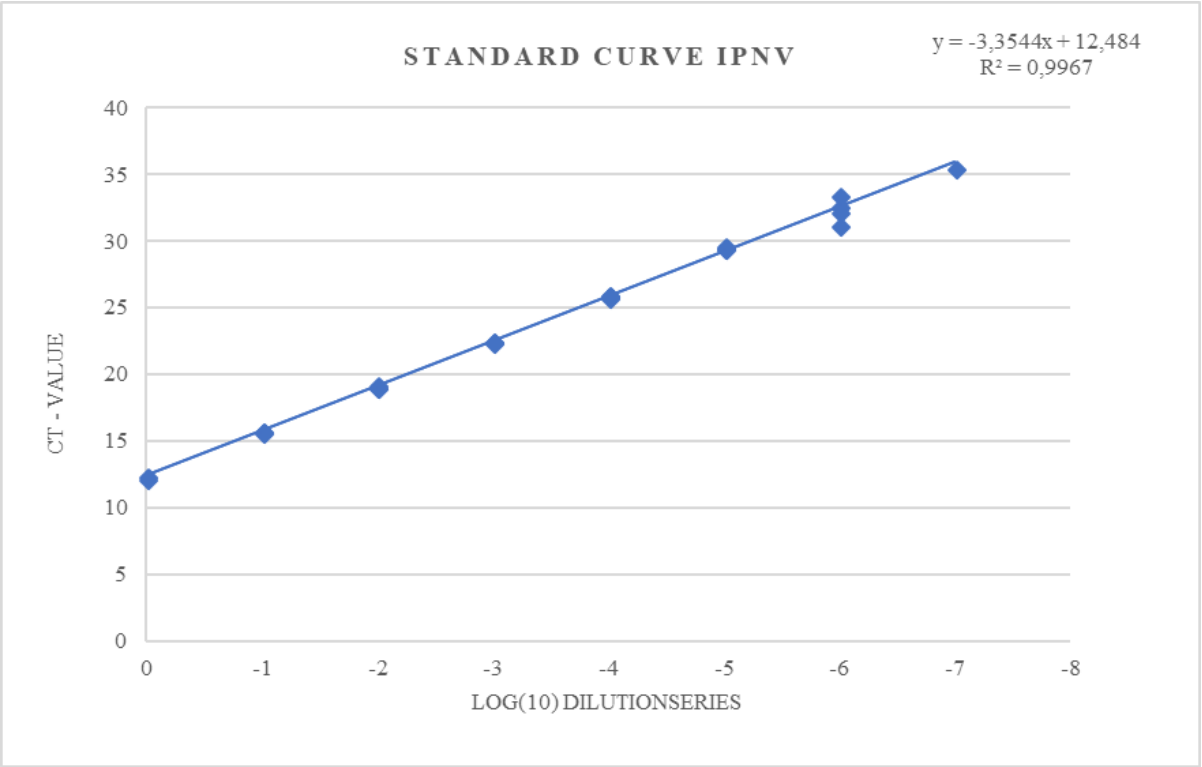

Figure 13: Standard curve for Infectious Pancreatic Necrosis Virus, IPNV, assay used for qPCR

Table 12: NE value calculated from the Ct value for Elongation factor 1α (RK Elfac) and *Nucleospora cyclopteri* 16S (NC16S). ND=Not detected, NE was set to 0 when NC16S was not detected.

Fish number	Ct-value		NE
	NC16S	RK Elfac.	
101	33,367	10,442	1,23966E-07
102	ND	9,853	0
103	36,195	11,339	1,88284E-08
104	ND	8,866	0
105	36,390	10,033	1,65398E-08
106	ND	7,472	0
107	ND	11,287	0
108	ND	8,681	0
109	20,918	7,740	0,000118686
110	37,154	10,193	1,12366E-08
111	10,783	10,565	0,607501224
112	26,570	10,167	1,27634E-05
113	10,582	11,081	0,962721843
114	14,230	11,405	0,104003725
115	28,969	10,362	2,9195E-06
116	30,730	9,574	5,4851E-07
117	32,198	8,548	1,0774E-07
118	5,888	9,439	7,771960098
119	ND	9,665	0
120	ND	13,348	7,64788E-08
121	ND	12,318	4,75602E-08
122	26,811	9,518	7,209E-06
123	10,313	9,052	0,318917242
124	30,733	13,723	7,56529E-06
125	30,189	9,798	9,06683E-07
201	ND	9,315	0
202	ND	10,072	0
203	ND	10,211	0

204	ND	8,608	0
205	ND	8,241	0
206	8,688	8,974	0,896141683
207	21,162	7,147	6,93199E-05
208	27,011	8,728	3,82722E-06
209	27,585	9,222	3,56963E-06
210	26,605	9,032	6,08022E-06
211	19,694	9,082	0,000627266
212	20,424	9,343	0,000454647
213	31,678	9,459	2,71243E-07
214	ND	8,897	0
215	34,420	9,169	3,6317E-08
216	ND	8,682	0
217	18,308	9,232	0,001735868
218	ND	7,416	0
219	35,408	7,948	8,6842E-09
220	ND	8,708	0
221	ND	9,024	0
222	ND	9,595	0
223	24,471	9,198	2,79889E-05
224	ND	9,858	0
225	ND	9,606	0
226	ND	8,937	0
227	ND	8,762	0
228	ND	8,650	0
229	18,230	8,695	0,001302338
230	10,693	8,852	0,21818459
231	26,233	8,689	6,27019E-06
232	10,010	9,980	0,702150686
233	20,855	8,591	0,000212105
234	20,417	8,498	0,000267699
235	ND	8,665	0
236	ND	9,250	0

