Diagnostic investigation into summer mortality events of farmed Chinook salmon (*Oncorhynchus tshawytscha*) in New Zealand.

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Abstract

Salmon farming is the second highest value aquaculture species in New Zealand and produces approximately 88% of the global market of farmed Chinook salmon, *Oncorhynchus tshawytscha* (Tucker, 2014). New Zealand salmon are free of many significant diseases affecting salmonids globally (Diggles, 2016). Therefore, disease is one of the greatest threats to this New Zealand aquaculture species. Biosecurity, early detection, and characterisation of new or emerging diseases is vital for management and sustainability of the aquaculture industry.

Elevated mortalities termed 'summer mortalities' with no cause identified have occurred in certain farmed Chinook salmon populations in the Marlborough Sounds since 2012. This study identified two potential bacterial pathogens involved in summer mortalities; New Zealand rickettsia-like organism (NZ-RLO) and *Tenacibaculum maritimum.* Distribution of NZ-RLO and *T. maritimum* within farmed Chinook salmon populations, phylogenetic analysis of these pathogens and the pathogenicity of two strains of NZ-RLO were assessed to provide an understanding of the role of NZ-RLO and *T. maritimum* in summer mortalities. Additionally, new diagnostic tests were developed to efficiently detect these pathogens.

Identification of NZ-RLO in the summer mortalities was the first detection in New Zealand. *Tenacibaculum maritimum* had been reported in New Zealand previously, however it had not been associated with mortalities. This study confirmed three strains of NZ-RLO with restricted geographical distribution. Two strains of NZ-RLO were found exclusively in areas where fish experienced summer mortalities and were associated with clinical signs of disease, indicating certain strains of NZ-RLO were likely primary pathogens. Widespread distribution of *T. maritimum* was detected within farmed salmon and no association was found with *T. maritimum* and clinical signs of disease in areas experiencing summer mortalities, indicating *T. maritimum* was unlikely to be a primary pathogen.

This study proves that laboratory exposure of salmon to two strains of NZ-RLO caused disease and mortalities however, the differences between the two strains suggest NZ-

RLO2 may be more pathogenic. This study suggests NZ-RLOs are likely to be involved in summer mortalities as primary pathogens however, the interaction between the pathogens and environment is likely to have amplified the levels of mortalities during these events.

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Chapter 1 : General Introduction

A plaque in the Dunedin botanic gardens to commemorate the first acclimatised fresh-water game fish (brown trout, *Salmo trutta*) to be liberated in Otago, New Zealand 1869.

1.1 Global seafood production

Fisheries and aquaculture are of critical importance for food, nutrition and employment of millions of people worldwide. In 2016, global aquaculture production reached 110.2 million tonnes, of which finfish comprised 54.1 million tonnes (FAO, 2018). This growth has led to aquaculture surpassing wild fisheries in the production of finfish available for human consumption (FAO, 2018). As the aquaculture sector grows, so too do threats from external factors such as adverse environmental events and disease. For example, in 2017 salmon aquaculture in Norway had an average increase in mortality of 3% in the years 2015 and 2016. This increase in mortality was mainly due to disease, but uncharacteristic storm events also played a major role (Moe et al., 2017).

1.2 New Zealand seafood sector

While New Zealand's seafood production is small at less than half a percent of the world's seafood supply (Plant and Food, 2013) it remains a significant industry for New Zealand with exports totalling 1.8 billion NZD in 2017 (Seafood New Zealand, n.d). Aquaculture plays a major role in this sector and in 2016 generated over 500 million NZD in revenue and employed over 3000 people and has a target of reaching 1 billion NZD in revenue by 2025 (Aquaculture New Zealand, 2011a). The three key aquaculture species produced in New Zealand are Greenshell™ mussels (*Perna canaliculus*), Chinook salmon (*Oncorhynchus tshawytscha*) and Pacific oysters (*Crassostrea gigas*) at 73%, 21% and 6% value respectively in 2011 (Aquaculture New Zealand, 2011b). As this sector seeks to increase its value, financial and social impacts caused by aquatic diseases will be a risk for New Zealand as seen in other parts of the world where diseases such as infectious salmon anaemia have either devastated salmon industries, cost millions of dollars to eradicate or resulted in millions of dollars of ongoing annual losses (Spickler, 2011). Chinook salmon are currently the only commercially viable salmon farmed in New Zealand and will be the focus of this thesis.

1.3 History of salmonids in New Zealand

Salmon are teleost fish, a group of ray-finned fish, in the family Salmonidae which also includes trout and char. Seven species of salmonids are present in New Zealand, all of which are non-native. These species include the following: brown trout (*Salmo trutta),* rainbow trout (*Oncorhynchus mykiss*), brook char (*Salvelinus fontinalis*), Lake char (*Salvelinus namaycush*), Chinook salmon, sockeye salmon (*Oncorhynchus nerka*)*, and* Atlantic salmon (*Salmo salar*). Salmonids were initially introduced into New Zealand by the English settlers in the mid to late 1800s to populate the waterways with sporting fish that were present in England. Subsequent salmonid introductions were overseen by the government, mainly for commercial purposes.

Brown and rainbow trout introductions were very successful. Brown trout ova were sourced from Tasmania in the mid to late 1800s and by 1900 these fish were well established and are currently found in all parts of New Zealand south of the

Coromandel Peninsula (Townsend, 1996). Rainbow trout ova were imported from California in the 1880s (Scott, Hewitson, & Fraser, 1978) and are now distributed from the top of the North Island to the bottom of the South Island (Scott and Poynter, 1991). Trout are prized sports fish which are targeted in many rivers around New Zealand. To maintain wild brown and rainbow trout stocks, private hatcheries and acclimatisation societies use captive adult fish to generate spawn. These eggs are then hatched and the juvenile fish are raised in captivity during their early life before being released to replenish lake and river populations for recreational fishing. Trout are not farmed commercially and it is illegal to sell trout or trout flesh in New Zealand.

Two species of char are present in New Zealand, brook char, also known as brook trout, and Lake char, also known as Mackinaw. Brook char ova were introduced from the Atlantic Coast of North America in the 1880s (McDowall, 1994). Lake char ova were imported on one occasion from Canada in 1906 (McDowall, 1994). These two species are self-sustaining and relatively small natural populations exist in New Zealand with brook char being present in both the North and South Islands and Lake char being present only in the South Island. These species are not actively recreationally fished or commercially farmed in New Zealand.

From the late 1800s, three salmon species were introduced into New Zealand, Chinook salmon became well established whereas sockeye and Atlantic salmon are present but in smaller populations with restricted distributions. Salmon farming was first established in New Zealand in the 1970s and attempts to farm all three salmon species have been made. However, farming of neither Atlantic nor sockeye salmon was successful and Chinook salmon is the only species currently farmed in New Zealand.

Chinook salmon, also known as Quinnat or King salmon, are the largest of the Pacific salmon. Pacific salmon have a native range that includes waters draining into the Pacific Ocean. Ova from this species were introduced to New Zealand from California on several occasions from the 1870s to the early 1900s by the government and acclimatisation societies (McDowall, 1994). The origin of these ova were the Baird Hatchery and Battle Creek on the McCloud River in California, a tributary of the Sacramento (McDowall, 1994). Initial introductions into New Zealand by the Hawkes

Bay, Auckland, North Canterbury and Southland acclimatisation societies with support from the government (McDowall, 1994) did not result in sustaining populations. This was likely due to the release of small batches of fish in many different river systems, some of which were unsuitable. The most successful introductions were in the early 1900s by the government under the direction of Mr Lake Falconer Ayson. These efforts concentrated on larger batches of fish in one river system, the Waitaki, and between 1901 and 1907 2.1 million ova were introduced (McDowall, 1994). Additionally, a hatchery was set up on the Hakataramea River, a tributary of the Waitaki River. Chinook salmon then naturally spread north and established runs in rivers along the East Coast of the South Island (McDowall, 1994) (Figure 1.1). In later years releases were made into other river systems including the Clutha, Wairau and Hokitika (McDowall, 1994). Wild Chinook salmon runs now occur in rivers from the Waiau to the Clutha and into a few West Coast rivers in the South Island (McDowall, 1994). As well as these South Island Rivers, Chinook salmon are also found in the Rangitikei River in the North Island, although no significant runs have been observed (Hicks and Watson, 1983). The majority of Chinook salmon populations remain anadromous meaning that they are born in freshwater, migrate to sea to grow and mature, then return to freshwater to spawn. Salmon generally return to the stream of their birth, however straying from these locations does occur and Chinook salmon in California have been recorded as having 10% to 13% straying rates (Snyder, 1931; Sholes and Hallock, 1979). Straying has been hypothesised to be most evident in populations of fish that spawn in unstable river systems or in areas where there is a high degree of similarity between rivers (Quinn, 1984). These features can be recognised in the East Coast Rivers of the South Island and Chinook salmon straying is likely to account for the wider distribution of these salmon in New Zealand than just the rivers in which ova were released (Quinn, Kinnison, & Unwin, 2001). As a result of the original successful establishment of this species in the Waitaki River system, the successful implementation of hatchery rearing and subsequent commercialisation of this species, there have been no further imports of Chinook salmon ova into New Zealand since 1907. New Zealand remains one of the only places in the world where this species has become successfully established outside of their natural range (McDowall, 1994).

Sockeye salmon is also a Pacific salmon. This species was introduced into New Zealand from a single introduction in 1902 from British Columbia, Canada. In British

Columbia, three stocks of sockeye salmon are recognised; sea-going stock, lake maturing progeny from sea-going parents and kokanee which live permanently in fresh water. New Zealand sockeye salmon originated from sea-going stock (Scott, 1984). This single introduction was of 500,000 ova that when hatched were released into the Hakataramea River and Lake Ohau (McDowall, 1994). Sockeye salmon populations in New Zealand did not establish migratory populations, despite the fact they originated from sea-going stock, and exist solely in landlocked populations within the Waitaki catchment including Lake Ohau and Lake Benmore (Graynoth, 1987). It has been suggested the reason these fish did not develop sea-going runs was because the fish that migrated to sea became disoriented and lost in the South Pacific Ocean (Graynoth, 1987). Construction of dams on the Waitaki after the introduction of these fish eliminated future possibilities of migration to sea.

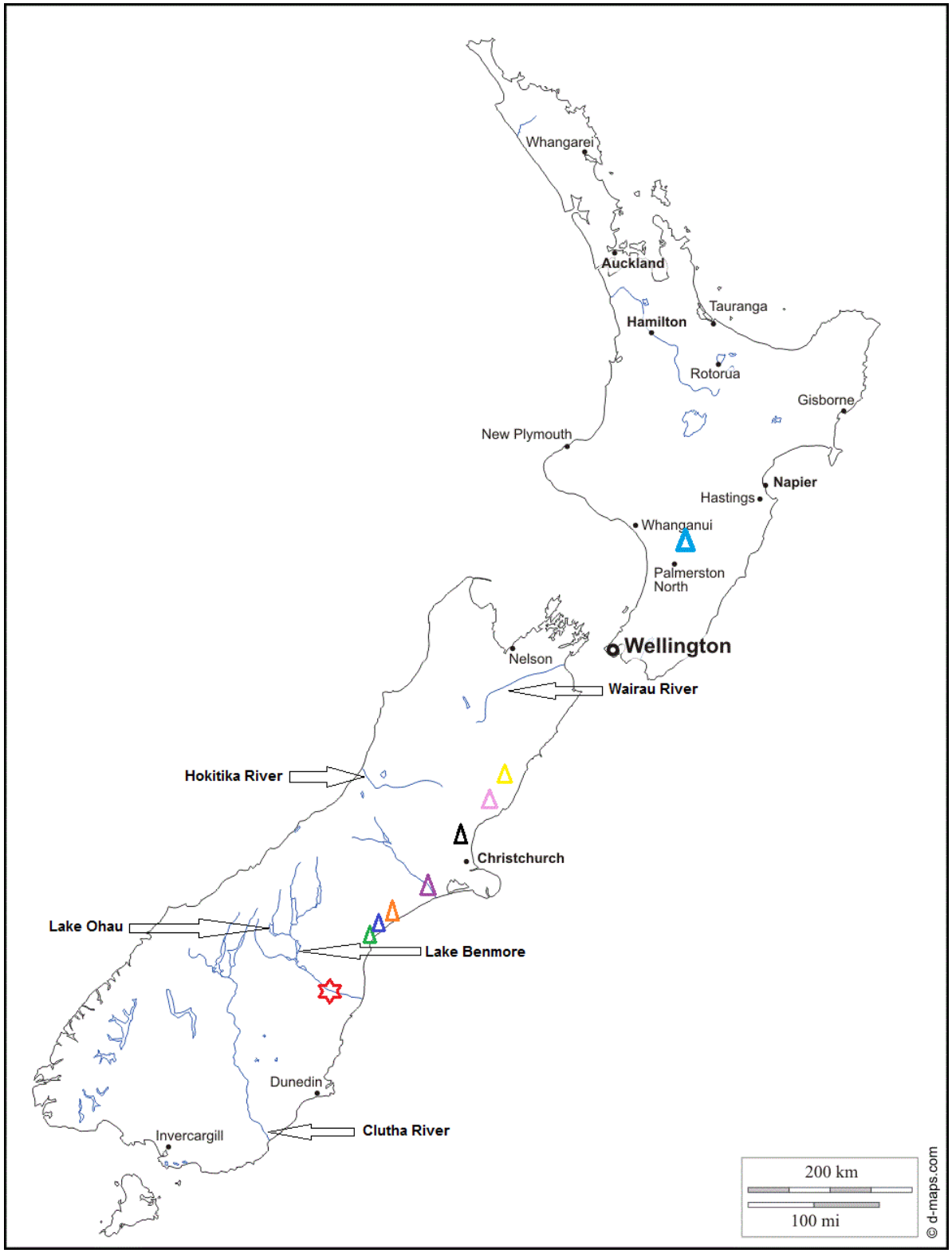

Figure 1.1. Map of New Zealand indicating rivers where wild Chinook salmon are found. Waitaki River (Star) site of successful introduction of Chinook salmon in 1901-1907. Rivers that Chinook salmon runs naturally extended to; Opihi (Green triangle), Rangitata (Blue triangle), Ashburton (Orange triangle), Rakaia (Purple triangle), Waimakariri (Black triangle), Hurinui (Pink triangle), Waiau (Yellow triangle), Rangitikei (Light blue triangle).

Atlantic salmon are the most common commercially produced species in other parts of the world with over 2 million tonnes produced for consumption in 2016 (FAO, 2018). Atlantic salmon was the preferred species for introduction into New Zealand and a concerted effort that included 24 shipments totalling five million ova from Canada, England, Scotland and Germany occurred from approximately 1864 to 1911 (McDowall, 1994). However, the establishment of Atlantic salmon in New Zealand was not successful. Early introductions saw ova that were live on arrival, hatched and the resulting fry scattered throughout a number of rivers not to be seen again. From 1898 onwards, a more controlled programme with larger numbers of introduced ova was carried out under the direction of Mr Lake Falconer Ayson. This introduction was into the Waiau River and a hatchery was set up on the Upukerora River. These salmon became voluntarily landlocked and the fish that did go to sea did not appear to return. To date, Atlantic salmon in New Zealand have a restricted distribution of landlocked populations in Southland's upper Waiau River and depend on the maintenance of hatchery stocks for survival (McDowall, 1994). Introductions of Atlantic salmon into Australia were carried out at the same time as New Zealand. These were also unsuccessful with no migratory populations establishing and only small landlocked populations in New South Wales maintained through hatchery rearing. However, these hatchery stocks were used in the 1980s to create the current successful Tasmanian Atlantic salmon industry (Llewellyn, 2015). It is unknown why the introduction of Atlantic salmon into New Zealand was unsuccessful. It is possible that the salmon were outcompeted by trout previously established in the river systems, which may have reduced the chances of acclimatisation (McDowall, 1994). Additionally, Atlantic salmon failed to successfully migrate to sea and back which also contributed to the failure of this species to become acclimatised. Landlocked fish could not take advantage of the nutrients that would be available in the sea further impacting their ability to thrive. The reason the fish did not return is uncertain, but they may have become disorientated in the Southern Hemisphere or potentially, the smaller fish may have been caught by predators in the Pacific Ocean not present in the Atlantic Ocean.

1.4 New Zealand Chinook salmon aquaculture

The first successful commercial Chinook salmon farm was established in 1976. This was an ocean farmed venture based in Pupu Springs, Takaka (Figure 1.2) (Knowles,

1983) in which juvenile fish were reared in a hatchery and released into the ocean to mature. Between one and four years later, these fish returned from the ocean to spawn and were harvested or used as brood-stock on entry to the river systems. Due to the success of this operation, sea-pen farming of Chinook salmon was attempted.

In 1983, the first sea-pen farm was established in Big Glory Bay, Stewart Island. Subsequently, a trial to assess the potential for salmon farming in Elie Bay in the Marlborough Sounds was performed at the end of 1984. Elie Bay was found to be too warm for the salmon during the summer months with temperatures exceeding 16°C. In search of cooler waters, the trial was then moved to Ruakaka Bay, again in the Marlborough Sounds which was subsequently found to be an appropriate site for salmon farming (Figure 1.3).

Over the following 40 years, salmon farming in New Zealand has continued to grow. In 2016 there were four commercial hatcheries, seven freshwater grow-out facilities and nine sea-pen farms in operation around the South and Stewart Island.

The most recent estimates are that New Zealand salmon production was worth 132.7 million NZD in 2011. This revenue was split approximately evenly between domestic and export revenue. In 2012, New Zealand salmon was exported to 30 countries; Japan, USA, Australia, Hong Kong, and Canada being the top five (Aquaculture New Zealand, 2012). New Zealand is the largest producer of farmed Chinook salmon worldwide and accounts for approximately 88% of the world market (Tucker, 2014). Chile is the only other country to have a significant Chinook salmon farming industry (FAO, 2010).

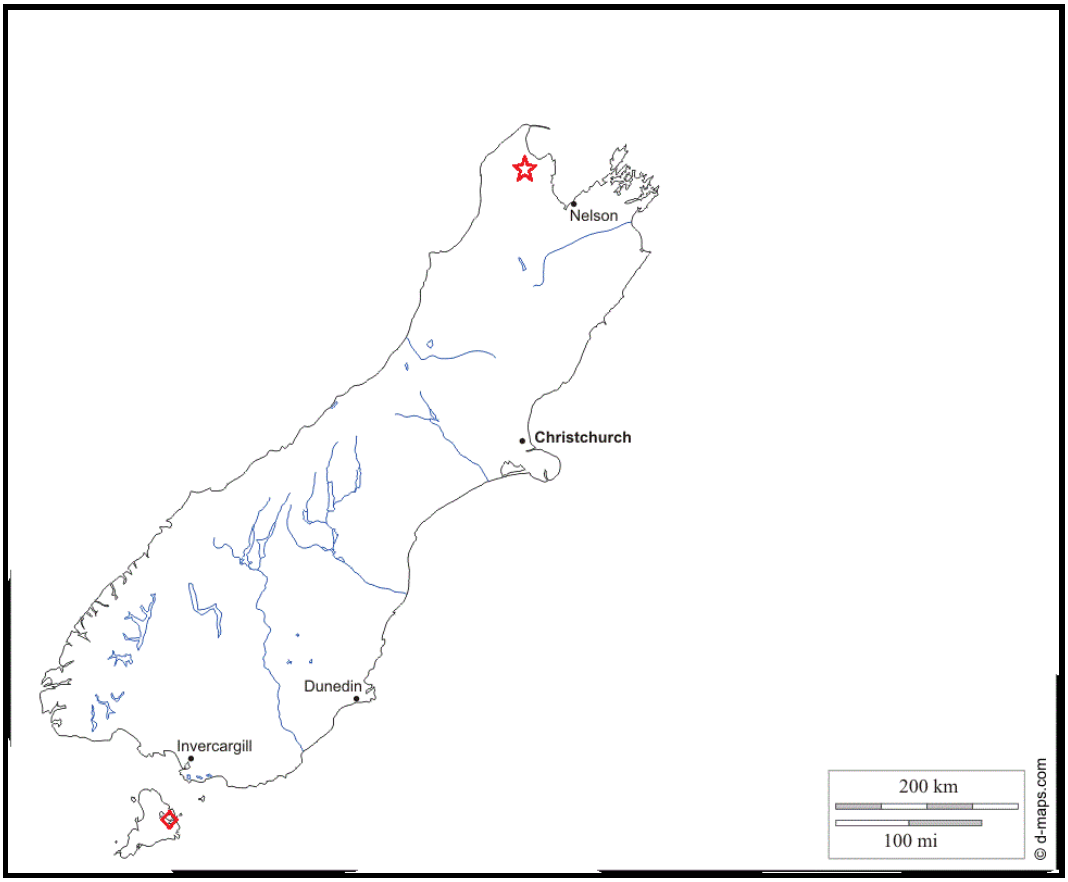

Figure 1.2. Locations of first salmon farming sites in South Island, Pupu Springs (red star) and Big Glory Bay (red triangle).

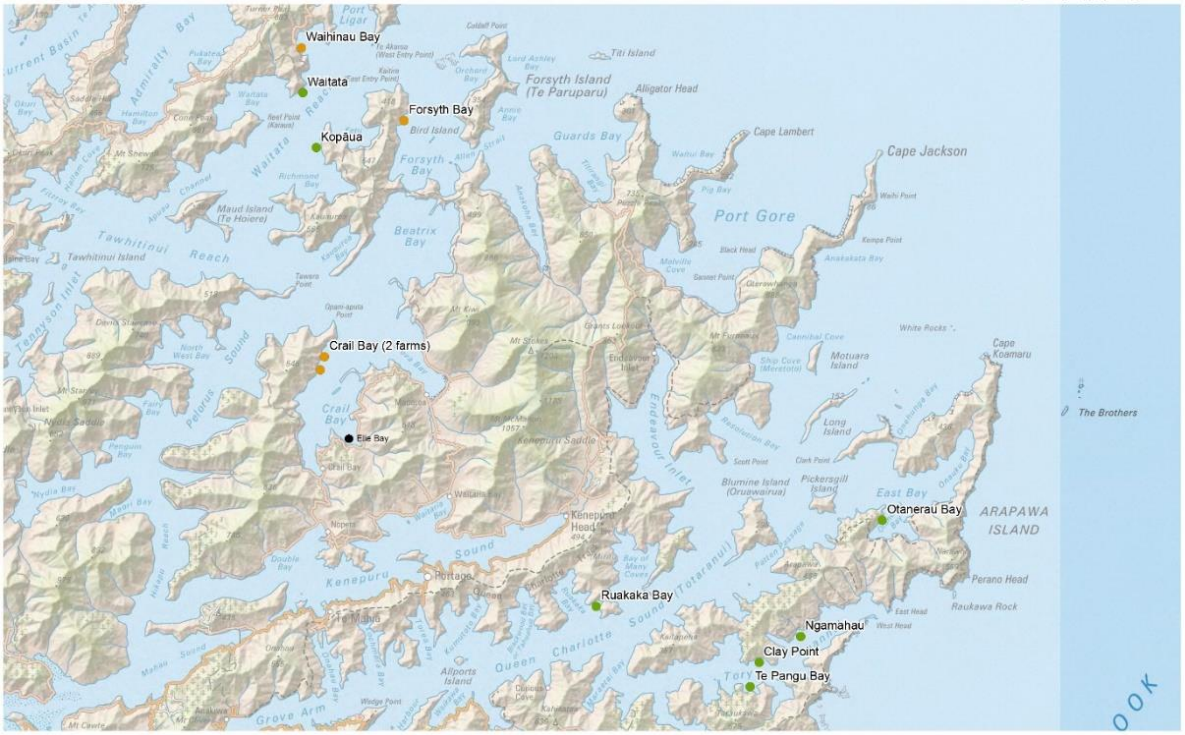

Figure 1.3. Marlborough sounds sea-pen trial sites. Elie Bay (Black) and Ruakaka Bay (green). Orange and Green circles showing current licenced sites of New Zealand King Salmon Company (as at 2016).

1.4.1 Chinook salmon life-cycle in New Zealand – natural and farmed

The Chinook salmon life-cycle begins with ova hatching in freshwater. The fish then develop from alevin to fry to fingerling (also known as parr), and then to smolt. Smolt go through a behavioural, morphological and physiological transformation to prepare for life in seawater, a process referred to as smoltification. Smoltification is critical for the successful movement of fish from freshwater into seawater. Due to the significant changes occurring in these fish, smoltification is a time in which fish are expected to be especially vulnerable to disease. Once in seawater, salmon remain at sea until they are mature and ready to spawn. Before spawning, mature Chinook salmon will migrate up rivers, usually the same rivers in which they were spawned. Chinook salmon do not feed when they are migrating and die after the spawning process. Some smolt do not migrate to sea and spend the rest of their life in freshwater lakes, migrating back to the rivers to spawn as their anadromous counterparts.

Chinook salmon aquaculture in New Zealand has both freshwater and sea-pen rearing operations. Farmed sea-pen culture begins with artificial spawning of freshwater female and male brood-stock. The fertilised ova then hatch in freshwater and are grown until the smolt stage (~200 days old). Smolt are then transferred into sea-pens to grow and reach maturity for harvest at approximately three years of age.

The process is the same for freshwater Chinook salmon culture however smolt are not transferred to saltwater and instead are raised to maturity in freshwater.

1.4.2 Diseases status of Chinook salmon in New Zealand

Passive and active surveillance programmes occur in New Zealand salmon to detect disease as early as possible.

Passive surveillance has occurred prior to the commercialisation of salmonid farming in New Zealand and relies on reporting from farmers, vets and the public of unhealthy fish or fish with unusual elevated mortalities. Once reported, unhealthy fish are sent to either government or commercial laboratories for disease testing.

Active surveillance of farmed Chinook salmon exported to Australia has been undertaken since 2000. As this programme is voluntary for farmers wanting to export Chinook salmon to Australia, it does not cover all salmon farms in New Zealand, nor does it include non-commercial salmon and trout hatcheries. This programme involves annual farm inspections and collection of samples as well as collection of samples at harvest from farms participating in the scheme. These samples are collected for bacteriology, virology, and specific molecular tests. Bacteriology is carried out to detect *Yersinia ruckeri* and *Aeromonas salmonicida*. Additionally, any bacteria that are cultured in dominant or pure growth from these samples will also be identified. Virology is used to determine the presence of any virus that produces a cytopathic effect on the following cell lines: Chinook salmon embryo (CHSE-214, ECACC 91041114) and Epithelioma papulosum cyprini (EPC, ECACC 93120820). Molecular tests, i.e. polymerase chain reaction (PCR), are used to detect the myxosporean parasite *Myxobolus cerebralis* and the intra-cellular bacteria *Renibacterium salmoninarum*.

Through these surveillance programmes, numerous potential pathogens have been detected. Pathogens that have been detected, but have not been reported to be associated with high mortalities include the bacterial species *Vibrio anguillarum* (Powell and Loutit, 1990), *Flavobacterium columnare* (Boustead 1989), *F. branchiophilum* (Diggles, Hine, Handley, & Boustead, 2002), the oomycete *Saprolegnia* species (Ministry for Primary Industries, unpublished), the virus Aquabirnavirus (Tisdall and Phipps, 1987), the amoeba *Neoparamoeba* species (Munday, Zilberg, & Findlay, 2001), an un-classified amoeba causing nodular gill disease (Tubbs, Wybourne, & Lumsden, 2010), the protozoan parasites *Ichthyophthirius multifiliis* (Boustead 1989) and *Chilodonella* species (Boustead, 1989), the isopod parasites *Cirolana* species (Boustead, 1982) and *Nerocila orbignyi* (Scott, 1964), and the copepod parasites *Paeonodes nemaformis* (Hine, Jones & Diggles, 2000; Hewitt, 1979) and *Caligus longicaudatus* (Hine et al., 2000).

Pathogens that have previously been detected in New Zealand salmon that have been associated with disease as well as elevated mortality include the bacterial species *Yersinia ruckeri*, *Flavobacterium psychrophilum, Nocardia* species and *Vibrio ordalii* and the parasite *Myxobolus cerebralis*. These are discussed in more detail below.

Yersiniosis is a contagious bacterial disease of salmonids and non-salmonids caused by *Yersinia ruckeri*, a bacteria of the family Enterobacteriaceae. *Yersinia ruckeri* is found in most countries where salmonids are present including Europe, North and South America, Australia and New Zealand (Carson and Wilson, 2009). *Yersinia ruckeri* is endemic to New Zealand where one serotype is known to occur; O1b, biotype 1 (Barnes et al., 2016). The virulent Hagerman strain (O1a, biotype 1), the cause of enteric red mouth, is exotic to New Zealand and is characterised by haemorrhage in and around the mouth. Yersiniosis, a milder disease caused by the serotype O1b, and is characterised by a bacterial septicaemia that most often presents as exophthalmos and pinpoint haemorrhage or reddening in the eyes, but can also be present as a sub-clinical infection with no obvious signs. *Yersinia ruckeri* was first detected in New Zealand from freshwater Chinook salmon hatcheries in 1989 (Boustead and Anderson, 1990). Yersiniosis in Chinook salmon in New Zealand is associated with reddening of the eyes and, less frequently, of the lower jaw (Boustead and Anderson, 1990; Anderson, Knowles, & de Lisle, 1994) and can cause elevated mortalities.

Flavobacterium species belong to the Cytophaga-Flexibacter-like bacteria (CFB) group, one of the most common bacterial groups found in the aquatic environment. These bacteria are often identified as a cause of disease in aquaculture due to poor husbandry conditions. *Flavobacterium psychrophilum*, previously *Cytophaga psychrophila* and *Flexibacter psychrophilus,* is the causal agent of bacterial cold-water disease. This disease typically affects fish reared in fresh water. Bacterial cold-water disease initially presents as discolouration on the body surface progressing to skin necrosis and open ulcers exposing the musculature. Commonly this bacteria affects the caudal area (Starliper, 2010). In more serious cases, severe cellulitis and muscle necrosis can occur (Cipriano and Holt, 2005). The first report of *F. psychrophilum* causing disease in New Zealand salmon was in 1989 (Boustead, 1989) where it was identified by clinical presentation and microscopic examination but was not confirmed by any other methods. In 2012, *F. psychrophilum* was identified in samples from a Chinook salmon hatchery in the South Island where fingerlings were experiencing elevated mortalities (Ministry for Primary Industries, unpublished). All fish tested in this case displayed degradation of the caudal fin and skin necrosis on the caudal peduncle. *Flavobacterium psychrophilum* was confirmed from these fish by bacterial culture,

biochemical tests, partial sequencing of the 16S rRNA gene and multi-locus sequence analysis.

Nocardia species are slow growing filamentous Gram-positive bacteria, ubiquitous within the environment predominantly in soil and plant matter, but also found in fresh and seawater environments (Brown-Elliot, Brown, Conville, & Wallace, 2006). Nocardiosis, caused by *Nocardia* species, is characterised by granulomas in the skin, gills, kidney, liver or spleen that can appear similar to mycobacteriosis, a disease cause by *Mycobacterium* species. In New Zealand, Nocardiosis was diagnosed in a Chinook salmon hatchery in 1972 (Boustead, 1982) based on histopathology, although no organism was able to be isolated for identification. In 2016, this pathogen was again reported from a freshwater Chinook salmon hatchery with cumulative mortalities in one pen of 3.5%. An isolate was cultured and subsequent molecular and biochemical analysis was carried out to determine the species. This isolate was determined to be a newly identified species that did not show high similarity with any previously reported *Nocardia* species using biochemical or molecular techniques (Brosnahan et al., 2017a).

Vibrio species are Gram-negative bacteria, ubiquitous in marine and estuarine environments. These bacteria can cause Vibriosis, a potentially fatal haemorrhagic septicaemic disease of fish. In New Zealand, *V. ordalii* has been reported twice; once from diseased farmed Chinook salmon in sea pens in Owaka, Stewart Island, and once from disease farmed Chinook salmon in the Marlborough Sounds (Diggles et al., 2002; Wards, Patel, Anderson, & de Lisle, 1991). On discovery of this disease, antibiotic medication and vaccination were used in an attempt to reduce mortalities, although whether or not these measures were effective is unknown. Vibriosis outbreaks have been rare in Chinook salmon farms since 1989 and antibiotics and vaccinations are no longer used (Anderson, 1996).

Myxobolus cerebralis is a myxosporean parasite first described by Hofer in 1903. The parasite requires an intermediate host, the oligochaete worm (*Tubifex tubifex*) (Markiw and Wolf, 1983) and has two complete infective spore-forming phases, myxospore and triactinomyxon-like actinospore. The triactinomyxon-like actinospore stage is formed in the worm host. *Myxobolus cerebralis* is the causative agent of whirling

disease which affects both wild and farmed freshwater salmonids (Baldwin Vincent, Silflow, & Stanek, 2000). Whirling disease primarily affects juvenile salmonids with fish developing symptoms of a whirling, tail-chasing behaviour caused by the parasite affecting the nervous system. This disease can be fatal. *Myxobolus cerebralis* infections can also cause spinal skeletal deformities due to the parasites effect on the nerves (Hewitt and Little, 1972; Markiw, 1992). Older salmon can also be affected, however this is rare (Gilbert and Granath, 2003). *Myxobolus cerebralis* was first detected in New Zealand by microscopy in 1971 in rainbow trout from a hatchery in Waitati, north of Dunedin (Hewitt and Little, 1972). It is likely this parasite had been present at this hatchery since the 1950s with reports of fish showing the same behaviour of whirling but no diagnostic testing performed (Hewitt and Little, 1972). Following initial confirmation of this pathogen, a large survey was conducted testing over 5000 wild and hatchery reared salmonids for the presence of *M. cerebralis*. In addition to this testing, sentinel rainbow trout were used in six locations in the South Island for detection of *M. cerebralis* (Boustead, 1993). *Myxobolus cerebralis* has also been detected in wild and farmed Chinook salmon in New Zealand (Boustead, 1993; Ministry for Primary Industries, unpublished), although no detections have occurred since 1989.

Incidences of outbreaks due to the pathogens listed above are uncommon and as of 2016, antibiotics or vaccines are not in regular use for management or control of disease in farmed salmon in New Zealand. None of the diseases identified in New Zealand Chinook salmon are listed by The Office International des Epizooties (OIE). The OIE is an intergovernmental organisation responsible for improving animal health worldwide. This organisation creates a list of diseases considered to have the greatest economic importance for terrestrial and aquatic industries. The OIE listed diseases for salmon are: epizootic haematopoietic necrosis, infectious haematopoietic necrosis, infectious salmon anaemia (HPR-deleted or HPR0), salmonid alphavirus disease, viral haemorrhagic septicaemia, disease due to *Oncorhynchus masou* virus, epizootic ulcerative syndrome caused by the oomycete *Aphanomyces invadans*, and disease due to the parasite *Gyrodactylus salaris* (OIE, 2016). To be free of these diseases is a significant benefit for trade, as well as benefits for production and animal welfare. To maintain this level of disease freedom, it is essential for industry to maintain good biosecurity practices to help prevent the entrance of these diseases and for regular

surveillance programmes for diseases to be conducted. It is equally important to understand the base line of pathogens to aid with early detection of any new or emerging disease issues including deciphering trends of endemic diseases.

Since 2012, elevated mortalities have occurred in a Chinook salmon farm in the Marlborough Sounds during the summer months, termed ‘summer mortalities’. Until 2015, no potential primary pathogens had been detected (Norman et al. 2013). In 2015, these mortalities were again reported and samples were investigated. This time, two potential pathogens were identified; a rickettsia-like organism, termed NZ-RLO, and *Tenacibaculum maritimum*. Due to the rickettsia-like organism being an unwanted organism in New Zealand, a controlled area notice was imposed by the Ministry for Primary Industries and has been placed around the Marlborough Sounds salmon farms to restrict the spread of an unwanted organism to other parts of New Zealand. This controlled area notice remains to this date (2019). These two organisms are the main focus of this thesis.

1.5 *Piscirickettsia salmonis* and NZ-RLO

Piscirickettsia salmonis is a Gram-negative, facultative intra-cytoplasmic coccoid bacteria and a member of the class Gammaproteobacteria. *Piscirickettsia salmonis* is phylogenetically related to the genus *Legionella, Francisella* and *Coxiella* (Fryer, Lannan, Giovannoni, & Wood, 1992). When first described, *P. salmonis* was classed as a *Rickettsia* species and placed within the Alphaproteobacteria (Cvitanich, Garate, & Smith, 1991) based on the morphology and the intracellular nature of the organism and termed rickettsia-like organisms (RLO). Subsequent phylogeny based on nucleotide sequencing of the 16S rRNA gene demonstrated that *P. salmonis* belongs to the class Gammaproteobacteria and was not a true *Rickettsia* species. The genus *Piscirickettsia* currently has only one type species, *P. salmonis* (Fryer et al., 1992). However, many different strains of *P. salmonis* have been identified that are similar but show genetic differences and are termed piscirickettsia-like organisms (PLO), RLO or *P. salmonis* (Mauel and Miller, 2002). More recently, two clusters of *P. salmonis* strains have been proposed based on genomic and phylogenetic analysis and have been named after the reference strains; LF-89-like and EM-90 like (Bohle et al., 2014; Mandakovic et al., 2016). Phylogenetic studies such as these reveal the great diversity

and complicated nature of *P. salmonis* taxonomy. An increased number of *P. salmonis* genomes from wider geographical areas and diverse host species will be required to better understand the classification in this genus.

Piscirickettsia salmonis primarily infects the inflammatory cells of teleost fish causing the disease piscirickettsiosis, also known as salmonid rickettsial syndrome. Piscirickettsiosis is predominantly characterised by the development of skin ulcers, beginning with white patches progressing to shallow ulcers, pale gills, swollen and discoloured kidneys, enlarged spleen, and pale livers that may have sub capsular multifocal nodules in heavily infected fish (Cvitanich et al., 1991; Corbeil, Hyatt, & Crane, 2005). Fish with milder infections may appear normal with no obvious clinical signs.

Piscirickettsia salmonis was first identified from Coho salmon in Chile in 1989. During this epizootic, mortalities of an estimated 1.5 million salmon from approximately 200 g to market size (~3 kg) occurred (Cvitanich et al., 1991). In 1990, *P. salmonis* was isolated in cell culture for the first time and was injected into naïve fish. Injected fish replicated the pathology seen in naturally infected fish of pale gills, swollen kidneys and enlarged spleens as well as cream nodules scattered through the liver. Additionally, injected fish resulted in severe mortalities (Cvitanich et al., 1991). Subsequently, *P. salmonis*, PLO, and RLO have been identified in both salmonid and non-salmonids on almost all continents, Europe, North America, South America, Asia, and Australia.

Transmission of *P. salmonis* is known to be horizontal from fish to fish without the requirement of a vector (Smith et al., 2004; Almendras, Fuentealba, Jones, Markham, & Spangler, 1997). Survivability of a facultative intracellular bacteria for extended periods of time outside of the host requires a protective environment for survival, potentially in a biofilm. Biofilms are a collection microorganisms that adhere to a surface for protection in adverse environments (Marshall, Gómez, Ramírez, Nilo, & Henríquez, 2012). Survivability of *P. salmonis* in seawater outside of host cells under laboratory conditions has been found to be up to two weeks (Lannan and Fryer, 1993) however, the mechanisms by which it can survive are undetermined. Under experimental conditions, *P. salmonis* is not found to survive in freshwater. However,

there is some evidence that *P. salmonis* can survive in freshwater after shedding from infected fish, and it is thought that this may occur by being protected within faeces (Almendras et al., 1997) or biofilms. *Piscirickettsia salmonis* is known to be transmitted vertically from parent to offspring under experimental conditions (Larenas et al., 2003) to date it has not been established if this occurs naturally.

1.5.1 Detection of *Piscirickettsia salmonis*

In 2003, *P. salmonis* was listed in the OIE manual as a disease of concern. Since this time, it has been removed due to the cosmopolitan detection and therefore reduced trade risk. When *P. salmonis* was OIE listed, visualisation of the organism by microscopy within inflammatory cells (macrophages) or hepatocytes either within histological sections or within tissue imprints was recommended for presumptive diagnosis. Confirmation required growth of *P. salmonis* in cell culture followed by indirect fluorescent antibody test (IFAT) or PCR (OIE, 2003). Microscopy to visualize organisms appears to be of low sensitivity with detection unlikely within mild infections.

Confirmation of the presence of *P. salmonis* and assessment of the viability of the bacteria requires growth in cell culture or on agar. *Piscirickettsia salmonis* was initially thought to be an obligate intracellular organism but more recently many cell free agar and nutrient broths have been developed to be able to support the growth of *P. salmonis* (reviewed in Makrinos and Bowden, 2017). For further confirmation of *P. salmonis*, immunofluorescence antibody tests (IFAT) or *in-situ* hybridization (ISH) can be carried out on histology slides or cell culture material. Immunofluorescence antibody tests are specific for the detection of the antigens produced by the pathogen and presents as a fluorescent signal when the antibody has bound to the *P. salmonis* antigen. *In-situ* hybridization is specific for the detection of genetic material using a labelled DNA probe to detect and localize the presence of bacterial DNA within a sample. Both IFAT and ISH are time consuming and require specialised antibodies or probes respectively. Additionally these techniques, including growth in cell culture or on agar, may not allow for strain recognition of *P. salmonis*. Determination of the strain is important as different strains have shown differences in pathogenicity (House, Bartholomew, & Winton, 1999). Additionally, strain determination has a biosecurity

role as some strains are not present in certain geographical areas for example, *P. salmonis* is exotic to Australia but Tasmanian-RLO is not (Corbeil et al 2005).

To determine the differences in *P. salmonis* strains, specific PCR or PCR followed by nucleotide sequencing is required. The protocol of PCR followed by nucleotide sequencing uses primers to amplify part of the 23S rRNA gene of *P. salmonis* (Corbeil, McColl, & Crane, 2003) followed by amplification and nucleotide sequencing of the internal transcribed spacer rRNA region and if possible the 16S rRNA gene (Marshall, Heath, Henriquez, & Orrego, 1998; Mauel, Giovannoni, & Fryer, 1996).

New Zealand Rickettsia-like organisms (NZ-RLO) are Gram-negative facultative intracytoplasmic coccoid bacteria closely related to *P. salmonis* both morphologically and genetically. The initial detection, characterisation and phylogenetic differences to *P. salmonis* of these novel organisms from clinically affected Chinook salmon is described further in Chapters 2 and 4 of this thesis. In addition to the work carried out in this thesis, initial isolation of NZ-RLO using cell culture was successfully achieved by other groups from the Ministry for Primary Industries, Animal Health Laboratory from the samples received in Chapter 2 and was confirmed as NZ-RLO in June 2015 using nucleotide sequencing and transmission electron microscopy (MPI, unpublished). Initial isolation of this bacteria in cell culture will not be discussed further, however the work carried out in Chapter 3 uses the isolate from this culture as the positive control material.

1.6 *Tenacibaculum maritimum*

Tenacibaculum maritimum, previously known as *Flexibacter maritimus,* is a Gram-negative bacteria in the family Flavobacteriaceae. This organism is ubiquitous in the marine environment and a common cause of disease in aquaculture. This bacteria causes tenacibaculosis in marine salmon, and other finfish, which is characterised by skin ulcers, mouth, gill, fin, and tail erosions (Avendaño-Herrera, Toranzo, & Magariños, 2006). *Tenacibaculum maritimum* had not been reported to be associated with mortalities in New Zealand farmed Chinook salmon until 2015 (Chapter 2).

Although *T. maritimum* is an important and moderately common disease in aquaculture, the mode and route of transmission of the bacterium are not well understood. Mitchell and Rodger (2011) reviewed the following routes of transmission: via seawater, horizontally from host to host, ingestion with food as well as the more unusual transmission route via a jellyfish vector. These have all shown to be valid routes of transmission, however future research is required to understand the reservoir of the bacterium, the survival strategy outside the host, and the mechanisms underpinning the virulence.