237	ND	8,839	0
238	25,681	8,287	7,02062E-06
239	27,269	9,347	4,76959E-06
240	ND	8,921	0
241	23,737	9,241	4,68957E-05
242	26,147	7,056	2,36161E-06
243	23,857	8,427	2,58731E-05
244	21,207	8,862	0,000199144
245	ND	6,662	0
246	ND	8,477	0
247	ND	8,749	0
248	29,250	9,175	1,14236E-06
249	14,443	9,251	0,023078406
250	ND	8,987	0
251	10,712	8,387	0,160490837
252	23,476	7,504	1,85916E-05
253	15,003	8,698	0,011200019
254	33,839	8,509	3,52171E-08
255	14,475	6,756	0,004661036
256	27,719	8,683	2,32161E-06
257	18,946	6,690	0,000227244
258	18,794	7,275	0,000364174
259	ND	8,420	0
260	ND	8,528	0

Table 13: Coccidia band score as a relative measure of quantity

Fish number	Coccidia	Band thickness
101	Yes	2
102	Yes	2
103	Yes	2
104	Yes	2
105	Yes	2
106	Yes	2
107	Yes	2
108	Yes	2
109	Yes	3
110	Yes	2
111	Yes	2
112	Yes	3
113	Yes	3
114	Yes	2
115	Yes	3
116	Yes	3
117	Yes	3
118	Yes	1
119	Yes	2
120	Yes	3
121	Yes	3
122	Yes	3
123	Yes	3
124	Yes	3
125	Yes	3
201	Yes	3
202	Yes	2
203	Yes	3
204	Yes	3
205	Yes	3

206	Yes	2
207	Yes	3
208	Yes	1
209	Yes	3
210	Yes	3
211	Yes	3
212	Yes	1
213	Yes	3
214	Yes	3
215	Yes	2
216	Yes	3
217	Yes	3
218	Yes	3
219	Yes	1
220	Yes	3
221	Yes	1
222	Yes	1
223	Yes	3
224	Yes	1
225	Yes	3
226	Yes	3
227	Yes	2
228	Yes	2
229	No	0
230	Yes	2
231	Yes	3
232	Yes	3
233	Yes	2
234	Yes	3
235	Yes	3
236	Yes	3
237	Yes	3
238	Yes	3

239	Yes	3
240	Yes	2
241	Yes	3
242	Yes	2
243	Yes	2
244	Yes	2
245	Yes	1
246	Yes	3
247	Yes	3
248	Yes	3
249	Yes	2
250	Yes	2
251	Yes	2
252	Yes	2
253	Yes	2
254	Yes	3
255	Yes	1
256	Yes	3
257	Yes	2
258	Yes	3
259	Yes	3
260	Yes	3

Table 14: Sample size data overview. F= Female, M=Male

Fish number	Sex	Length (cm)	Weight (g)
101	F	43	1959
102	F	44,5	3015
103	F	47	2368
104	F	47,7	3330
105	F	42,8	2145
106	F	46,2	3474
107	F	42,2	2360
108	F	41,2	2097
109	F	48	3603
110	F	41	1600
111	F	51,6	3840
112	F	43	2184
113	F	46	3580
114	F	46,5	2588
115	F	36	1800
116	F	41	1785
117	F	40,5	2111
118	F	41	2290
119	F	41	2102
120	F	41	1792
121	F	43	2809
122	F	48,5	3323
123	F	48	3140
124	F	45	2724
125	F	47	3065
201	F	46	2714
202	M	35,7	1728
203	F	47,8	4623
204	F	47,6	3337
205	F	42	2280
206	F	46,5	3009

207	F	40,5	2301
208	M	36,2	1622
209	F	40,6	2209
210	F	48	5196
211	F	49,5	4116
212	F	47,2	2920
213	F	46,2	3790
214	F	45,5	3972
215	F	44,5	3586
216	M	38,3	1882
217	M	33,6	1221
218	F	43,5	2317
219	F	40,7	2751
220	F	49,3	3921
221	M	41,8	2101
222	M	36,1	1472
223	M	34	1335
224	M	30,6	875
225	M	31,1	936
226	M	34,5	1618
227	M	34	1494
228	F	44,5	2378
229	F	42,5	2567
230	F	41,5	2716
231	F	44,8	3616
232	F	44,5	4303
233	F	46,2	3442
234	F	49	4882
235	F	45,4	3090
236	F	47	3684
237	F	46,5	3327
238	F	41	2022
239	F	42	2700

240	F	41	2578
241	F	53,3	4979
242	F	48,1	4150
243	F	45,1	3068
244	F	50	4284
245	F	45,2	3094
246	F	48,3	3052
247	F	42,5	3164
248	F	45,3	3410
249	F	44,2	2555
250	F	44,5	3557
251	F	39	2099
252	F	45,3	2952
253	F	50,9	4174
254	F	43	2516
255	F	45,5	3738
256	F	40,8	2834
257	F	46,7	3230
258	F	44,5	2369
259	F	42,5	2759
260	F	44,5	3136