1.6.1 Detection of *Tenacibaculum maritimum*

Tenacibaculum maritimum is presumptively diagnosed through clinical signs and wet mounts of affected tissues showing long bacterial rods with gliding motility under microscopic examination. However, confirmation of the species is required as there are currently five recognised species of this genus (Fernández-Álvarez and Santos, 2018). Confirmation of *T. maritimum* is carried out by traditional bacterial culture techniques on specialised agar, biochemical analysis, and PCR analysis. These methods have advantages and limitations. Culture techniques ensure the cells detected are viable and replicating, and therefore infectious. However, this organism is fastidious, slow growing, and requires specialised media for growth. As *T. maritimum* often infects fish on areas of the body that have high loads of environmental bacteria, such as the skin and gills, the presence of *T. maritimum* within the sample can be masked by faster growing environmental bacteria. This is one reason molecular techniques are commonly used for detection as they are specific and sensitive and there are many well validated assays available (Cepeda and Santos, 2002; Fringuelli et al., 2012). The use of molecular methods allows detection of *T. maritimum* at low levels in a background of other bacteria however traditional molecular techniques detect all DNA and so cannot discriminate between live and dead bacteria.

To overcome the disadvantage of not being able to differentiate between live and dead bacteria, a technology using intercalating dyes coupled with PCR, termed viable PCR (vPCR) was developed (Nogva, Dromtorp, Nissen, & Rudi, 2003). This technique was first described in 2003 using ethidium monoazide (EMA) (Nogva et al., 2003). This dye

primarily only crossed the membranes of dead bacteria but it was also was found to cross the membrane of some live bacteria (Nocker and Camper, 2006). It was theorised that in live bacteria, where EMA crossed the membrane, the dye would be expelled as this is what occurs in the dye EMA is derived from, ethidium bromide (Codony, Agusti, & Allue-Guardia, 2015). In 2006 propidium monoazide (PMA) was described. This dye was adapted from propidium iodine, a common membrane impermeant dye used extensively in live-dead determination by flow cytometry and fluorescent microscopy (Nocker and Camper, 2006). PMA was designed to overcome the limitation of the lack of specificity of EMA. A limitation of PMA is that it will not penetrate dead bacteria that still have an intact membrane, for example bacteria that have died due to treatment with UV. In 2006 another dye was introduced, PEMAX, a mixture of PMA with lower concentrations of EMA. This dye was designed to overcome the limitations of both EMA and PMA (Nocker and Camper, 2009; Cangelosi and Mescheke 2014; Codony et al., 2015). All these vPCR dyes work by passing through cell membranes of dead cells and intercalating with the nucleic acid. This nucleic acid/dye molecule is then exposed to light of a certain wavelength (446 to 474 nm) which crosslinks the nucleic acid, thereby preventing amplification by PCR. The use of the technique in tissue samples containing *T. maritimum* is discussed in Chapter 8 of this thesis.

The characterisation of the New Zealand strain of *T. maritimum* and the investigation of this pathogen as a cause of disease in farmed salmon is described in Chapters 2 and 4 of this thesis.

1.7 Teleost Immune system

Teleost fish possess both innate and acquired immune systems as defence mechanisms against the infection and subsequent multiplication of pathogens, including bacteria. These two immune systems are interconnected with the innate immune system acting rapidly on any foreign agent and the acquired immune system taking longer to be initiated but being targeted for specific foreign agents.

1.7.1 Innate immune system

The innate or non-specific immune system is activated by any foreign agent or pathogen. As this system has no immunological memory a second encounter with the same pathogen does not result in enhanced immunity. In teleost fish, innate immunity is considered to be the most important system protecting against diseases with skin and mucus playing a key role. As well and skin and mucus, the innate immune system also includes cellular and humoral components.

1.7.1.1 Skin and mucus

The physical barrier for protection of teleost fish is the skin. Skin consists of mucus, scales and epidermis. As teleost fish are immersed in an aquatic environment containing a soup of foreign agents including bacteria, the physical barrier between the internal organs and the outside environment is integral to defence and osmotic balance. Mucus is present over the surface of the skin, gills, and gastrointestinal tract and is constantly produced by epithelial surface cells. This constant production of mucus can trap pathogens preventing colonisation. Mucus also contains immunological factors that defend against pathogens. These immunological factors include antibacterial peptides, proteases, lectins, lysozyme, and antibodies (immunoglobulin M) (Ellis, 2001; and reviewed by Magnadóttir, 2006; Uribe, Folch, Enriquez, & Moran, 2011). In areas of poor water quality, for example areas of high silt, additional mucus will be produced to provide added protection from the external environment (Shephard, 1994).

The epidermis is the outermost layer of the teleost skin which sits under the mucus layer. Unlike terrestrial animals, all of the epidermal cells in teleost fish are live allowing for rapid healing of wounds by sliding of surrounding epidermal cells over the lesion (Hickey 1982). Maintaining integrity of this epidermal layer is crucial to prevent entry of pathogens and maintaining osmotic balance. Scales form in shallow pockets within the stratum spongiosum of the dermis, under the epidermis. Scales provide a flexible, epidermal covered armour protecting the dermal layer. Salmon do not have scales on the top of their head or over the fins, so the epidermis is thicker in these areas to provide greater protection.

1.7.1.2 Cellular

Phagocytosis is the most important cellular component of the innate immune system and is an early stage of the inflammatory response. Phagocytic cells most commonly consist of white blood cells (WBCs) including neutrophils and macrophages. On pathogen recognition, WBCs will migrate from the blood or surrounding tissues to the site of infection. White blood cells engulf foreign particles including bacteria and kill them by processes such as lysozyme activity or respiratory burst. Lysozyme is an enzyme that attacks the peptidoglycan layer that makes up the cell wall of both Gram-positive and Gram-negative bacteria, causing it to break down (Ellis, 2001). This process is also known to enhance phagocytosis and activate the complement system (reviewed by Magnadóttir, 2006). Respiratory burst is the rapid release of reactive oxygen species such as the superoxide free radical and hydrogen peroxide as well as nitric acid and other factors which degrade the engulfed particles. Non-specific cytotoxic cells are also involved in the cellular components of innate immunity (Evans, Leary, & Jaso-Friedmann, 2001) mediating cytotoxicity through apoptosis, programmed cell death, and necrosis (Greenlee, Brown, & Ristow, 1991). These cells are found in the blood, lymphoid tissues and intestine of teleost fish (Evans and Jaso-Friedmann 1992).

1.7.1.3 Humoral

As well as physical barriers and cellular components, the innate immune system also consists of humoral components that inhibit the growth and survival of pathogens. Humoral immunity is mediated by macromolecules found in body fluids and include cytokines, natural antibodies, antimicrobial peptides, and the components of the complement system.

Cytokines are small proteins important in both innate and adaptive immune systems. The main teleost cytokines include tumour necrosis factor (TNF), interferons (INF) and interleukins (IL). The cytokine TNF are important in the activation and recruitment of macrophages during an inflammatory response. This allows macrophages to migrate to the areas containing pathogens and also promotes killing of these pathogens by

respiratory burst (reviewed by Uribe et al., 2011). The cytokine INF are produced by many cell types, primarily macrophages, in response to viral double-stranded RNA infections (Ellis, 2001) and work by inhibiting viral nucleic acid replication within infected cells (Roberts, 1978). Interleukins also help in regulation of both the innate and acquired immune system through recruitment of pathogens for elimination.

Natural antibodies are present in the humoral innate and adaptive immune system, however they differ between the two systems. In the innate immune system, natural antibodies are produced in the absence of antigen stimulation (Whyte, 2007) and are produced by B-lymphocytes (Hamilton, Lehuen, & Kearney, 1994). Natural antibodies are found in high levels in the serum of teleost fish and have been found to provide defence against viral and bacterial pathogens in rainbow trout and goldfish (reviewed by Magnadóttir, 2006) and are important in the first line of defence in the teleost fish immune system. Due to the natural antibodies not requiring antigen stimulation, they are activated more rapidly than those of the adaptive immune system (Sinyakov, Dror, Zhevelev, Margel, & Avtalion, 2002).

Antimicrobial peptides are another important feature of the humoral innate immune system in providing a first line of defence for teleost fish. They have a broad range of target organisms, including bacteria and viruses, and have predominantly been found in fish mucus (Cole, Weis, & Diamond, 1997; Masso-Silva and Diamond, 2014). Due to the small size of these peptides, they can move quickly to the site of infection for defence (reviewed by Shabir et al., 2018). Generally they kill bacteria by targeting the bacterial cell membrane and causing disintegration of the lipid bilayer (Bahar and Ren, 2013).

The complement system is well developed in teleost fish (Boshra, Li, & Sunyer, 2006) and eliminates pathogens through pathogen recognition and activation of phagocytes (Rauta, Nayak, & Das, 2012). Teleost fish possess three complement systems; classical, alternative, and lectin systems. The classical complement can be triggered by antibodies binding to the cell surface (Holland, 2002) or directly by pathogens (reviewed by Whyte, 2007). The alternative pathway is activated directly by foreign microorganisms and is independent of antibodies (reviewed by Uribe et al., 2011). The alternative pathway can also be directly activated by lipopolysaccharides, an important

component of the cell membranes of Gram-negative bacteria. The alternative pathway is prominent in teleost fish compared to mammals and has therefore been suggested as being highly important (Rauta et al., 2012). The lectin pathway is activated by the binding of mannose-binding lectins to mannans on bacterial cell surfaces (reviewed by Whyte, 2007). All three pathways lead to opsonisation and direct killing of the pathogen.

1.7.2 Acquired immune system

The acquired, or specific immune system, is effective for specific foreign agents or pathogens. This system has immunological memory and repeated exposure to the same antigen from a pathogen results in increased immune response. The acquired immune system of teleost fish is divided into cellular and humoral components.

1.7.2.1 Cellular

Cellular components of the acquired immune system are mediated by T-lymphocytes that are primarily produced in the thymus of fish (Roberts and Pearson, 2005) before being distributed through the other lymphoid tissues including the kidney and spleen (Nakanishi, Shibasaki, & Matsuura, 2015). T-lymphocytes detect and kill host cells that have been infected by, or phagocytised by, pathogens (Evensen, 2016). T-lymphocytes are separated into CD4+ helper T-lymphocytes (CD4+ cells) and CD8+ cytotoxic T-lymphocytes (CD8+ cells). The CD4+ cells produce cytokines, directly stimulating the immune response to kill infected host cells and CD8+ cells directly kill infected host cells (Laing and Hansen, 2011). An important part of initiation of the acquired immune system is the recognition of the antigens of the foreign agent. The main antigen presenting cells are dendritic cells, macrophages, and B-lymphocytes. These cells present the antigen to the T-lymphocytes along with a class of major histocompatibility complex (MHC), cell surface proteins. MHC class I bind peptides of pathogens produced within the cells, for example virus or intracellular bacteria, and present the antigens at the surface of the cell for CD8+ cells to produce a cytotoxic response. MHC class II bind peptides of pathogens outside the host cells, which include most bacteria, and present these antigens at the cell surface for CD4+ cells,

resulting in a humoral response of a release of cytokines, attracting other killing cells to overcome the foreign body (Miller et al., 2014).

1.7.2.2 Humoral

The humoral components of the acquired system consist of antibodies produced by B-lymphocytes, also known as immunoglobulin (Ig) (Press and Evensen, 1999). B-lymphocytes are predominantly found in the kidney, blood, and spleen (Overland, Pettersen, Ronneseth, & Wergeland, 2010) and are activated by antigens of foreign bodies or pathogens presented to them by CD4+ helper T-lymphocytes. Antibodies bind specifically to a single type of antigen from a foreign agent, blocking entry into host cells and promoting phagocytosis of the antibody-antigen complex (Schroeder and Cavacini, 2010). B-lymphocytes clone the specific antibody producing large quantities for defence, as well as retaining memory B-lymphocytes for a repeat encounter with that pathogen. Fish are known to produce the following antibodies: IgM, IgT and IgD. IgM is the primary antibody and important in systemic immunity (Hordvik, 2015), IgT is important in mucosal immunity in the intestine and in the skin (Zhang et al., 2010) and IgD is important in gill immunity (Hordvik, 2015). A summary of the main features of the innate and adaptive immune system in teleost fish is presented in Figure 1.4.

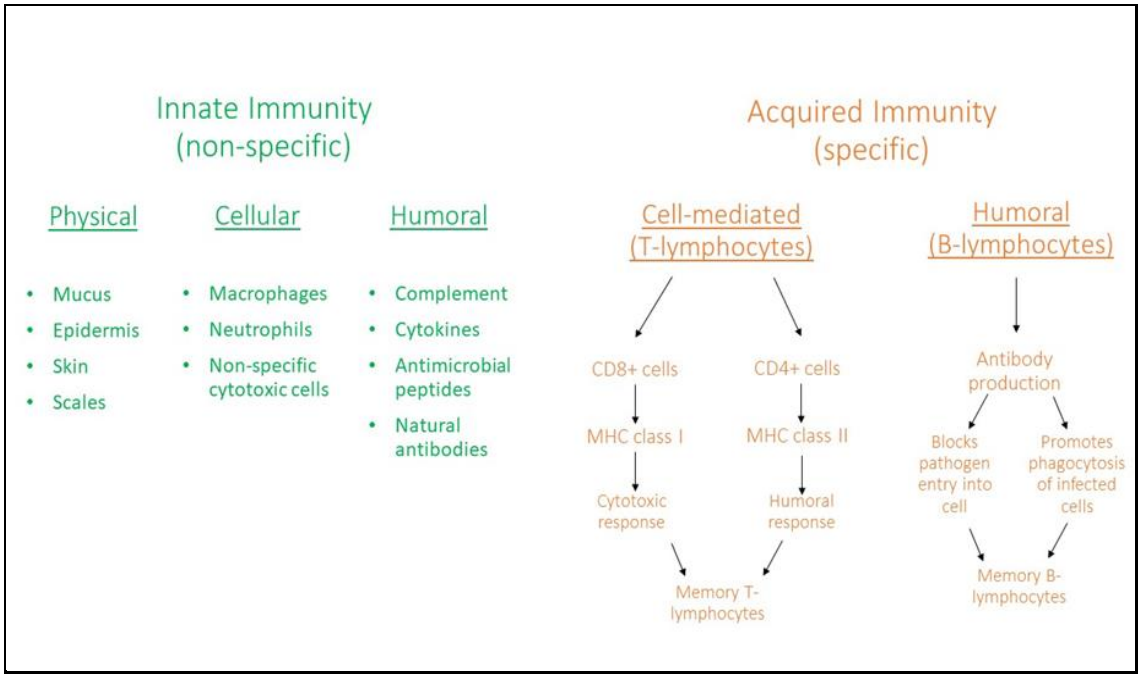

Figure 1.4. Summary of the main features of the innate and acquired immune system in teleost fish.

Mechanisms of how pathogens evade the host immune system and therefore their virulence factors are often not well understood. This is the case for both *P. salmonis* and *T. maritimum*, however in recent years many studies have focused on the virulence factors of *P. salmonis* primarily to improve the efficacy of treatments for this important disease.

It has been established that *P. salmonis* primarily infects and multiplies within macrophages (McCarthy et al., 2008; Rojas, Galanti, Bols, & Marshall, 2009) and induces apoptosis of these host cells for increased infection (Rojas et al., 2010). In teleost fish immunity, the primary role of macrophages is to engulf and kill foreign particles including bacteria so being able to evade this system is key for both survival and dissemination of pathogens within the host. The means by which *P. salmonis* is able to survive this is less clear and it is likely multiple strategies are involved. Multiple studies have been reported since 2016 exploring the virulence factors and mechanisms of *P. salmonis*, many of which have focused on assessing virulence factors of other closely related bacterial pathogens such as *Coxiella burnetti, Francisella tularensis, Leigonella pneumophila,* and *Edwardsiella tarda* to determine if *P. salmonis* also possess these factors.

One virulence factor found to be important for *P. salmonis* is the presence of a Dot/Icm Type IV-B secretion system. Secretion systems are common in pathogenic bacteria to deliver molecules from the bacteria to the environment or to the host. The most common secretion system is type III (Segal, Feldman, & Zusman, 2005). The Dot/Icm type IV-B system has only been detected in two other bacterial pathogens, *L. pneumophila* and *C. burnetti* and is essential for their pathogenesis (Segal et al., 2005). A study by Gómez et al., (2013) confirmed the presence of this system in *P. salmonis* as well and confirming four components of the Dot/Icm type IV-B secretion system that were essential for pathogenesis, *dot*B, *dot*A, *icm*K and *icm*E. The importance of this system for pathogenesis in *P. salmonis* was confirmed in a study by Mancilla et al., (2017) who found that an additional gene is essential for pathogenesis of *P. salmonis*, the *icm*B gene.

Another virulence factor important for *P. salmonis* to be able to enter host cells and evade the host cellular immune response involves clathrin. Clathrin is a structural

membrane protein that is involved in endocytosis, a cellular process where materials are invaginated into cells. A study by Ramírez, Gómez, & Marshall (2015) found *P. salmonis* uses a clathrin-dependent pathway to enter salmonid cells and to protect itself from the host cellular response. This clathrin-depended endocytosis pathway has also been described to be used by other pathogenic bacteria and viruses (Veiga and Cossart, 2006).

More recently, the possibility that *P. salmonis* is able to modify the expression of interleukins (IL-10, IL-12) to reduce the activation of macrophages and explain how *P. salmonis* survives inside the infected cells has been assessed (Álvarez, Gómez, Mercado, Ramírez, & Marshall, 2016). This study also evaluated the involvement of the antimicrobial peptide hepcidin in the infection of salmonid macrophages by *P. salmonis*. As previously described, IL regulates the innate and acquired immune system by recruitment of pathogens for elimination. Therefore, the ability of a pathogen to modify IL would enable the manipulation of host macrophages. Through *in-vitro* infection models, a study by Álvarez et al., (2016) showed IL-10 was upregulated during infection with *P. salmonis* which in turn deactivated macrophages and prevented stimulation of the inflammatory response allowing for bacterial growth. Levels of hepcidin expression decreased in *P. salmonis* infected cells suggesting *P. salmonis* can also manipulate this expression to allow successful survival and replication within the host.

The mechanisms underlying these virulence factors have also been explored. A recent study by Oliver et al., (2016) investigated outer membrane vesicles (OMV) as a possible mechanism for *P. salmonis* to deliver these virulence factors to the host. Production of OMV have been described for bacteria closely related to *P. salmonis* and other fish pathogens such as *Vibrio anguillarum* (Hong et al., 2009). This study showed that by isolating OMV and subjecting them to cell culture, cytopathic effect was observed, as seen in *P. salmonis* infection, indicating these vesicles contain factors involved in pathogenesis. The use of molecular methods found that proteins such as outer membrane protein A and heat shock protein 60 were also detected in these OMV which are virulence molecules involved in biofilm formation and adhesion to host cell membrane respectively.

The expression of high affinity iron uptake mechanisms have also been suggested for *P. salmonis* (Calqúin et al., 2018). Iron is key to the pathogenicity of most bacteria as it is essential for bacterial multiplication within hosts (Brown and Holden, 2002).

Finally, small RNA and chaperone proteins have been detected in *P. salmonis* (Marshall, Flores-Herrera, Henríquez, & Gómez, 2017). These proteins, hfq-1 and hfq-2, are important in controlling gene expression in bacteria and the study by Marshall et al., (2017) showed that the hfq-2 gene is likely to be directly involved in pathogenicity of *P. salmonis*.

Studies into the mechanisms by which *T. maritimum* is able to infect and survive within the host and evade the immune system are scarcer. However, some strategies have been suggested including attaching to hydrophobic surfaces or fish mucus, the production of extracellular products, and iron uptake mechanisms.

The ability of an organism to attach to fish skin, a hydrophobic surface covered in mucus, is essential in overcoming the initial immune response of teleost fish. A study by Magariños, Pazos, Santos, Romalde, & Toranzo (1995) demonstrated *T. maritimum* can strongly attach to glass slides, a hydrophobic surface. Burchard, Rittschof, & Bonaventura (1990) also reported the adhesion of *T. maritimum* to hydrophobic surfaces and demonstrated *T. maritimum* produced extracellular polymers to adhere more firmly. The ability of pathogens to attach strongly to fish skin is important so as not to be sloughed off by the mucus the fish is continuously producing. Additionally Magariños et al., (1995) demonstrated that *T. maritimum* can grow in the presence of mucus from turbot (*Scophthalmus maximus*), seabream (*Sparus aurata*), and seabass (*Dicentrarchus labrax*) indicating the fish mucus of these species did not contain antimicrobial compounds that limit *T. maritimum* growth.

The extracellular products (ECP) of *T. maritimum* have been investigated to determine their virulence. Van Gelderan, Carson, & Nowak, (2009) tested the ECP from *T. maritimum* both *in-vivo* and *in-vitro.* Fish were injected intraperitoneally with *T. maritimum* ECP and they showed severe toxicity with internal necrosis and haemorrhage. However, *in-vitro* testing with these ECP showed contrasting results. The reasons for this are unknown as are the precise toxins and enzymes present in

the ECP. However, it is known that lipopolysaccharides (LPS) are involved in the signs of disease caused by *T. maritimum*. Mabrok et al., (2016) recently reported that *T. maritimum* has the presence of an LPS O-chain compound with linkages unique to *T. maritimum* and may enhance biofilm formation in *T. maritimum*.

Finally, as with other pathogenic bacteria, *T. maritimum* has been shown to have the capacity to express high-affinity iron-uptake mechanisms that compete with the host for iron (Avendaño-Herrera, Toranzo, Romalde, Lemos, & Magariños, 2005). Iron is essential in the growth and replication of bacteria so is an essential part of pathogenesis.

The whole genome of *T. maritimum* has recently been fully sequenced (Pérez-Pascual et al., 2017) allowing for the prediction of genes relevant to the pathogenesis of this bacteria and a greater understanding of the mechanisms underlying the virulence factors described above. Analysis of the whole genome revealed genes associated with iron uptake, adhesion, toxin genes, and virulence factors including membrane-damaging enzymes. Additionally, genes such as multiple superoxide dismutases were detected suggesting *T. maritimum* uses a sophisticated mechanism to handle oxidative stress produced by host macrophages. This study highlights the complexity of the virulence mechanisms of *T. maritimum*.

1.8 Conclusion

As reviewed, farmed Chinook salmon in New Zealand appear to be free of the major notifiable infectious agents that affect fish elsewhere in the world. This suggests that disease due to infectious agents may be rare in New Zealand. However, the detection of the novel NZ-RLO as well as *T. maritimum* in fish with high levels of mortality and clinical signs of disease requires investigation. As described in this review, these pathogens, or ones closely related, have been shown to be virulent and have had significant impacts on aquaculture globally with variable success of the management of the diseases they induce. Chinook salmon is the only farmed species of salmonid in New Zealand whereas the majority of farmed salmonids globally are Atlantic salmon. Most global research into salmon pathogens is therefore conducted on

Atlantic salmon which many not necessarily mirror how pathogens will act in a different host species.

The overall aim of this thesis was to determine if pathogens were involved in the summer mortalities of chinook salmon in Marlborough Sounds. Two potential pathogens were detected in Chapter 2; New Zealand rickettsia-like organism (NZ-RLO) and *Tenacibaculum maritimum*. These pathogens were detected from moribund fish sampled from a summer mortality event and were identified using histopathology, bacteriology and molecular methods. The following chapters tested the hypothesis that either NZ-RLO or *T. maritimum* were involved in summer mortalities of farmed Chinook salmon. In Chapter 3, the aim was to determine if the organisms observed under histology within areas of inflammation in Chapter 2 were NZ-RLO, which would provide more evidence that this pathogen was directly involved in the summer mortalities. This was carried out by the use of *in-situ* hybridization and transmission electron microscopy. In Chapter 4, the aim was to understand which of the two potential pathogens identified in Chapter 2 were more likely to be involved in the summer mortalities in the Marlborough Sounds. This was carried out by assessing the distribution of both pathogens in both healthy and affected salmon in all marine farmed populations in New Zealand. In Chapters 6 and 7, the aim was to determine the pathogenic potential of NZ-RLOs in chinook salmon smolt and understand how infection with NZ-RLOs presents in chinook salmon smolt. This was carried out by experimental infection trials using NZ-RLO1 or NZ-RLO2.

Understanding if NZ-RLO or *T. maritimum* are involved in summer mortalities of farmed Chinook salmon is crucial for ongoing understanding of the pathogens occurring in New Zealand aquatic animals as well as for the ongoing management and sustainable growth of the New Zealand salmon aquaculture industry.

Chapter 2 : First report of a rickettsia-like organism from farmed Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), in New Zealand

Queen Charlotte Sound, Marlborough Sounds.

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2.1 Introduction

Piscirickettsia salmonis is a Gram-negative intracellular bacteria and the causative agent of piscirickettsiosis or salmonid rickettsial syndrome. Piscirickettsiosis is a severe bacterial disease affecting salmonids in aquaculture with a global distribution (Rozas and Enriquez, 2014), not including New Zealand or Australia.

Piscirickettsia salmonis was first described from Coho salmon, *Oncorhynchus kisutch* (Walbaum), in Chile (Bravo and Campos, 1989) and was characterised as a rickettsia-

like organism (RLO) based on morphology. Subsequent characterisation using molecular tools determined it belonged in the order Gammaproteobacteria, and was not a true *Rickettsia* species (Fryer et al., 1992).

Since the first detection, *P. salmonis* and similar organisms referred to as *piscirickettsia*-like organisms (PLO) or RLO have been described from a wide geographic range, infecting salmonid species in the Pacific and Atlantic coasts of Canada (Brocklebank, Evelyn, Speare, & Armstrong, 1993), Norway (Olsen, Melby, Speilberg, Evensen, & Hastein, 1997), Ireland (Rodger and Drinan, 1993), Scotland (Grant, Brown, Cox, Birkbeck, & Griffen, 1996), and Tasmania (Corbeil et al., 2005). Detections have also been found in non-salmonid species in southern California (M.F., Chen et al., 2000; Mauel et al., 2005), the French Mediterranean (Comps, Raymond, & Plassart, 1996), Greece (Athanassopoulou, Groman, Prapas, & Sabatakou, 2004), Columbia (Iregui, Vasquez, Rey, & Verjan, 2011), Hawaii (Mauel et al., 2003), Florida, South Carolina (Mauel et al., 2005), and Taiwan (Chen, Wang, Tung, Thompson, & Adams, 2000). These infections have been detected in fish predominantly from seawater environments, but have also been identified in Atlantic and Pacific salmonids in freshwater (Almendras et al., 1997; Gaggero, Castro, & Sandino, 1995).

Outbreaks of *P. salmonis* in farmed fish can cause large mortalities resulting in significant financial loss (McCarthy et al., 2008). Mortalities have been reported to be as high as 90% in Coho salmon in Chile (Branson and Diaz-Munoz, 1991) or as low as 8% in Atlantic salmon and negligible mortality rates in Chinook salmon in British Columbia (Brocklebank, Speare, Armstrong, & Evelyn, 1992; Brocklebank et al., 1993). The severity of the disease caused by *P. salmonis* varies between geographic location, host species, and strain of the organism (House et al., 1999).

In the austral summer of 2015, cumulative mortalities of up to 70% occurred among Chinook salmon at one sea-pen farm in the Marlborough Sounds. A sample size of 10 moribund Chinook salmon displaying signs of disease were submitted to the Ministry for Primary Industries, Animal Health Laboratory for disease testing in April (austral autumn), after the peak of the mortality. A full range of diagnostic testing was carried out including: necropsy, histopathology, general bacteriology, and PCR testing. Initial

testing by PCR was carried out for the following exotic diseases to New Zealand: infectious pancreatic necrosis virus, infectious salmon anaemia virus, totivirus, aquareovirus, infectious hematopoietic necrosis virus, *Aeromonas salmonicida* and, *Piscirickettsia salmonis*. All PCR tests for viruses and *A. salmonicida* returned negative results and will not be described further in this report. Bacteriology culture revealed the presence of *Tenacibaculum maritimum*, the causative agent of tenacibaculosis. Subsequent testing was carried out on tissues for detection of *T. maritimum* by PCR targeting the 16S rRNA gene. Testing of *P. salmonis* by PCR targeted three genetic areas; the 16S rRNA gene, the 23S rRNA gene, and the 16-23S rRNA internal transcribed spacer (ITS) region. Initial genetic characterisation of the first described NZ-RLO, bacteriology, and PCR testing for *T. maritimum* from farmed Chinook salmon in New Zealand will be discussed.

2.2 Materials and methods

2.2.1 Post mortem examination and sample collection.

Sampled fish were approximately two-years-old with an average weight of approximately 2 kg. Fish had been transferred from a freshwater hatchery into sea-pens approximately 11 months prior to sampling. The water temperature throughout the mortality events was consistently above 17°C.

Moribund fish were selected for disease testing by farm staff. Fish were netted out of pens experiencing mortalities and euthanised immediately. Fish were then individually bagged, placed on ice and shipped to the Animal Health Laboratory overnight. Fish were received and processed in the laboratory 24 hours post-mortem.

Following measurement of the fork length, each fish was examined both externally and internally for gross abnormalities. Tissue samples were collected for histopathology, bacteriology, and PCR tests.

2.2.2 Histopathology

Gills, skin and skeletal muscle taken at the lateral line, skin ulcer (if present), spleen, mid-kidney, liver, heart, pyloric caeca, and mid-intestine were collected and fixed in 10% neutral buffered formalin for up to one week. Fixed tissues were processed using standard methods to produce histological sections stained with haematoxylin and eosin (H&E). Further special stains of Giemsa were requested on liver, kidney, and spleen tissue from all fish.

2.2.3 Bacteriology

Liver, kidney, and skin ulcers were subjected to bacteriology. Liver and kidney from all 10 fish were sampled by making an incision into the organs using a sterile scalpel blade and inserting a sterile swab. This swab was then inoculated onto Columbia sheep's blood agar (BA) and Thiosulfate-citrate-bile salts-sucrose agar (TCBS) (both sourced from Fort Richard, Auckland, New Zealand).

Skin ulcers, where present (Fish 1 to 6 and 8 to 10), were sampled by first surface sterilising the ulcers using 70% ethanol to reduce the growth of potential external contaminating bacteria. A sterile incision was then made at the leading edge of the lesion and a sterile swab was inserted. The swab was then inoculated onto marine Anacker & Ordal agar (M-AO), Tryptic Soy agar with sheep's blood and 3% NaCl (TSA + 3%; both sourced from Fort Richard), and TCBS. All inoculated media was incubated at 22°C for 7 days. Inoculated media was checked for growth on day three and finally on day seven. Any common or dominant bacterial colonies were sub-cultured for purity on days three or seven and identification was carried out by biochemical and molecular methods.

Pure isolates were initially subjected to the following biochemical tests: Gram stain, cytochrome oxidase test (BD DIFCO BBL, New Jersey, USA), catalyse test (BD DIFCO BBL), spot indole test (BD DIFCO BBL), oxidative-fermentative tube test to determine metabolises of carbohydrates (O/F) (Fort Richard), motility (Fort Richard), and sensitivity to the vibriostatic agent 0/129 (Oxoid, Hampshire, UK). Following

biochemical tests, PCR and nucleotide sequencing was carried out for identification of isolates of interest.

2.2.4 DNA extraction

Thirty-six tissues comprising skin ulcer (if present), spleen, liver, and mid-kidney were individually and aseptically collected from each fish for DNA extraction immediately after collection.

DNA was extracted from tissues using an automated Qiagen QIAXtractor with the DX reagent kit (Qiagen, Valencia, USA). Briefly, 20 mg of mid-kidney, liver, and skin ulcer tissue, and 10 mg of spleen tissue were lysed overnight at 56°C in a lysis buffer with 10% digest enzyme. Lysed tissue was then centrifuged at 2,500 rpm for 5 min and 220 µL of the supernatant was transferred to a lysis block. This material was then subjected to extraction following the manufacturer's protocol for tissue. The concentration of DNA was measured by Qubit fluorometer (Life technologies, Oregon, USA) using the dsDNA HS Assay Kit (Life technologies) as per the manufacturer's protocol. DNA from bacterial isolates were extracted using Instagene matrix (Bio-rad, Hercules, USA) as per the manufacturer's protocol and DNA measured as above.

2.2.5 PCR

The presence of amplifiable DNA within tissues was confirmed using an internal 18S rRNA control (Ribosomal 18S rRNA Endogenous Control; Life technologies).

2.2.5.1 Conventional Piscirickettsia salmonis ITS rRNA PCR

A conventional PCR assay targeting the ITS rRNA region of *P. salmonis* (Marshall et al., 1998) was carried out on all DNA samples. Positive control material for *P. salmonis* was not available in the initial stages of testing so the PCR was performed as described by Marshall et al., (1998) with minor modifications. Molecular grade water was run as a no template control (NTC) with each PCR. DNA was added to a mixture of 12.5 µL Kapa2G Fast ReadyMix (2X) (Kapa Biosystems, Wilmington, USA) and 0.5 µM of each primer (RITS1 and RITS4) to a total volume of 25 µL with nuclease free water. All conventional PCR were performed on a Veriti Dx Thermal Cycler (Applied

Biosystems, Massachusetts, USA). The cycling conditions used were as follows: 1 cycle of 95°C for 3 min, followed by 35 cycles of 95°C for 15 sec, 50°C for 15 sec, 72°C for 1 sec, followed by 1 cycle of 72°C for 1 min. The PCR was further optimised once positive control material was available under different concentrations of primers and cycling conditions (data not shown). The final PCR reaction consisted of 0.56 µM of each primer, 12.5 µL of Kapa2G Fast Readymix (2X), and DNA to a final volume of 25 µL with nuclease free water. The cycling conditions were as above but with an increased annealing temperature of 55°C. All DNA from tissues were tested with a 2 µL and 5 µL template volume to be within the range of a final DNA concentration of 50 to 200 ng. All resulting products from the conventional PCR were resolved by electrophoresis in 1.5% agarose gel (Promega, Madison, USA) and stained with GelRed (Biotium, Fremont, USA).

2.2.5.2 Quantitative Piscirickettsia salmonis 23S rRNA PCR

A quantitative PCR (qPCR) assay targeting the 23S rRNA gene of *P. salmonis* (Corbeil et al., 2003) was implemented and all extracted DNA was tested. With each PCR, molecular grade water was run as a no template control (NTC). The PCR reaction consisted of 10 µL SsoAdvanced Universal Probes Supermix (Bio-Rad), 0.5 µM of each primer (F-760 and R-836) and 0.2 µM of probe (PS23S). The amount of DNA and nuclease free water were adjusted to make a total volume of 20 µL. The PCR was performed on a CFX 96 real-time PCR detection system (Bio-Rad) with the following cycling conditions: 1 cycle of 95°C for 2 min followed by 45 cycles of 95°C for 15 sec and 60°C for 30 sec, acquiring to the FAM channel. All samples were performed with both a 1 µL and 3 µL template volume to be within the range of a final concentration of DNA of 50 to 150 ng.

2.2.5.3 Conventional Piscirickettsia salmonis 16S rRNA PCR

A nested conventional PCR assay targeting the 16S rRNA gene of *P. salmonis* was performed on samples that tested positive by the *P. salmonis* 23S rRNA qPCR and *P. salmonis* ITS rRNA PCR. The assay was adapted from Mauel et al., (1996) with minor modifications. With each PCR, molecular grade water was run as a no template

control (NTC). In the primary round, DNA was added to a mixture of 12.5 µL Kapa2G Fast ReadyMix (2X) and 1 µM of each primer (Eub-A and Eub-B) to a total volume of 25 µL with nuclease free water and the following cycling conditions: 1 cycle of 95°C for 3 min, followed by 40 cycles of 95°C for 15 sec, 50°C for 15 sec, 72°C for 1 sec, followed by 1 cycle of 72°C for 1 min. The primary PCR round DNA template was used at a final concentration of 50 to 200 ng. In the nested round, 2 µL of PCR product from the primary round was added to a mixture of 12.5 µL Kapa2G Fast ReadyMix (2X) and 1 µM of each primer (PS2S and PS2AS) to a total volume of 25 µL with nuclease free water with cycling conditions as above but with an annealing temperature of 61°C. All resulting PCR products were resolved by electrophoresis in 1.5% agarose gel and stained with GelRed.

2.2.5.4 DNA sequencing and analysis of Piscirickettsia salmonis 16S rRNA and ITS rRNA

PCR products of the expected size from the *P. salmonis* 16S rRNA and ITS rRNA PCR were gel purified using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, USA). Purified PCR products were then submitted to EcoGene (Landcare Research, Auckland, New Zealand) for nucleotide sequencing. Sequencing was performed in the forward and reverse direction using the *P. salmonis* 16S rRNA and ITS rRNA PCR primers. The resulting sequences from the forward and reverse direction were aligned, manually edited and trimmed using Geneious version 7.1.5 (Kearse et al., 2012). Consensus sequences were subjected to Blast analysis using the National Centre for BioTechnology Information (NCBI) nucleotide blast. Returned sequences from the 16S rRNA gene and ITS rRNA region (Table 2.1) were aligned using the Geneious alignment and default parameters.

Table 2.1. *Piscirickettsia salmonis* and piscirickettsia-like organism strains used to compare with the New Zealand rickettsia-like organism in the 16S rRNA gene and internal transcribed spacer region (ITS rRNA). Strain name is followed by the GenBank accession in brackets.

***Piscirickettsia salmonis* strains**		**Piscirickettsia-like organism strains**	
16S rRNA	**ITS rRNA**	**16S rRNA**	**ITS rRNA**
LF-89 (U36941)	LF-89 (U36943)	SC-2004 (AY578984)	SC-2004 (AY578985)
NOR-92 (U36942)	NOR-92 (U36946)		SBPLO (AY607584)
ATL-4-91 (U36915)	ATL-4-91 (U36945)		
SLGO-94 (U55015)	EM-90 (U36944)		
EM-90 (U36940)	C1-95 (U62103)		
AL-10015 (EU289216)	AL-10015 (EU289216)		
	IBM-019 (KF831146)		

2.2.5.5 Bacterial identification PCR and nucleotide sequencing

2.2.5.5.1 Vibrio species identification.

Suspect *Vibrio* species (Gram-negative and 0/129 sensitive isolates) were subjected to the *Vibrio* species PCR targeting the atpA gene as previously published (Thompson, Thompson, Vicente, & Swings, 2007) with minor modifications. Briefly, DNA of concentration 1 ng was added to a mixture of 12.5 µL Kapa2G Fast ReadyMix (2X) and 0.5 µM of each primer (atpA-06F and atpA-04R) to a total volume of 25 µL with nuclease free water. All conventional PCR were performed on a Veriti Dx Thermal Cycler. Cycling conditions for this assay were as follows: 1 cycle of 95°C for 2 min, followed by 35 cycles of 95°C for 15 sec, 58°C for 15 sec, 72°C for 30 sec. With each PCR, molecular grade water was run as a no template control (NTC) and a positive control using DNA extracted from a pure culture of *Vibrio splendidus* (ATCC 33871) at 1 ng was used.

2.2.5.5.2 16S rRNA identification.

Bacteria that were not suspect *Vibrio* species (Gram-positive or Gram-negative and resistant to the vibriostatic agent 0/129) were subjected to a bacterial species PCR targeting the 16S rRNA gene as previously published (Lane, 1991) with minor modifications. Briefly DNA of concentration 2.5 ng was added to a mixture of 12.5 µL Kapa2G Fast ReadyMix (2X) and 0.5 µM of each primer (27f and 1525r) to a total

volume of 25 µL with nuclease free water. Cycling conditions for this assay were as follows: 1 cycle of 95°C for 2 min, followed by 35 cycles of 95°C for 15 sec, 50°C for 15 sec, 72°C for 1 sec. With each PCR, molecular grade water was run as a no template control (NTC) and a positive control using DNA extracted from a pure culture of *Escherichia coli* (ATCC 25922) at 0.1 ng was used.

All resulting amplicons from the conventional atpA gene or 16S rRNA gene PCR were resolved by electrophoresis in a 1.5% agarose gel and stained with GelRed. PCR products of the expected size were gel purified using a Zymoclean Gel DNA Recovery Kit (Zymogen Research). Purified PCR products were then submitted for nucleotide sequencing (Landcare Research). Sequencing was performed in the forward and reverse direction using the PCR primers. The resulting sequences from the forward and reverse direction were aligned, manually edited, and trimmed using Geneious. Consensus sequences were subjected to Blast analysis using the National Centre for BioTechnology Information (NCBI) nucleotide blast.

2.2.5.6 Quantitative Tenacibaculum maritimum PCR

A qPCR targeting the 16S rRNA gene of *T. maritimum* was performed as previously described (Fringuelli et al., 2012). Any amplification <40 cycle threshold (Ct) resulting from this PCR was considered positive based on validation work carried out at the Animal Health Laboratory (data not shown). All DNA from tissues were used in the PCR with both a 1 µL and 3 µL template volume to be within the range of a final concentration of DNA of 50 to 150 ng. With each PCR, molecular grade water was run as a no template control (NTC) and a positive control using DNA extracted from a pure culture of *Tenacibaculum maritimum* (W15_494#9) at 1 ng was used.

2.3 Results

2.3.1 Gross pathology

The fork length of the 10 fish submitted ranged from 47 to 57 cm. On necropsy, eight of the 10 fish displayed multiple skin ulcers over the body, nose, jaw, and fins (Figure 2.1A). Skin ulcers ranged from 4 mm in diameter to 80 mm width x 20 mm in length,

accompanied in some cases with loss of the dermis exposing the musculature (Figure 2.1B). In addition, seven of the 10 fish presented with skin lesions that had healed (Figure 2.1C). Minor petechial haemorrhage of the ventral surface was also seen in four fish (Fish 2, 3, 8, and 10) and ecchymosis was seen in six fish (Fish 1, 4, 5, 6, 8, and 10). Four fish (Fish 1, 2, 4, and 5) had isopod parasites present in the gills or mouth.

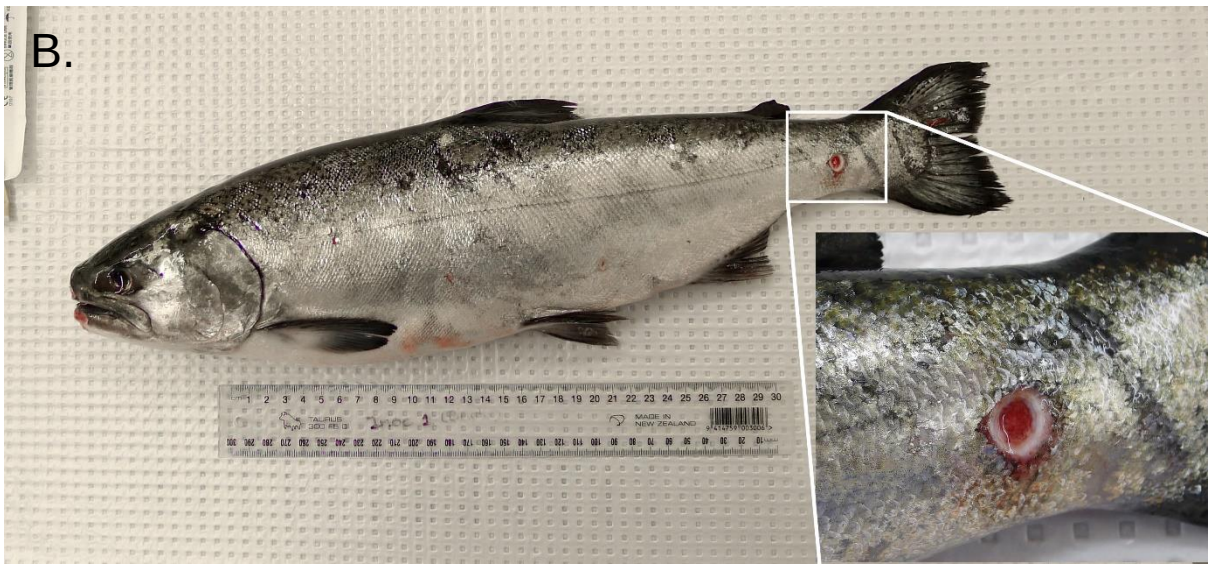


Figure 2.1. Gross pathology of the sampled Chinook salmon (**A**) showing multiple skin ulcers over the body surface, (**B**) a representative skin ulcer observed in these fish extending through the dermis exposing the musculature (**C**) an example of a healed ulcer, above the lateral line.

When examined internally, four fish displayed pale liver and five fish displayed mottled, pale kidney. Reddening of the intestine was observed in five fish and five fish presented with petechial haemorrhage on the internal adipose tissue surrounding the pyloric caeca (Table 2.2).

Table 2.2. Summary of the gross lesions and histological lesions observed from the 10 fish analysed during the present study

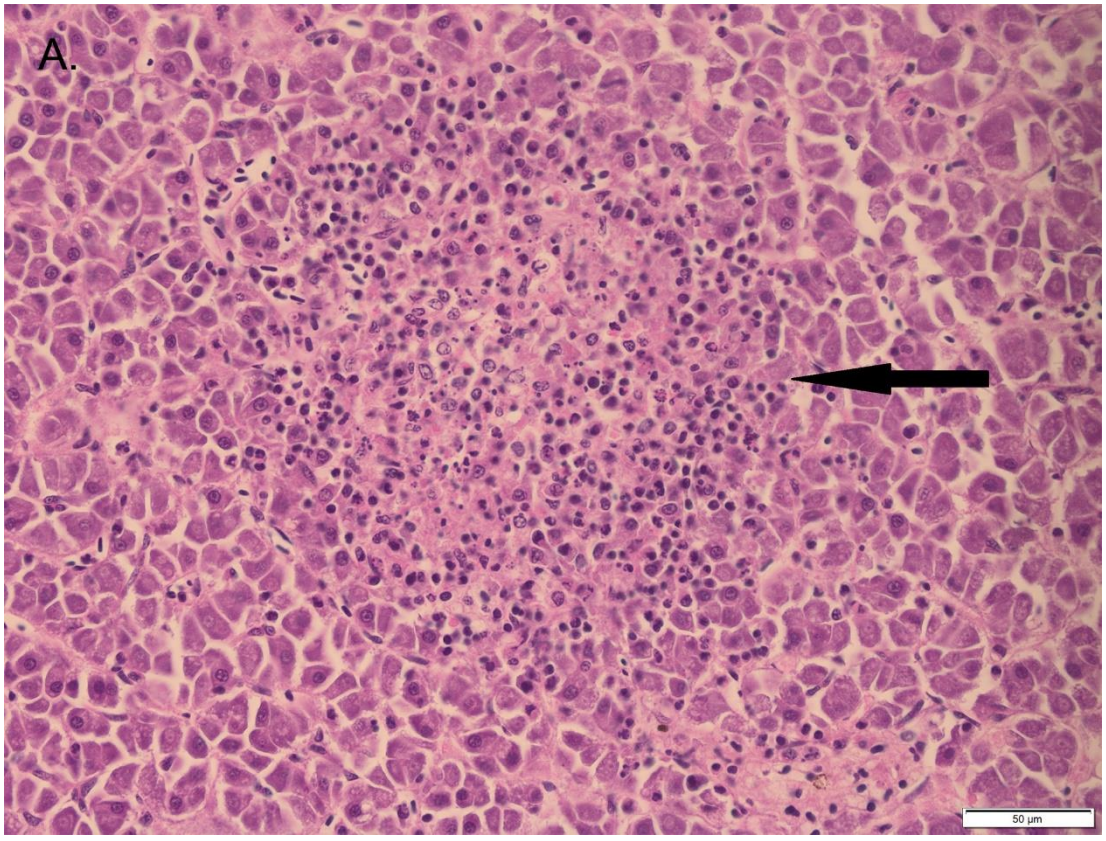
	Gross lesions							Histological lesions					
Fish No.	**Skin ulcers**	**Petechiae in skin**	**Ecchy-mosis**	**Pale liver**	**Mottled kidney**	**Intestine reddening**	**Haemorrhage in the internal adipose tissue**	**Spleen**	**Kidney**	**Heart**	**Liver**	**Skin**	**Peri-pancreatic adipose tissue**
1	+	-	+	+	+	-	+	-	Glomerulo -nephritis	I	+ PBC, focal necrosis.	I	-
2	+	+	-	+	+	-	-	+ PBC, F	-	-	+ PBC	I	-
3	+ (and healing ulcer)	+	-	-	-	+	-	F, necrosis, depletion of lymphatic cells.	+ PBC	-	-	I	-
4	+ (and healing ulcer)	-	+	+	+	+	+	-	Glomerulo -nephritis	-	+ PBC	Myositis.	-
5	+	-	+	+	-	+	-	F	-	-	-	I	-

	Gross lesions							Histopathology lesions					
Fish No.	**Skin ulcers**	**Petechiae in skin**	**Ecchy-mosis**	**Pale liver**	**Mottled kidney**	**Intestine reddening**	**Haemorrhage in the internal adipose tissue**	**Spleen**	**Kidney**	**Heart**	**Liver**	**Skin**	**Peri-pancreatic adipose tissue**
6	+ (and healing ulcer)	-	+	-	-	+	+	F	+ PBC	-	-	Myositis.	I
7	Healing ulcer.	-	-	-	+	-	-	F	-	-	+ PBC, I, necrosis.	-	-
8	+ (and healing ulcer)	+	+	-	+	-	+	Depletion of lymphatic cells.	-	-	-	I	I
9	Healing ulcer.	-	-	-	-	-	-	Depletion of lymphatic cells.	-	-	-	-	-
10	+ (and healing ulcer)	+	+	-	-	+	+	+ PBC	-	I	+ PBC	I	-

KEY: + = present, - = absent, PBC = pleomorphic basophilic cocci-like organisms, I = inflammation, F = fibrosis.

2.3.2 Histopathology

Small pleomorphic basophilic intra-cytoplasmic cocci-like bacteria approximately 1 - 1.4 µm in size, were visible within inflammatory cells in the following tissues: the liver of five fish (50%), the spleen of one fish (10%), and the kidney of two fish (20%). These bacteria were consistent with previous descriptions of RLO. Lesions with inflammatory infiltrate and necrosis were commonly found in tissues where RLO where observed (Figure 2.2A). Under Giemsa stain, bacteria resembling RLO stained dark blue (Figure 2.2B). Overall, bacteria consistent with RLO were detected by histology in seven fish (70%) examined in either the spleen, kidney, or liver (Table 2.2).



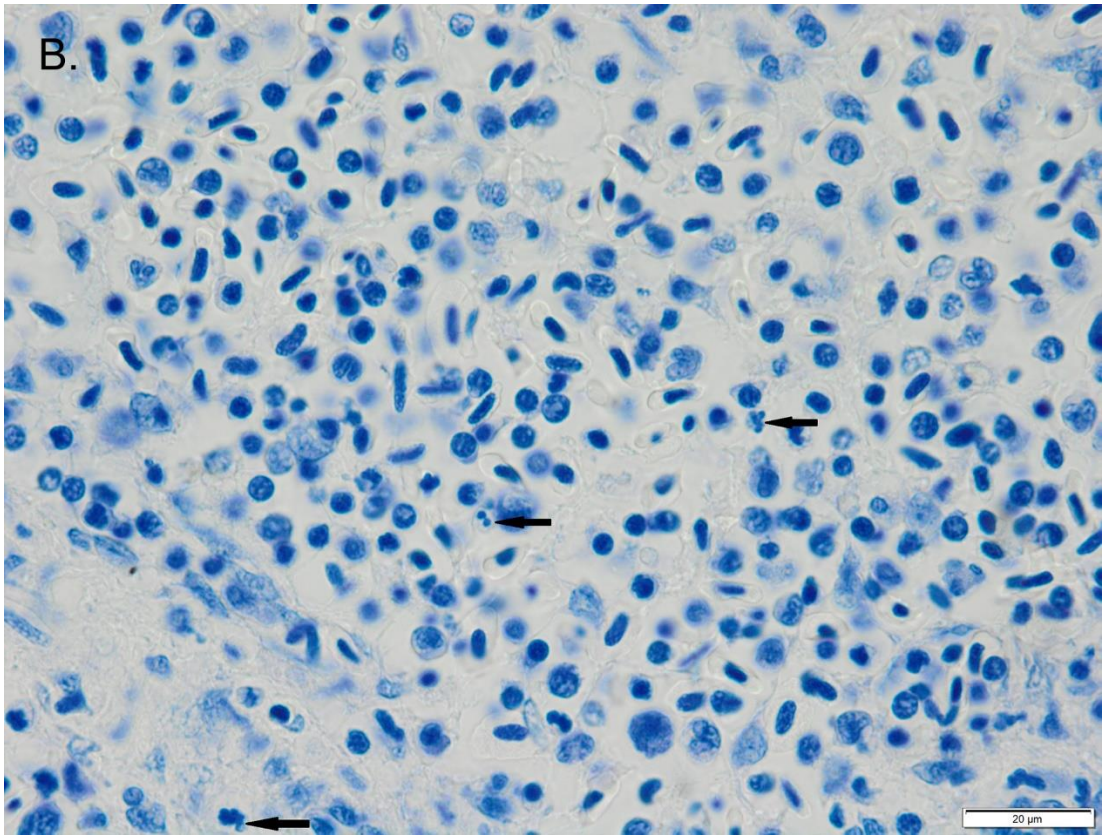


Figure 2.2. Liver from a fish affected by summer mortalities. **A.** Note the area of hepatocyte depletion, inflammation and necrosis (arrow), H&E, 400 x magnification. **B.** Liver from a fish affected by summer mortalities. Note the dark blue pleomorphic intra-cytoplasmic cocci-like bacteria ~1-1.4 µm in size (arrows), Giemsa, 1000 x magnification.

Examination of the kidney from two fish (20%) revealed glomerulonephritis with the main lesion being peri-glomerula epithelial thickening, fibrosis of Bowman's capsule, and occlusion of Bowman's space (Figure 2.3A). Examination of the spleen of three fish (30%) revealed depletion of the lymphatic cells and five fish (50%) revealed perivascular fibrosis. Randomly scattered focal necrosis and degeneration of the hepatocytes was visible in the liver of two fish (20%). Additionally, focal areas of inflammation in the heart ventricle was observed in two fish (20%). Histological examination of the pyloric caeca revealed mild neutrophilic inflammation of the peri-pancreatic adipose (Figure 2.4) in two fish (20%).

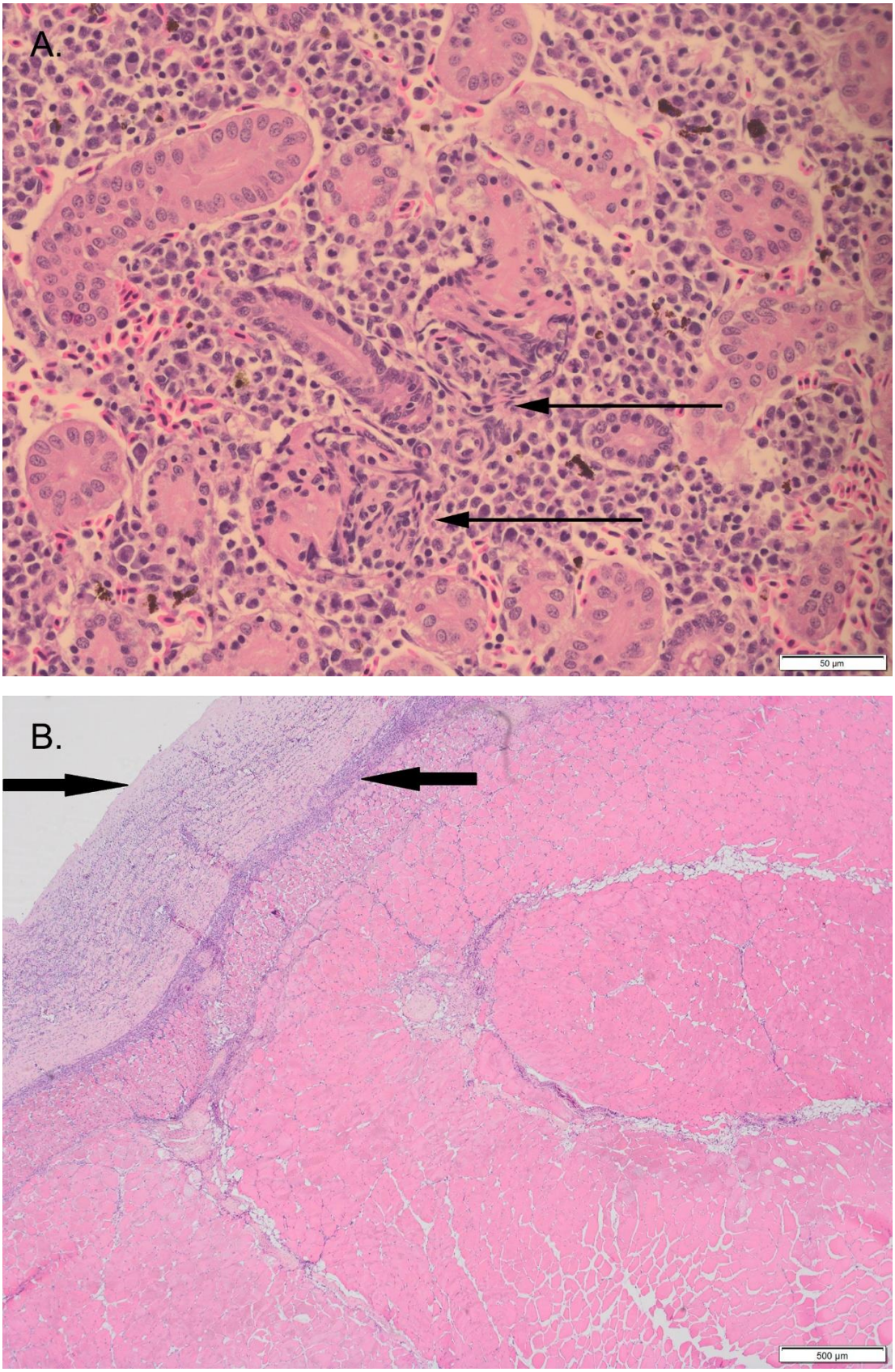


Figure 2.3. Kidney from fish affected by summer mortalities. **A.** Note glomerulonephritis with thickening of the basement membrane (arrows), H&E, 400 x magnification. **B.** Skin ulcer from fish affected by summer mortalities. Note the inflammation of the dermal layer and hypodermis extending into the musculature (arrows), H&E, 40 x magnification.

Histological examination of the skin ulcers revealed loss of the epidermis, scales, and stratum spongiosum as well inflammation of the stratum compactum and hypodermis (Figure 2.3B) with inflammation extending into the musculature. Myositis was observed in two fish (Table 2.2).

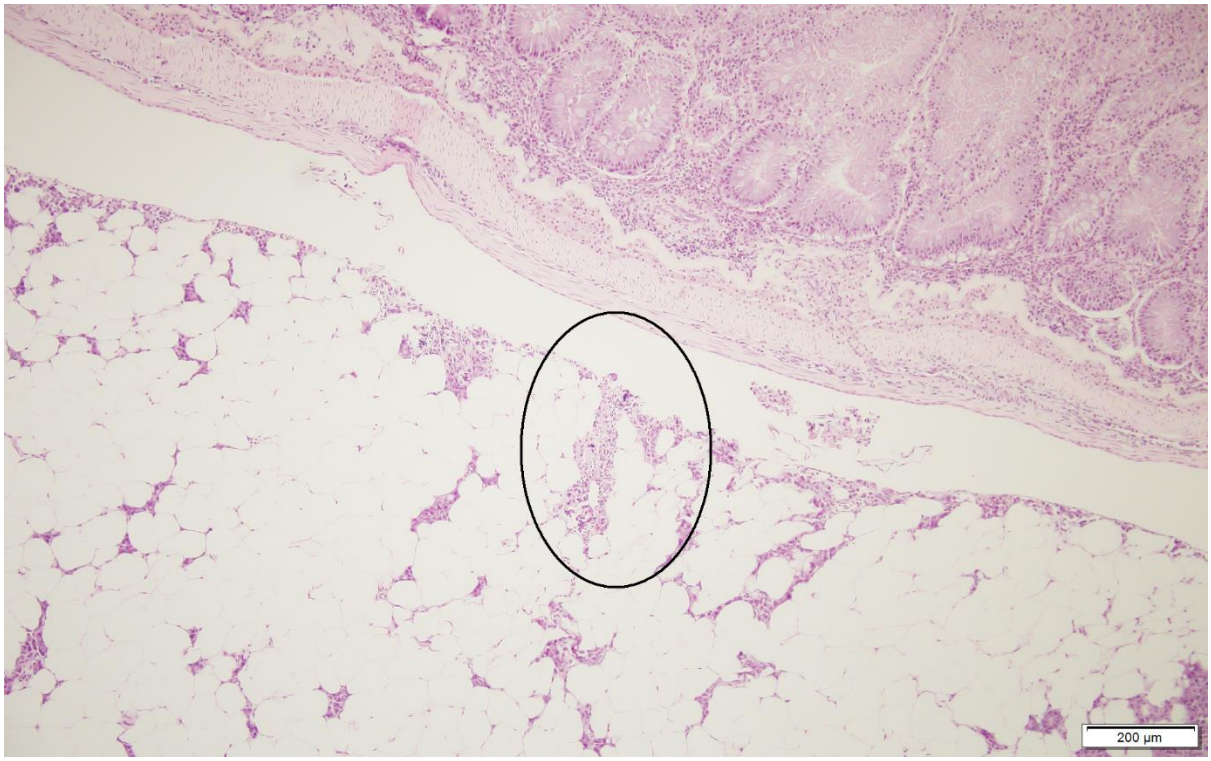


Figure 2.4. Pancreas from a fish evaluated during the summer mortalities. Note the mild infiltrate of inflammatory cells within the surrounding adipose tissue (circle), 100 x magnification.

Histological examination of part of the mid-intestine revealed thickening in the smooth muscle layer, congestion of the blood vessels and a loosening of the stratum compactum (Figure 2.5). Thickening of the muscle and congestion of the blood vessels was observed in all four fish that presented with reddening of the intestine on necropsy.

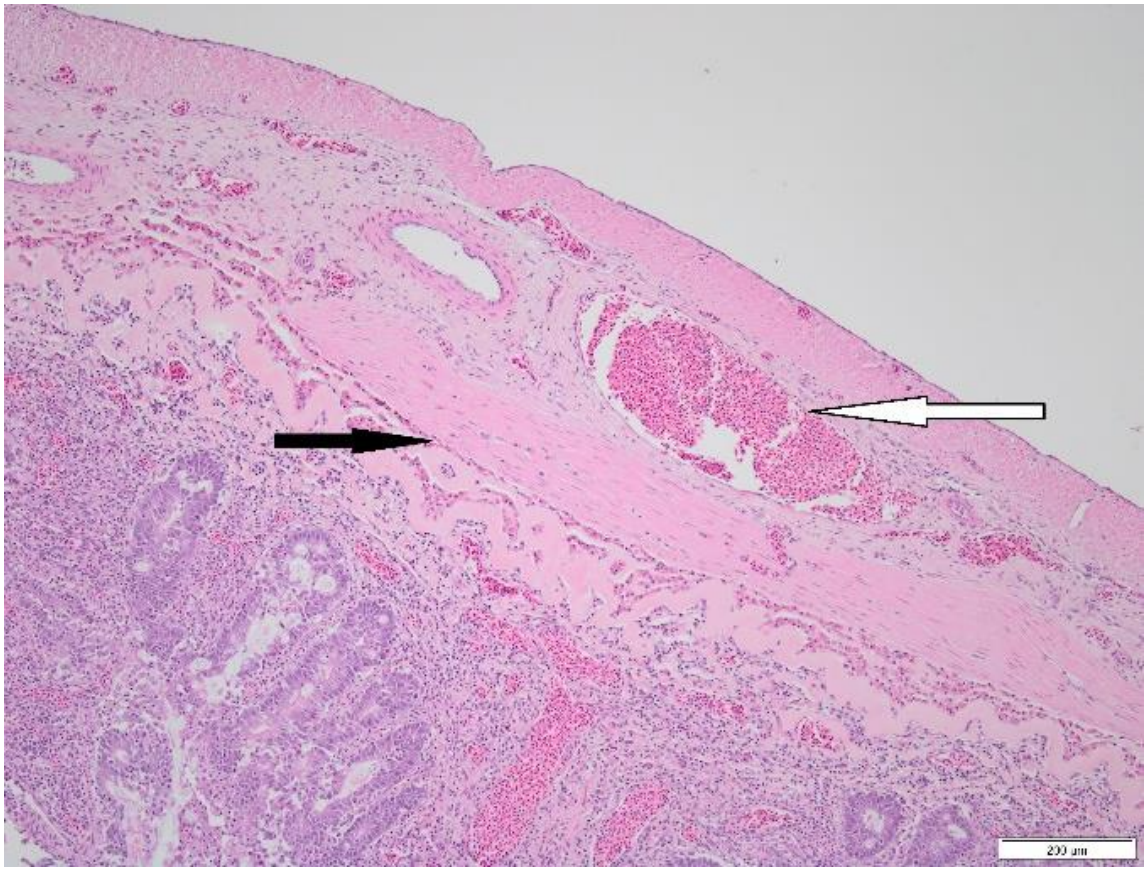


Figure 2.5. Distal intestine from fish affected by summer mortalities. Note the thickening of the longitudinal muscle (black arrow) with congestion of the blood vessels (white arrow). H&E, 200 x magnification.

2.3.3 Bacteriology

Kidney tissue subjected to bacteriology revealed no growth from four fish on TCBS or BA (Fish 1,2,4,6). Light mixed growth was recovered from four fish on BA (Fish 3,5,10) or TCBS (Fish 7) including common growth of *Vibrio scophthalmi* (Fish 7 and 10). Moderate mixed growth was recovered from two fish on TCBS and BA (Fish 8), or BA (Fish 9) including common growth of *Vibrio harveyi* (Fish 8; Table 2.3).

Growth from liver tissues subjected to bacteriology revealed no growth from eight fish on TCBS or BA (Fish 1,2,3,4,5,6,7,9), light mixed growth from one fish on TCBS and BA (Fish 10), and moderate mixed growth from one fish on TCBS and BA (Fish 8), including common growth of *V. harveyi*.

Skin ulcers revealed no growth from one fish on TCBS, TSA + 3%, or M-AO (Fish 2). Light mixed growth was recovered from six fish on TSA + 3%, and M-AO (Fish 1),

TCBS, M-AO, or TSA + 3% (Fish 3 and 8), or TSA +3% (Fish 4, 5, 6) including common growth of *Olleya* species (Fish 4), *Pseudomonas* species and *Tenacibaculum maritimum* (Fish 6), and *V. harveyi* (Fish 8). Moderate mixed growth with no significant or common growth was recovered from Fish 10 on TSA + 3%. Results of the biochemical tests and nucleotide sequencing results for each identified bacteria can be seen in Table 2.4.

Table 2.3. Summary of general bacteriology results from the ten fish analysed including names of the common bacterial species present.

	Tissue tested		
Fish Number	**Kidney**	**Liver**	**Skin ulcer**
1	NG	NG	LMG
2	NG	NG	NG
3	LMG	NG	LMG
4	NG	NG	LMG, *Olleya* species
5	LMG	NG	LMG
6	NG	NG	LMG, *Pseudomonas species, T. maritimum*
7	LMG, *V. scopthalmi*	NG	NA
8	MMG, *V. harveyi*	MMG, *V. harveyi*	LMG, *V. harveyi*
9	MMG	NG	NA
10	LMG, *V. scopthalmi*	LMG	MMG

NG = no growth, LMG = light mixed growth, MMG = moderate mixed growth, NA = not available.

Table 2.4. Identification of the common or dominant bacterial isolated was carried out by biochemical testing and nucleotide sequencing. Biochemical and nucleotide sequencing results from each of the identified bacteria isolated from analysed fish.

Test	*Vibrio scopthalmi*	*Vibrio harveyi*	*Olleya* species	*Pseudomonas* species	*Tenacibaculum maritimum*
Gram stain	Gram-neg. curved rods	Gram-neg. small rods	Gram-neg. long rods	Gram-neg. rods	Gram-neg. long filamentous rods
Cytochrome oxidase	+	+	+	+	+
Catalase	+	+	+	+	+
Indole	-	+	-	-	-
Motility	+	+		+	Gliding
O/F	+/+	-/+	NG	-/-	-/-
0/129 sensitivity	S	S	S	R	NT
16S rRNA gene sequencing	NT	NT	99% identity to GenBank accession JX844463	99% identity to GenBank accession JQ995152	99% identity to GenBank accession KJ651988
atpA gene sequencing	99% identity to GenBank accession EF601365	100% identity to GenBank accession JF23507	NT	NT	NT

NG = no growth, S = sensitive to the vibriostatic agent 0/129, R = resistant to the vibriostatic agent 0/129, NT = not tested due to Gram morphology discounting isolate as *Vibrio* suspect.

2.3.4 PCR and DNA sequencing

2.3.4.1 Conventional Piscirickettsia salmonis ITS rRNA PCR and nucleotide sequence analysis

Under initial *P. salmonis* ITS rRNA PCR conditions prior to optimisation, 13 of 36 tissue samples from six fish (Fish 3 kidney, lesion, liver, and spleen; Fish 5 spleen; Fish 6 liver and lesion; Fish 8 kidney; Fish 9 kidney, liver, and spleen; Fish 10 kidney and spleen), produced amplicons of the expected molecular weight, approximately 300 base pairs (bp). Nucleotide sequencing of these amplicons revealed 12 of 13 samples to have highest similarity to Chinook salmon, 98% to 99% similarity with 86 to 95% coverage.

One of these samples (Fish 10, spleen) returned a result of 99% similarity to Tasmanian-RLO strain SC-2004 (AY578985). This sample was then used as a positive control for further optimisation of the *P. salmonis* ITS rRNA PCR.

Following optimisation of the *P. salmonis* ITS rRNA PCR, DNA samples were repeated in the ITS rRNA PCR. Results from the repeated PCR revealed amplicons of the expected size in the kidney and spleen samples from two fish (Fish 8 and 10). Nucleotide sequencing of these amplicons all returned 99% similarity to Tasmanian-RLO strain SC-2004 (AY578985) (Table 2.5).

Genetic analysis of these sequences revealed a consecutive 19 bp deletion at the 3'-end of the ITS rRNA sequence compared with other *P. salmonis* strains deposited in GenBank (KU523537). This deletion also occurred in the Tasmanian-RLO strain SC-2004 (AY578985) from Atlantic salmon in Tasmania and strain SBPLO (AY607584) from European seabass in Greece (Figure 2.6).

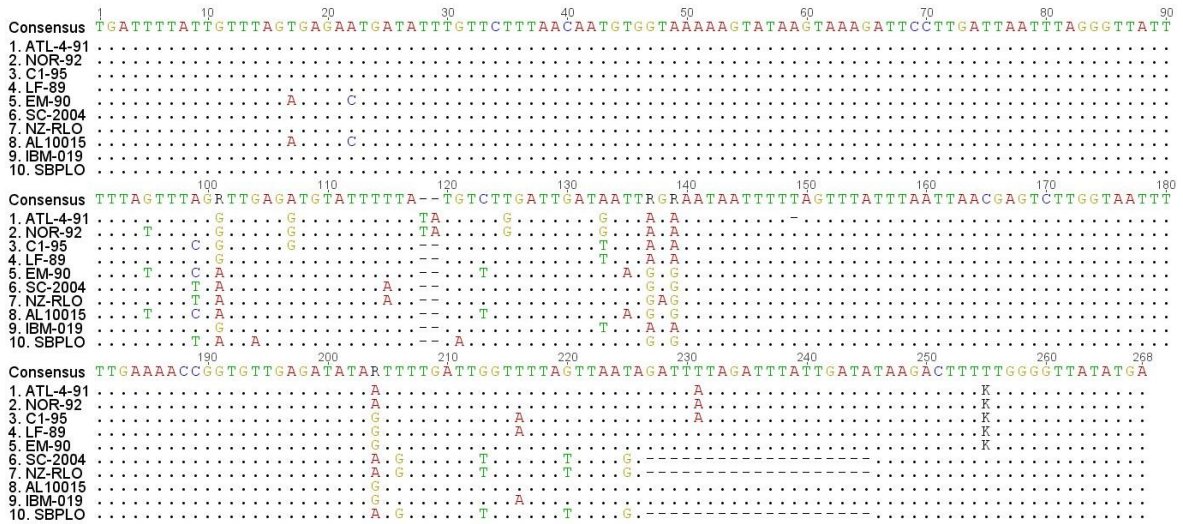


Figure 2.6. Partial ITS rRNA region nucleotide sequence alignment of the NZ-RLO aligned with sequences of other *Piscirickettsia salmonis* and PLO strains available in GenBank.

2.3.4.2 Quantitative Piscirickettsia salmonis 23S rRNA qPCR

Fish 6, 8, and 10 produced amplification in the qPCR. The results were as follows: kidney and spleen from Fish 8 had a Ct value of 35.05 and 33.36 respectively; kidney, liver, and spleen from Fish 10 had Ct values of 31.98, 36.37, and 33.28 respectively; and liver and spleen samples from Fish 6 had Ct values of 36.77 and 37.17 respectively (Table 2.5). Attempts were made to confirm the qPCR results by nucleotide sequencing from the liver and spleen of Fish 6 and liver sample of Fish 10, however no positive amplicons were able to be produced by the conventional ITS rRNA or 16S rRNA PCR.

2.3.4.3 Conventional Piscirickettsia salmonis 16S rRNA PCR and sequence analysis

A nested conventional PCR targeting the 16S rRNA gene of *P. salmonis* was performed on the liver and spleen samples from Fish 6, kidney and spleen samples from Fish 8, and kidney, liver, and spleen samples from Fish 10. Of these samples, only the spleen from fish 10 produced an amplicon. The nucleotide sequence analysis of this amplicon showed 100% similarity to Tasmanian-RLO strain SC-2004 (AY578984) (Table 2.5).

Table 2.5. Summary of *Piscirickettsia salmonis* PCR results from the 10 fish analysed during the present study.

	***Piscirickettsia salmonis* PCR**		
Fish No.	**Kidney**	**Spleen**	**Liver**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	+ 23S (Ct 37.17)	+ 23S (Ct 35.50)
7	-	-	-
8	+ 23S (Ct 36.15), ITS	+ 23S (Ct 33.55), ITS	-
9	-	-	-
10	+ 23S (Ct 31.96), ITS	+ 23S (Ct 33.98), ITS, 16S	+ 23S (Ct 36.56)

KEY: 23S = qPCR targeting the 23S rRNA gene, 16S = conventional PCR targeting the 16S rRNA gene, ITS = conventional PCR targeting the internal transcribed spacer region, Ct = cycle threshold. Note, no NZ-RLO DNA was detected in skin ulcers from any fish so were not included in the table.

Sequence analysis of the *P. salmonis* 16S rRNA gene showed single nucleotide polymorphism variations to other *P. salmonis* strains including the reference strain LF-89 (Figure 2.7), and were identical to that of the variations displayed by the Tasmanian-RLO strain SC-2004 (AY578984) in the partial 16S rRNA nucleotide sequence analysed.

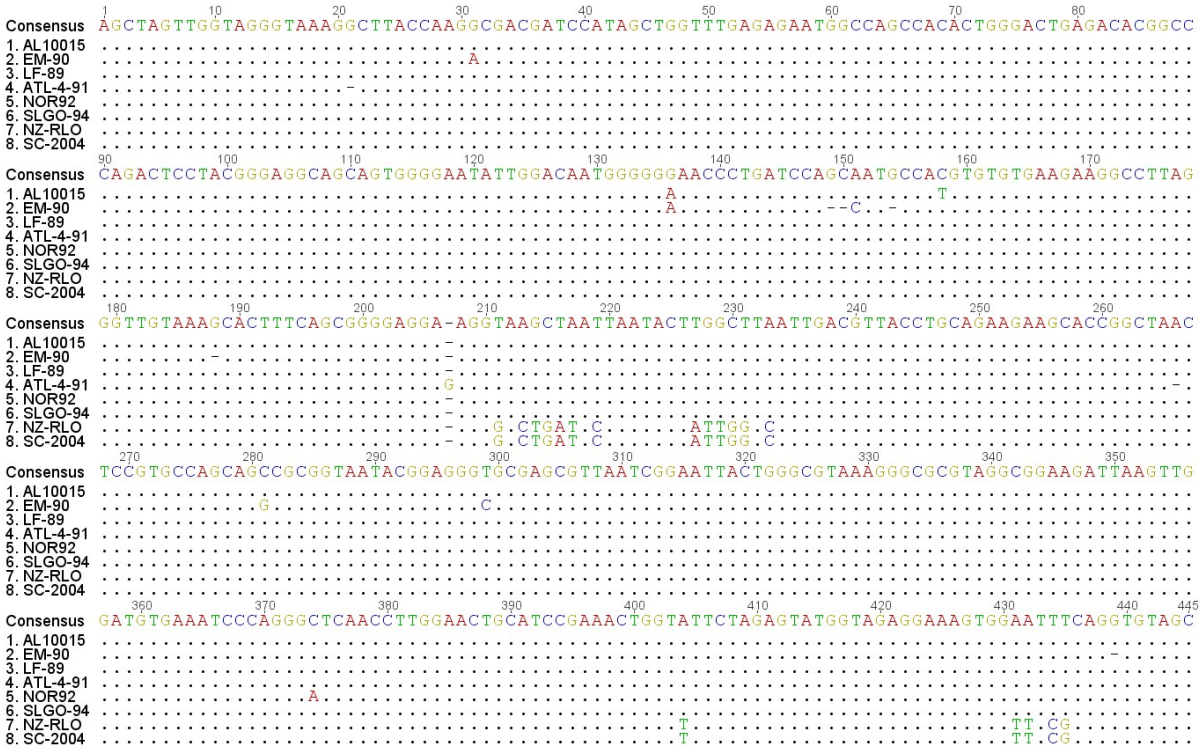


Figure 2.7. Partial 16S rRNA gene nucleotide sequence alignment of the NZ-RLO aligned with sequences of other *Piscirickettsia salmonis* and PLO strains available in GenBank.

2.3.4.4 Quantitative Tenacibaculum maritimum PCR

Seven of the 10 fish (70%) produced amplification in the qPCR. The results were as follows: skin ulcer from Fish 1, 3, 4, 6, and 8 had Ct values of 34.65, 34.1, 36.11, 36.4 and 28.14 respectively; kidney from Fish 7 had a Ct value of 37.47; and spleen from Fish 7 and 10 had Ct values of 36.67 and 36.61 respectively (Table 2.6).

Table 2.6. Summary of *Tenacibaculum maritimum* qPCR results from the 10 fish analysed. - = no Ct value produced in the qPCR.

	***Tenacibaculum maritimum* qPCR (Ct values)**			
Fish No.	**Kidney**	**Liver**	**Spleen**	**Skin ulcer**
1	-	-	-	34.65
2	-	-	-	-
3	-	-	-	34.1
4	-	-	-	36.11
5	-	-	-	-
6	-	-	-	36.4
7	37.47	-	36.67	-
8	-	-	-	28.14
9	-	-	-	-
10	-	-	36.61	-

2.4 Discussion

Piscirickettsia salmonis, PLO, and RLO are associated with severe bacterial diseases affecting aquaculture globally (Mauel and Miller, 2002), including Australia (Corbeil et al., 2005). These bacteria have not previously been in detected New Zealand. Necropsy, histopathology, and PCR tests in the present study revealed a newly detected rickettsia-like organism (NZ-RLO) that was identified from fish analysed from this mortality event. Another potential pathogen, *T. maritimum,* was isolated from these fish by bacteriology and PCR. Due to the small number of fish examined and the low levels of these pathogens in the fish, the significance of the NZ-RLO and *T. maritimum* from the present study could not be determined.

Gross lesions observed in the fish examined in the present study were consistent with those reported previously for piscirickettsiosis (Bravo and Campos, 1989; Cvitanich et al., 1991; Branson and Diaz-Munoz, 1991). In recent years, fish reported with *P. salmonis* have often presented with multiple diffuse skin ulcers over the body surface (Rozas and Enriquez, 2014) as was most commonly seen in the fish from the present study. Petechiae or ecchymosis in the skin was noted on eight of the fish analysed. Petechiae or ecchymosis are common clinical signs of diseased fish, often resulting from a systemic infection due to a bacterial or viral cause. Petechiae in the skin is a true finding and not a post-mortem artefact. However, ecchymosis or reddening can be a post mortem artefact, or livor mortis. As these fish were analysed 24 hours post mortem and the areas of skin were not examined histologically the findings of reddening in these fish in relation to disease cannot be confirmed. A characteristic, but not always present clinical sign of piscirickettsiosis is pale, sub-capsular nodules, or ring-shaped foci within the liver observed on necropsy (Branson and Diaz-Munoz, 1991, Cvitanich et al., 1991; Olsen et al., 1997). Gross liver lesions were not observed in any of the fish analysed in the present study. Studies carried out on Chilean Coho, Atlantic, and Chinook salmon in 1991 (Cvitanich et al., 1991) noted that the presence of these lesions in the liver had diagnostic significance and were always present in heavily infected fish. However, gross pathology of this disease can vary with some infected fish exhibiting no external symptoms (McCarthy, 2005).

Histological examination showed the presence of bacteria resembling RLO in the liver, kidney, and spleen consistent with piscirickettsiosis as previously described (Branson and Diaz-Munoz, 1991; Rodger and Drinan, 1993). These RLO were found in tissues of seven of 10 fish examined. However, DNA was only detected in two fish by conventional PCR and three fish by qPCR. It is possible that due to the focal distribution of NZ-RLO within the tissue, DNA extraction failed to select the tissue with NZ-RLO thus subsequent PCR testing did not amplify any DNA. Alternatively, it remains possible that another intracellular organism may be present in these affected fish. Use of *in-situ* hybridization methods using specific probes would aid in this confirmation*. In-situ* hybridization was not available during the present study but is described further in Chapter 3.

Inflammation and necrosis of the intestine have been reported in Chilean Coho salmon affected with piscirickettsiosis (Branson and Diaz-Munoz, 1991; Cvitanich et al., 1991) including visualisation of basophilic granules. Histological examination of the intestine in the present study is consistent with changes seen related to the diet of the salmon, and it is suspected not to have a pathogenic cause (Dale, Torud, Kvellestad, Koppang, & Koppang, 2009; Forgan and Forster, 2007). An investigation into NZ-RLO affected and unaffected fish on the same diet may provide some insight into whether there is any correlation of the intestine pathology and the disease agent present. Histological examination of kidney tissue revealed glomerulonephritis in two fish. Glomerulonephritis has previously been described in Chinook salmon (Lumsden et al., 2008) and attributed, by them, to an immune-complex glomerulonephritis of unknown aetiology after careful examination for pathogens, including by transmission electron microscopy. This therefore is unlikely to be associated with NZ-RLO as glomerulonephritis has been observed in New Zealand for a long time whereas NZ-RLO is a recently detected pathogen. Alternatively, it is possible that NZ-RLO has been present, but has gone undetected, long before being detected in the present investigation. Due to the detection of NZ-RLO in the present study, historical sampling of tissues from the 2012 summer mortality (Normal et al., 2013) was carried out which revealed the presence of NZ-RLO1 DNA (Fischer and Appleby, 2017), proving this pathogen has been present in New Zealand since at least 2012.

Testing by PCR of three genes followed by nucleotide sequencing confirmed the presence of NZ-RLO DNA in three fish. Nucleotide sequencing carried out on the ITS rRNA region and 16S rRNA gene, showed variations to the reference strain of *P. salmonis* (LF-89) and other *P. salmonis* strains from Chile and Norway. The alignment of the partial *P. salmonis* ITS rRNA region and 16S rRNA gene with different strains showed NZ-RLO aligned to the SBPLO strain, isolated from European Sea Bass in Greece (GenBank accession number AY607584) and the strain SC-2004 (GenBank accession number AY578985 and AY578984) isolated from Atlantic salmon in Tasmania (Corbeil et al., 2005). The levels of mortality these PLO and RLO induced in the host species were low in Greece (McCarthy et al., 2005), and 26.8% in Tasmania (Zainathan, 2012). These levels of mortality are much lower than those reported in the present study as well as in outbreaks from Chile and Norway of fish infected with *P. salmonis* which can be greater than 90% (Mauel and Miller, 2002). This may suggest that NZ-RLO is not as virulent as the strains from Chile and Norway and is not the primary cause of summer mortalities. The lower levels of mortality may also be related to host susceptibility or environmental conditions. The outbreaks in Tasmania in association with Tasmanian-RLO often occur as co-infections with Tasmanian reovirus (TSRV) or elevated seawater temperatures (Zainathan, 2012) which may suggest NZ-RLO infections are more likely a multifactorial event potentially including other infectious agents and environmental conditions resulting in the high mortality rate seen in the present study.

During the present study, three molecular assays were used to detect NZ-RLO that did not have validation data for the diagnostic specificity and sensitivity. Previous validation of analytical specificity and sensitivity showed high specificity when tested with other related bacteria and fish DNA (Corbeil et al., 2003, Marshall et al., 1998, Mauel et al., 1996). However, in the present study it was observed that the *P. salmonis* ITS rRNA PCR lacked specificity when tested with DNA extracted from New Zealand Chinook salmon and carried out under the published conditions. This was illustrated by 12 of 13 samples producing amplicons of the correct molecular weight but nucleotide sequencing returning highest similarity to Chinook salmon DNA. Furthermore, a loss of sensitivity had been observed by Corbeil et al., (2003) when DNA from *P. salmonis* was inoculated into a homogenate of un-infected fish tissue and tested by the qPCR. Work conducted during the present study also showed a

reduction of sensitivity in the *P. salmonis* 16S rRNA PCR compared with the *P. salmonis* ITS rRNA PCR and 23S rRNA qPCR when NZ-RLO DNA was spiked into un-infected Chinook salmon DNA and performed on the two PCR assays (data not shown) suggesting that host DNA is interfering with the PCR.

The present study demonstrated that when DNA is subjected to the *P. salmonis* 23S rRNA qPCR assay using conditions described in this study, a sample with a Ct value greater than 35 did not produce an amplicon in the *P. salmonis* ITS rRNA PCR. The same was not observed with the *P. salmonis* 16S rRNA assay as a sample from fish tissue with a Ct value of 33.28 produced an amplicon in the 16S rRNA assay but a sample with a Ct value of 31.98 did not. This further suggests that the Chinook salmon DNA may be impacting on the sensitivity of the *P. salmonis* 16S rRNA PCR generating inconsistent results when testing the pathogen in DNA extracted from New Zealand Chinook salmon tissue. Developing specific assays for NZ-RLO may help for more specific detection in Chinook salmon tissues.

Pathogenicity of NZ-RLO in farmed Chinook salmon is currently unknown. Based on the low prevalence of NZ-RLO in the fish tested (three of 10 fish positive by PCR) and the limited amount of evidence to prove association and causation based on the Bradford Hill criteria (Hill, 1965) it is suggested that this organism may not be the primary cause of the high mortalities. However, these fish were sampled after the peak of the mortality, thus after the pathogen numbers had peaked. The 10 fish tested may have been immunocompromised due to the immunological defence of a pathogen and the low level of NZ-RLO may be indicative of a recovering infection.

Bacteriology revealed the presence of *Tenacibaculum maritimum,* the aetiological agent of tenacibaculosis in marine fish. *Tenacibaculum maritimum* was isolated from the skin ulcer in one fish on TSA + 3% agar. *Tenacibaculum maritimum* reportedly does not grow in the presence of sodium chloride and requires seawater as the salt source for growth (Wakabayashi, Hikida, & Masumura 1986). The growth of the New Zealand isolate in the presence of sodium chloride suggests some strains may not have an obligate requirement for seawater as the salt source. The use of qPCR to detect *T. maritimum* revealed a low abundance and prevalence in the internal organs and a higher prevalence with a low abundance in the skin ulcers tested. Due to this

low abundance of bacteria, predominantly detected on the skin ulcers as a mixed culture and only detected systemically in two fish, *T. maritimum* was not considered a primary pathogen in these mortalities but is not ruled out as a potentially serious contributing pathogen.

No other common or dominant bacteria were detected across any of the fish evaluated. *Vibrio* species, *Pseudomonas* species, and *Olleya* species were detected as dominant growth in five of the fish analysed. *Vibrio* species are a common resident of salmon microbiota with both pathogenic and probiotic species present (Egerton, Culloty, Whooley, Stanton, and Ross, 2018). *Vibrio scopthalmi* was identified from the kidney of two fish. The isolation of this organism in the kidney of the salmon is potentially significant, but its true significance in the morbidity of the infected fish is unknown. It is most likely this organism was either a secondary invader, taking advantage of an immunocompromised host, or potentially may have been a post-mortem invader due to the high numbers likely to be residing in the intestine. A study by Hatje, Neuman, Stevenson, Bowman, & Katouli (2014) found that in Atlantic salmon cultured in Tasmania, the levels of *Vibrio* species increased as the water temperatures increased and that the *V. ichthyoenteri* and *V. scophthalmi* group were the most abundant species identified (71%). *Vibrio harveyi* was identified in all organs tested from one fish as pure growth and is likely to have played a part in the ill-health of this fish. *Vibrio harveyi* is a known pathogen of many invertebrate and vertebrate hosts, including salmonids (Zhang et al., 2010; Won and Park, 2008). However, as it was not found in any other fish evaluated, the likelihood of its significance in summer mortalities remains low.

Olleya species and *Pseudomonas* species were also identified as dominant isolates from the skin lesion in one fish and are suspected environmental contaminants or secondary pathogens invading already immunocompromised fish. *Olleya* species are a genus of bacteria in the family *Flavobacteriaceae;* part of the Cytophaga–Flexibacter–Bacteroides group (Garrity and Holt, 2001) constituting one of the most dominant bacterial groups of the marine environment (Suzuki, Nakagawa, Harayama, & Yamamoto, 2001). There have been no reports of this organism being implicated in disease naturally or when host animals are challenged (Pratheepa, Silva, & Vasconcelos, 2014; Kiselev, Ageenko, & Kurilenko, 2013). *Pseudomonas* species are

also ubiquitous in the marine environment however, there are some known pathogenic species of salmonids; *P. anguilliseptica, P. chlororaphis*, *P. fluorescens, P. alcaligenes,* and *P. putida* (Buller, 2014). The *Pseudomonas* species identified in the present study were genetically distinct from the pathogenic *Pseudomonas* species and is likely to be a previously unidentified environmental isolate.

It is likely there are a number of other environmental factors that influenced the occurrence of disease in these fish. Virulence of *P. salmonis* and other teleost RLO around the world is affected by host susceptibility, environmental conditions, or co-infections with other pathogens (House et al., 1999). Possible reservoirs of this organism are wild fish or shellfish which are in contact with the farmed salmon. Previous studies have demonstrated that isolates of *P. salmonis* recovered from non-salmonid marine fish can induce disease and mortality of up to 80% in Coho salmon populations (M.F., Chen et al., 2000). Shellfish are also known hosts of RLO in New Zealand (Hine and Diggles, 2002; Hine, 2002) however recent molecular testing of these shellfish RLO have determined them to be distinct from those found in salmon (H. Lane, pers. com.).

It is possible that NZ-RLO is an endemic resident in the New Zealand marine environment and the stress of a farmed environment, seawater transfer, less than optimal environmental conditions, co-infections with other pathogens, or nutritional related stress could have caused this disease to manifest in these fish.

2.5 Conclusion

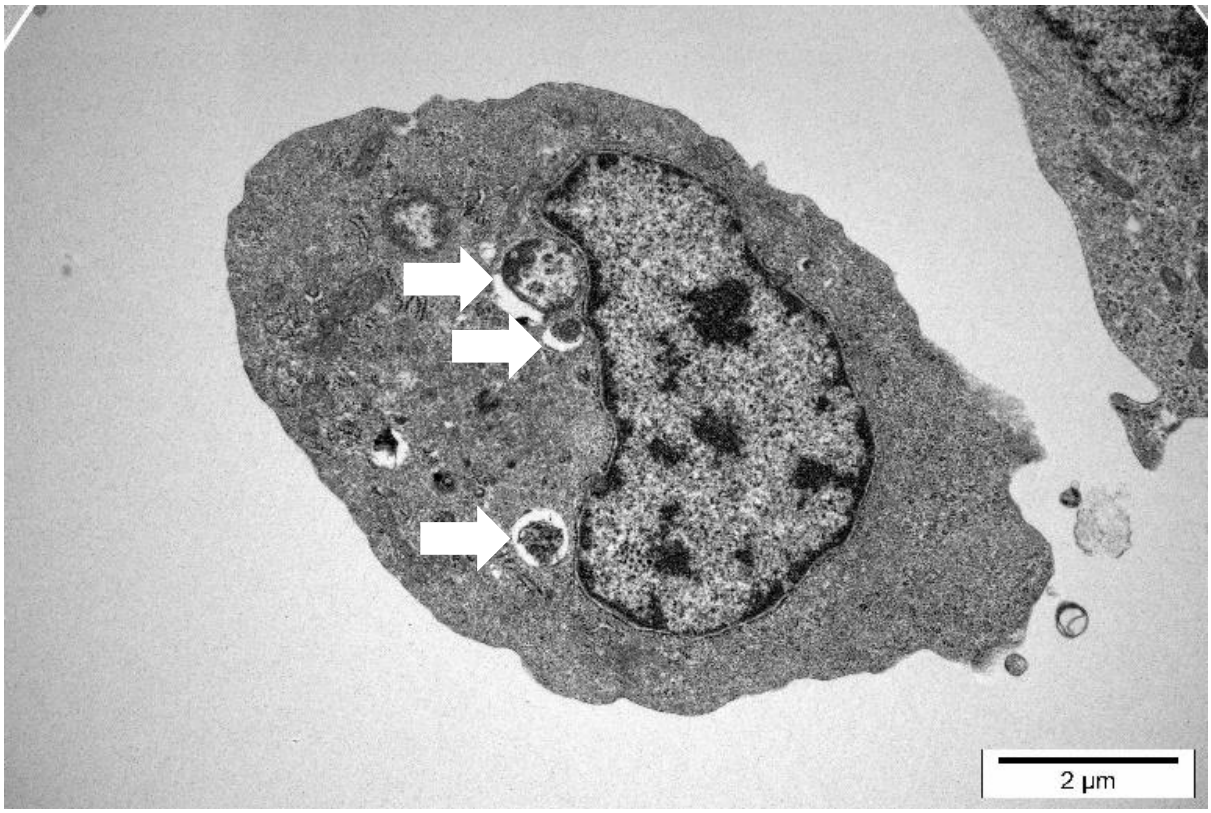
This study identified two potential pathogens from moribund fish in populations experiencing summer mortalities, New Zealand rickettsia-like organism (NZ-RLO) and *Tenacibaculum maritimum.*

Organisms interpreted as NZ-RLO were observed within areas of necrosis from the liver, kidney, and spleen using histology of seven fish analysed. New Zealand rickettsia-like organism was confirmed by PCR and nucleotide sequencing in three fish analysed. *Tenacibaculum maritimum* was identified using culture from the skin

ulcer in one fish and using PCR in seven fish analysed, predominantly from skin ulcers.

The relationship of these bacteria with summer mortalities remains unknown and further work will be required to determine this.

Chapter 3 : Techniques to visualise NZ-RLO in naturally infected samples: transmission electron microscopy and *in-situ* hybridization



Transmission electron microscope image of intra-cytoplasmic NZ-RLO (arrows) within vacuoles in the cytoplasm in Epithelioma papulosum cyprini cell line. Photo courtesy of Della Orr, Ministry for Primary Industries and St John Wakefield, Wellington School of Medicine.

3.1 Introduction

The bacteria New Zealand rickettsia-like organism (NZ-RLO) was detected in New Zealand in 2015 by PCR and histopathology from farmed Chinook salmon experiencing elevated mortalities (Chapter 2). Mortalities occurred during the summer months at farmed sites in the Marlborough Sounds and were termed 'summer mortalities'. Following initial detection of NZ-RLO by PCR and histopathology, NZ-RLO was isolated in cell culture from one fish investigated from these summer mortalities (Fish 10) and the identification was confirmed by quantitative PCR (qPCR) as well as transmission electron microscopy (TEM) from growth in cell culture material (Ministry for Primary Industries, unpublished data). This detection provided no information on the pathogenicity of NZ-RLO in infected fish and the pathogenicity of NZ-RLO remained unknown. One method used to validate if an organism is causing disease is

to localise the organism within the host tissue. If the organism is present within areas of necrosis or inflammation, this would strongly suggest the organism is responsible for the tissue damage observed. Conversely, if the organism is present in normal tissue, this suggests the organism is less likely to be responsible for disease and mortalities. Tissues examined from fish that died during the summer mortalities revealed organisms that appeared histologically similar to NZ-RLO within areas of inflammation and necrosis. If the identity of these possible NZ-RLO could be confirmed this would provide additional evidence that these bacteria were responsible for causing this tissue damage and subsequent mortalities.

Two tools commonly used to visualise organisms within tissues are *in-situ* hybridization (ISH) and TEM. *In-situ* hybridization was first described in 1969 (Gall and Pardue, 1969) and combines molecular biology and histology to target DNA or RNA within fixed tissues. *In-situ* hybridization uses a probe consisting of a nucleotide sequence complementary to the DNA sequence of the target pathogen. Binding of the probe is detected using a chromogen and the location of the DNA within the histological section can be evaluated using light microscopy. *In-situ* hybridization is widely used to visualise aquatic animal pathogens including *P. salmonis* in fish tissue (Venegas, Contreras, Larenas, & Smith, 2004).

Electron microscopes were developed in the 1930s and allow a magnification of up to 1000 times greater than light microscopy. This magnification allows visualisation of small bacterium and viral particles making electron microscopy an important diagnostic tool for new and emerging diseases (Goldsmith and Miller, 2009). There are two main types of electron microscopy; TEM and scanning electron microscopy. Transmission EM transmits beams of electrons through thin sections of sample. An images is then formed from the electrons that progress through the tissue section. Scanning EM allows the visualisation of three-dimensional objects by scanning the surface of the object with a beam of electrons with detection resulting from the electrons that are reflected from that samples surface. Transmission EM was used in the present study. The main advantages of TEM in diagnostics is to confirm the presence of an organism and to aid in the direction of other tests such as PCR. This is also important when exploring pathogens that do not grow using traditional methods or are too small to visualise under light microscopy (Hyatt et al., 1997). Transmission

EM allows the cellular structure of the organism to be visualised, often enabling a pathogen to be classified to a family level. Once family level is confirmed, generic PCR tests can be undertaken for agreement to family level and finally identification to species level.

Transmission EM was used to visualize NZ-RLO from a pure cell culture pellet (Ministry for Primary Industries, unpublished data). This visualisation provided a description of NZ-RLO by TEM which could be used to identify NZ-RLO within naturally-infected tissues. Visualisation of cultured NZ-RLO by TEM revealed that NZ-RLO is bound by two membranes with the outer membrane being undulated and the organism was enclosed in a membrane bound vacuole within the cytoplasm of the host cells. This chapter describes the methods used for both ISH and TEM performed on salmon tissue from the summer mortalities.

3.2 Methods

3.2.1 *In-situ* hybridization

3.2.1.1 DNA cloning and purification

In-situ hybridization was carried out using a digoxigenin (DIG) labelled probe with the sequence between the primer pair F-760/R-836 from the *Piscirickettsia salmonis* qPCR (Corbeil et al., 2003). *In-silico* analysis of the sequence between these two primers using the National Centre for BioTechnology Information (NCBI) nucleotide blast tool showed this probe is specific for *P. salmonis*, including NZ-RLO, DNA.

To prepare this probe, cloned NZ-RLO DNA from cell culture-positive material was used as the DNA template. Cloning was carried out using the TOPO TA Cloning kit (Invitrogen, Waltham, USA) as per the manufacturer's protocol. A summary of the process was as follows: a PCR reaction was carried out using the published PCR protocol targeting the 23S rRNA gene (Corbeil et al., 2003) to produce a PCR product of the correct size. This PCR product was purified using Sureclean plus (Bioline, London, UK), and the resulting product was used in the TOPO TA cloning reaction. The reaction consisted of the following: 4 µL PCR product, 1 µL salt solution, and 1 µL

plasmid vector pCR 4-TOPO. The reaction was incubated at RT for 5 min then placed on ice. Two µL of the cloning reaction was placed into a vial of one shot chemically competent *Escherichia coli* cells (Invitrogen) and incubated on ice for 5 min. Post incubation, the vial was heat shocked at 42°C for 30 sec then placed on ice for 5 min. After 5 min, 250 µL super optimal broth with catabolite repression (SOC) was added to the vial and incubated at 37°C while shaking at 200 rpm for 1 hour. Transformed *E. coli* cells were plated onto Luria–Bertani (LB) (Sigma, Missouri, USA) agar containing 50 µg mL^{-1} ampicillin (Sigma) and incubated at 37°C overnight. After incubation, approximately 10 individual colonies were selected from the LB agar and individually incubated in LB broth with 50 µg mL^{-1} of ampicillin overnight at 37°C. Once grown, the transformed cells were purified.

Purification of the transformed cells was carried out using the Purelink Quick Plasmid Miniprep kit (Invitrogen) as per the manufacturer's protocol. A summary of the purification was as follows: 1 mL of the transformed cells in LB broth were added to a 1.5 mL microfuge tube and centrifuged at 8,000 rpm for 3 min. Supernatant was removed and the pellet was re-suspended in 250 µL resuspension buffer until homogenous. Lysis buffer (250 µL) was added to the re-suspended cells and the tube inverted then incubated for 5 min. Precipitation buffer (350 µL) was added to the tube and mixed gently by inversion until a homogenous solution was reached. The tube was centrifuged at 12,000 rpm for 10 min and the supernatant placed into a spin column. The spin column was then centrifuged at 12,000 rpm for 1 min. Wash buffer (500 µL) was added to the column and incubated for 1 min prior to centrifuging at 12,000 rpm for 1 min. A second wash buffer was added to the spin column (700 µL) and centrifuged at 12,000 rpm for 1 min. Tris-EDTA buffer was then added to the spin column (75 µL) and incubated for 1 min prior to centrifuging at 12,000 rpm for 2 min and retaining the flow-through. The resultant flow-through was analysed by PCR and nucleotide sequencing. Both the M13 forward and reverse primers (5'-GTAAAACGACGGCCAG-3' and 5'-CAGGAAACAGCTATGAC-3') and the *P. salmonis* F-760/R-836 primers were used to screen for the presence of the insert containing plasmid and the product insert itself.

3.2.1.2 NZ-RLO DIG probe labelling

Probe labelling was carried out as per the PCR DIG Probe Synthesis kit (Roche, Penzberg, Germany). Three probes were created; DIG-labelled probe with primer pair F-760/R836 (NZ-RLO DIG-labelled probe), unlabelled NZ-RLO control (NZ-RLO unlabelled probe), and a labelled kit control (control). The reaction mixtures were performed as per Table 3.1.

Table 3.1. Reaction mix components for DIG-labelled and unlabelled probes in a 50 µL reaction.

Reagent	NZ-RLO DIG-labelled probe	NZ-RLO Unlabelled probe	Control
Molecular grade water	24.25 µL	24.25 µL	24.25 µL
PCR buffer	5 µL	5 µL	5 µL
PCR DIG labelling mix	5 µL	-	5 µL
dNTP stock solution	-	5 µL	-
Forward/Reverse primer	5 µL each	5 µL each	10 µL
Enzyme mix	0.75 µL	0.75 µL	0.75 µL
Template DNA	5 µL (~100 ng plasmid DNA)	5 µL (~10 ng plasmid DNA)	5 µL

PCR reactions were performed in a Veriti 96 well Thermal Cycler (Applied Biosystems, Massachusetts, USA) with an initial denaturation of 2 min at 95°C followed by 38 cycles of 30 sec at 95°C denaturation, 30 sec at 60°C annealing and 40 sec at 72°C elongation. Final elongation was 1 min at 72°C. PCR products were analysed by gel electrophoresis on a 1.5% gel stained with GelRed (Biotium, Fremont, USA).

The concentration of the NZ-RLO DIG-labelled probe was measured by Qubit Fluorometer (Life Technologies, Oregon, USA) and stored at -20 °C until use.

3.2.1.3 In-situ hybridization controls and samples

Positive control material from previously confirmed infected tissue was not available at the time of this experiment. In the absence of this material, NZ-RLO cell culture positive smears were prepared from a pure cell culture stock. These controls were used with each run in triplicate; one slide was subjected to the NZ-RLO DIG-labelled probe and anti-DIG alkaline phosphatase (AP)-conjugate (Roche), one slide was not

subjected to the NZ-RLO DIG-labelled probe (no probe control), and one slide was subjected to the NZ-RLO DIG-labelled probe but not to the anti-DIG AP-conjugate (no conjugate control).

Salmon tissue without any evidence of NZ-RLO by histopathology or qPCR was used as a negative control. These negative samples originated from salmon populations with no previous history of clinical signs associated with NZ-RLO. NZ-RLO DIG-labelled probe and anti-DIG AP conjugate were both added to the negative control slides.

Four fish from the summer mortalities (Chapter 2 and 4) were selected for the ISH protocol due to the severity of the histological lesions or the lowest Ct values obtained in the qPCR, indicating a high load of NZ-RLO (Table 3.2). Histological sections of both liver and kidney were evaluated from each fish.

Table 3.2. Summary of diagnostic test results from samples used for ISH and TEM.

Sample	Site^	qPCR result (Ct value)	Strain of NZ-RLO detected	Histopathology consistent with NZ-RLO infection	Test
W15_494-4	1	Negative	NA	Yes	ISH and TEM
W15_494-10	1	Liver = 38, Kidney = 32	NZ-RLO1	Yes	ISH
W15_735-20	3	Liver = 33, Kidney = 29	NZ-RLO1	Yes	ISH
W16_237-3	2	Kidney = 38	NZ-RLO1	Yes	ISH
W15_1255-83	1	Negative	NZ-RLO2*	Yes	TEM
W15_1255-84	1	Negative	NA	Yes	TEM

*NZ-RLO2 detected in the skin lesion tissue only.
^See Chapter 4

3.2.1.4 In-situ hybridization method

In situ hybridization procedures were adapted from published protocols for *P. salmonis* in salmon (Venegas et al., 2004) and Ostreid herpesvirus in Pacific oysters, *Crassostrea gigas* (Bueno, Perrott, Dunowska, Brosnahan, & Johnston, 2017).

Samples originating from cell culture positive smears were trialled initially. NZ-RLO positive cell culture was prepared by inoculating NZ-RLO into Epithelioma papulosum cyprini (EPC, ECACC-93120820) cell lines until cytopathic effect (CPE) was observed. Once approximately 80% CPE was observed, cells were scraped off the flask using a pipette tip and 100 µL of the cell culture material was smeared onto a positively charged slide. Slides were then air-dried followed by methanol fixation.

The protocol for ISH performed on the cell culture positive smears was as follows: three slides were incubated with 100 µg mL^{-1} proteinase K (Roche) and overlaid with a coverslip and incubated in a humid chamber at 37°C for 15 min. Protein hydrolysis was stopped and tissues were post fixed by submerging the slides in 4% formalin (Sigma) for 5 min before washing in 2 x saline sodium citrate (SSC; Roche). Slides were air dried before the addition of a pre-hybridization buffer (50% formamide (Sigma), 10% dextran sulphate (Sigma), 5 X SSC (Roche), 0.02% sodium deoxycholate (Sigma)). Hybri-slips (Sigma) were used to cover the sections and slides were incubated in a humid chamber at 42°C for 30 min. The coverslip was taken off and excess buffer removed using a lint free tissue paper. Two of the slides were flooded with 80 µL of hybridization solution (pre-hybridization buffer with NZ-RLO DIG-labelled probe added at a concentration of 3 ng μL^{-1}) and one slide was re-flooded with the same amount of pre-hybridization buffer (no probe control). After placement of the hybri-slip, denaturation of the DNA was carried out by incubating the slide on a flat solid metal platform at 95°C for 5 min. Post incubation, all slides were quenched on ice for 3 min and hybridized overnight inside a humid chamber at 42°C. Non homologous pairs of nucleic acids were removed by decreasing concentrations of salt washing buffers at 42°C. Washes were as follows: 2 x 5 min of 2 X SSC, 2 x 5 min of 1 X SSC, 2 x 5 min of 0.5 X SSC. After the final wash, tissue sections were equilibrated with Buffer 1 (100 mM maleic acid (Roche), 150 mM sodium chloride (Sigma), pH 7.5)

for 5 min at 37°C, followed by a wash in Buffer 2 (Buffer 1 with 1% blocking solution (DIG nucleic Acid Detection Kit, Roche)) for 30 min at 37°C.

All slides were drained and hybridization of the target DNA to the labelled probe was detected by adding 100 µL of the anti-DIG AP-conjugate (diluted 1:500 in buffer 2) to two slides (no probe control and probe and conjugate added) and Buffer 2 was added to the no conjugate control slide. All slides were incubated for 30 min in a humid chamber at 37°C. Excess antibody was removed by washing with Buffer 3 (100 mL 1M Tris-HCl (Sigma), 100 mL 1M NaCl (Sigma), 50 mL 1M MgCl (Sigma) for 5 min. To visualise the hybrid molecules, 100 µl 5-bromo-4-chloro-3-indolyl phosphate with nitroblue tetrazolium salt (BCIP/NBT, Roche) diluted 1:50 in Solution 2 (10 X detection buffer 1:10 in distilled water), was added to the slide and incubated for 1 hour at RT in the dark. Colour development was stopped by a brief rinse in distilled water followed by phosphate buffered saline (PBS). Following this, a counterstain of 0.5% Bismarck Brown Y (Sigma) was used for 5 min. Finally, slides were dehydrated by a series of increasing ethanol concentrations (96% ethanol (2 x 1 min; absolute ethanol (Merck, Massachusetts, USA; 3 x 15 sec) then cleared twice with xylene (Sigma) and tissue sections were mounted with a drop of Eukitt resin (Sigma) and cover-slipped. Slides were examined under light microscopy.

Slides containing salmon tissues were trialled under the same conditions as the cell culture positive smears after dewaxing. Sections of liver and kidney were evaluated as these tissues most frequently contained changes on histopathology suspicious of an NZ-RLO infection. Additionally, tissues from three of the four fish evaluated contained NZ-RLO DNA (Table 3.2). Five micron-thick sections were cut from previously embedded tissues and mounted on positively charged microscope slides. Slides were dewaxed and rehydrated as follows: slides were immersed in xylene (1 x 5 min and 2 x 3 min) followed by a series of decreasing ethanol concentration solutions (absolute ethanol 2 x 1 min; 96% ethanol 1 x 1 min; 70% ethanol 1 x 1min; 50% ethanol 1 x 1 min). Slides were then rinsed with distilled water for 1 min and equilibrated in PBS (Life Technologies, NY, USA) for 1 min. Failure of any detectable hybridization in these tissues samples resulted in optimisation of different parameters as follows: proteinase K solution (75, 100 or 150 µg mL^{-1}), incubation time with proteinase K (15

or 20 min) and hybridization temperature (42 °C or 45 °C). Each of the four samples were treated as described in Figure 3.1.

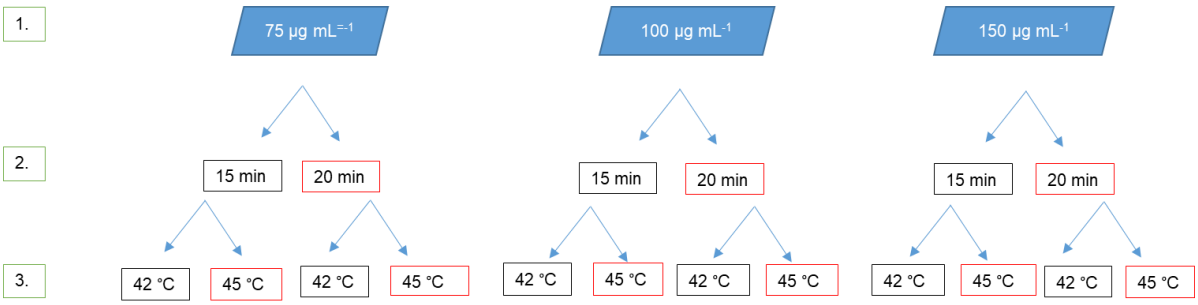


Figure 3.1 Schematic of tissue sample optimisation process trialled the different parameters; 1. Proteinase K concentration, 2. Incubation time with proteinase K, and 3. Hybridization temperature.

3.2.2 Transmission electron microscopy

3.2.2.1 Sample selection and initial fixation

Kidney and liver from three fish sampled from the summer mortalities were selected for TEM based on histology and the presence of organisms consistent with NZ-RLO (Table 3.2). One fish was selected from the initial investigation into summer mortalities (Chapter 2; W15_494-4) and two were from subsequent summer mortality investigations (W15_1255-83 and W15_1255-84; Ministry for Primary Industries, unpublished data). Tissue samples from W15_494-4 were initially fixed in 10% neutral buffered formalin then transferred to 70% ethanol after approximately 1 week. Tissue samples from W15_1255-83 and W15_1255-84 were fixed in 2.5% glutaraldehyde immediately on necropsy (Table 3.2).

3.2.2.2 Transmission electron microscopy methods

For TEM processing, kidney and liver tissue from three fish (W15_494-4, W15_1255-83, and W15_1255-84) were trimmed to the correct size and shape and fixed in modified Karnovsky's fixative (3% Glutaraldehyde (v/v) 2% formaldehyde (w/v) in 0.1 M phosphate buffer (pH7.2)) for at least 2 hours.

After fixation in modified Karnovsky's fixative, samples were washed in 0.1 M sodium cacodylate (pH 7.2) three times for 10 min each time and post fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 hour. Following this, samples were washed in 0.1 M sodium cacodylate as above.

Tissues were then dehydrated through a graded acetone series (25%, 50%, 75%, 95%, 100%) for 10 to 15 min each followed by two changes of 100% acetone for 1 hour each and put into 50:50 resin:acetone and placed on a stirrer overnight. Following this, the 50:50 resin:acetone was replaced by fresh 100% resin (Procure 812, ProSciTech, Australia) and left for 8 hours on the stirrer and repeated twice (overnight in 100% resin, 8 hr in 100% resin). Samples were embedded in moulds with fresh resin and cured in a 60°C oven for 48 hours.

Light microscope sections were cut at 1 µm using a glass knife on an ultra-microtome (Leica EM UC7) and heat-fixed onto glass slides. These were stained with 0.05% toluidine blue for 12 sec and viewed under the light microscope to identify areas to be examined by TEM. The tissue was trimmed to the selected area and cut using a diamond knife (Diatone, Switzerland) at 100 nm. These were stretched with chloroform and mounted onto a grid using a Quick Coat G pen (Daido Sangyo, Japan). Grids were stained in saturated uranyl acetate in 50% ethanol for 4 min, washed with 50% ethanol and milliQ water and stained in lead citrate (Venable and Coggeshall, 1965) for a further 4 min, followed by a wash in MilliQ water. Samples were viewed using a FEI Tecanai G^2 Spirit BioTWIN (Czech Republic).

3.3 Results

3.3.1 *In-situ* hybridization

3.3.1.1 Cloned cells

All 10 colonies of the transformed *E. coli* cells selected from the LB agar contained amplifiable NZ-RLO DNA by qPCR. Nucleotide sequencing from both primer sets confirmed NZ-RLO DNA. One of these purified transformed *E. coli* colonies were selected for use in DIG probe labelling.

3.3.1.2 DIG labelled probes

The NZ-RLO DIG-labelled probe produced a clearly visible amplicon on the gel that had a higher molecular weight than the unlabelled PCR product. The control DIG labelled probe produced an amplicon of approximately 550 bp (Figure 3.2).

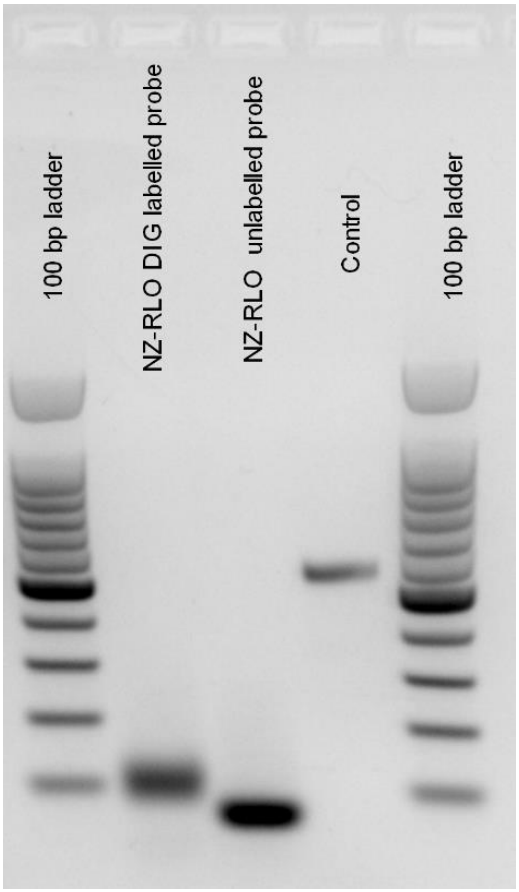


Figure 3.2 Electrophoresis gel (1.5%) of NZ-RLO DIG labelled probe, NZ-RLO unlabelled probe and control labelled probe.

3.3.1.3 In-situ hybridization

Evaluation of cell culture positive smears subjected to the NZ-RLO DIG-labelled probe and anti-DIG AP-conjugate showed dark blue to purplish-black-labelled cells as a consequence of the complementary hybridization of the NZ-RLO DIG-labelled probe (Figure 3.3). No probe labelling was detected within slides that had been incubated either in the absence of the NZ-RLO DIG-labelled probe of the absence of the anti-DIG AP-conjugate nor from the negative control tissue samples.

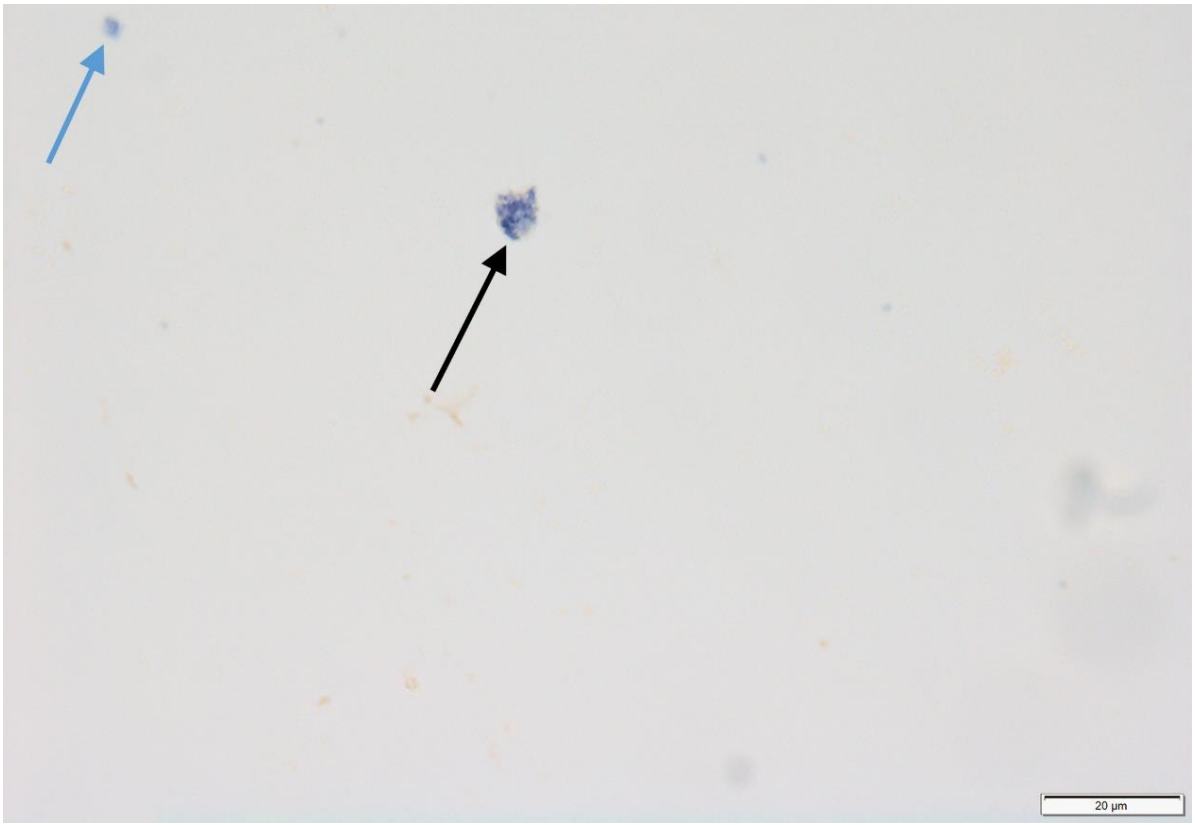
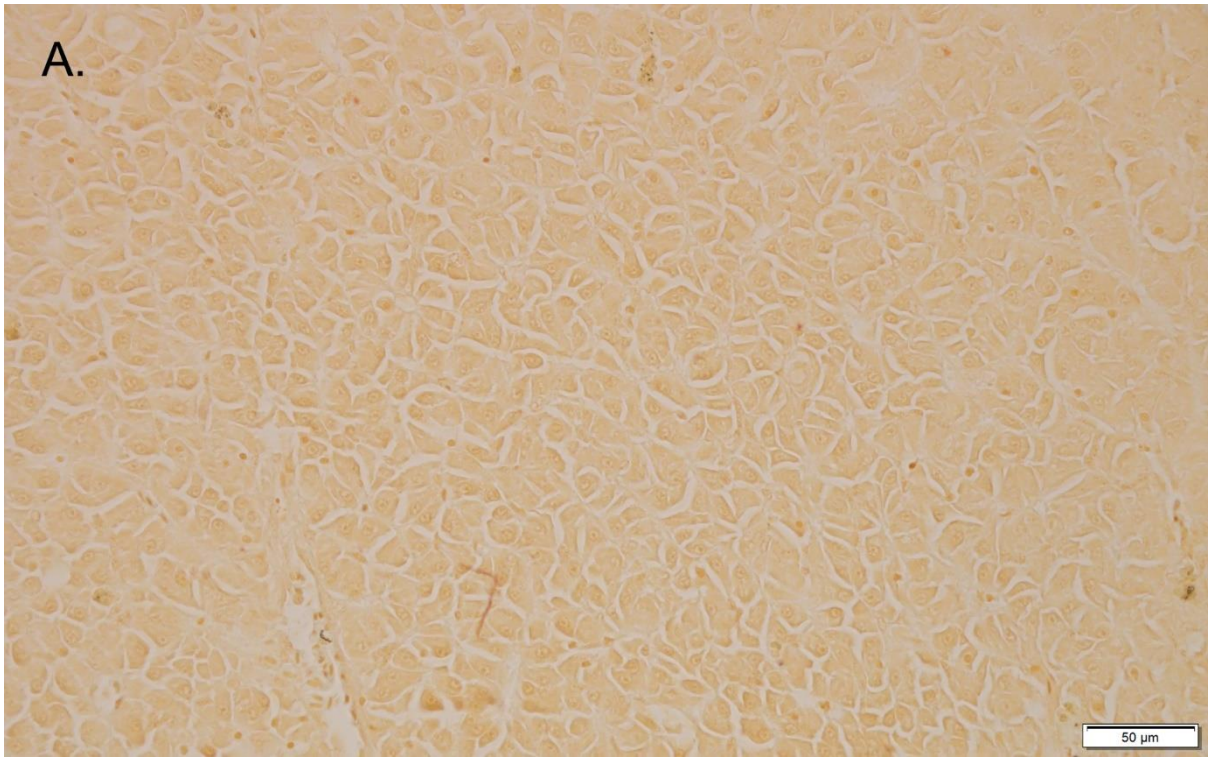
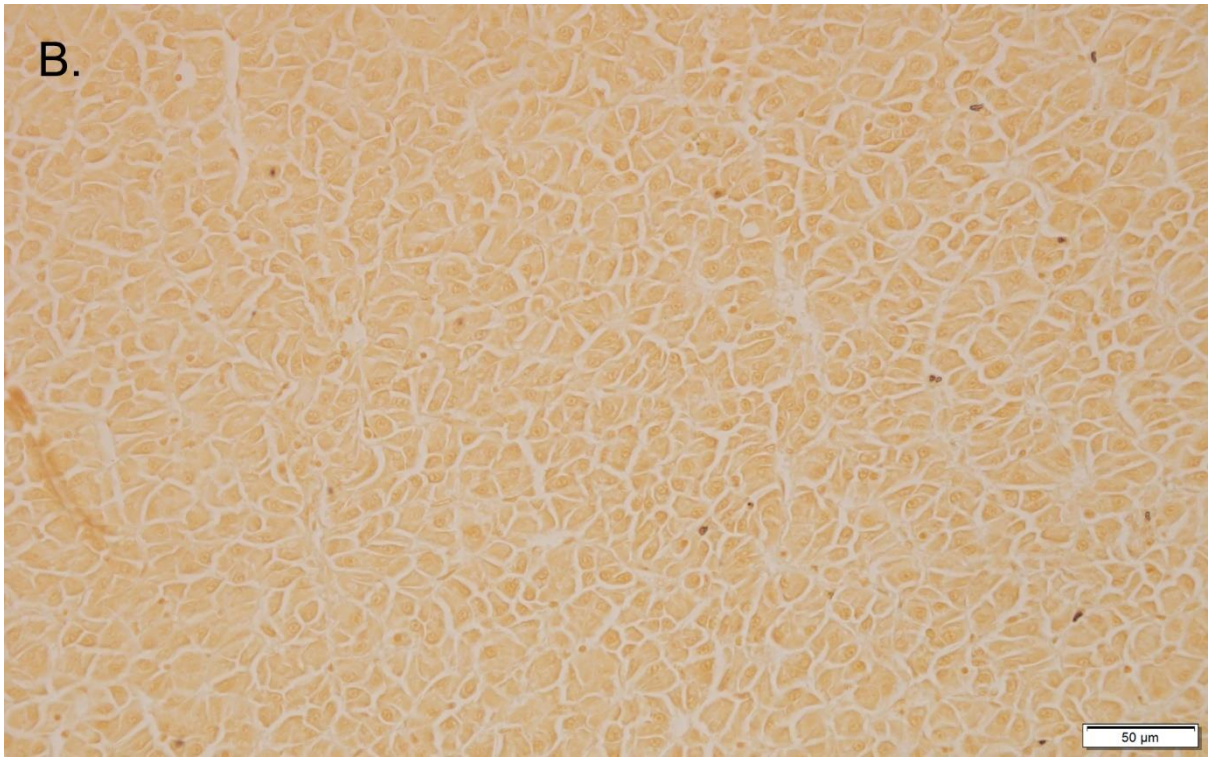


Figure 3.3. NZ-RLO cell culture positive material revealing hybridization under light microscopy using 100 µg mL-1 proteinase K, 15 min incubation at 37°C and 42°C hybridization. NZ-RLO cells labelled dark blue within cells (black arrow) and outside of cells (blue arrows). 1000 x magnification.

Labelling was not detected in any of the tissue slides evaluated (W15_494-4, W15_494-10, W15_735-20, W16_237-3). The absence of any labelled cells were consistent throughout all proteinase K concentrations, incubation or hybridization temperatures. While there was no difference in hybridization between the different proteinase K concentrations trialled, there was a noticeable difference in the structure of the host cells. Increasing the amount of proteinase K resulted in a shrunken and disfigured appearance to the host cells (Figures 3.4 A to C).


A.
50 µm


B.
50 µm

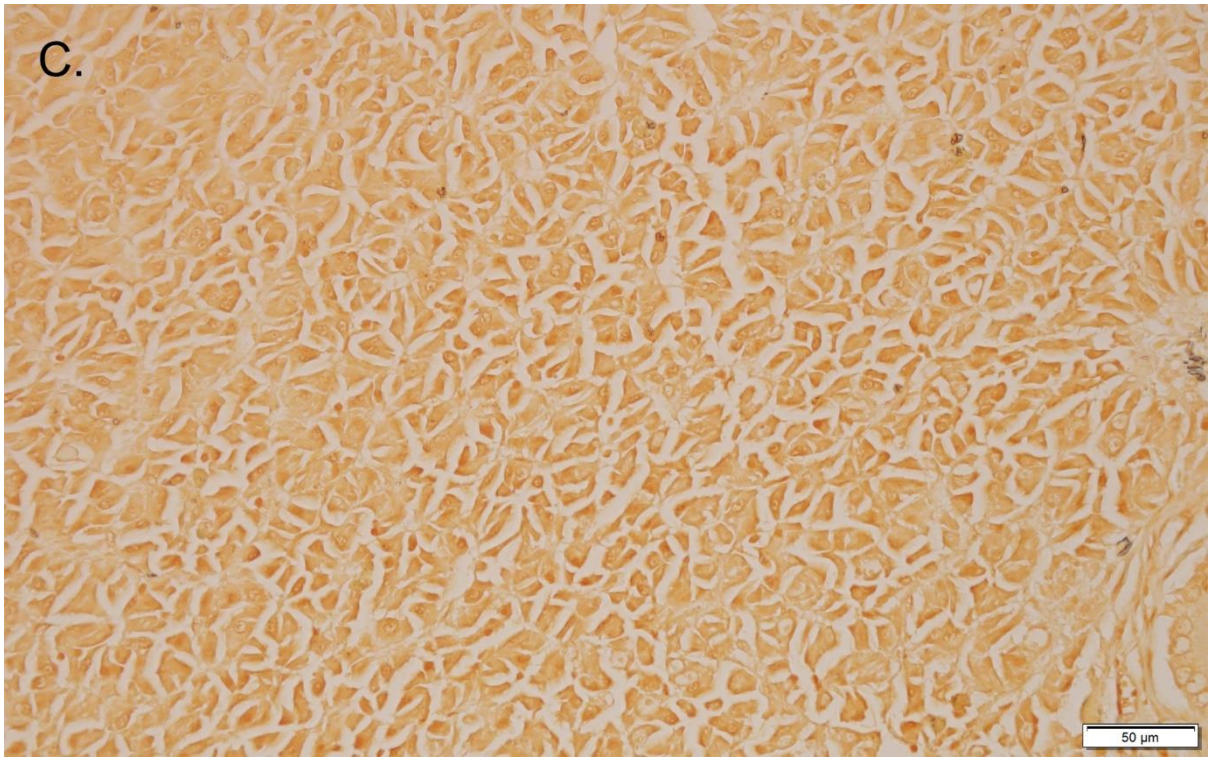


Figure 3.4. Histological sections of liver **A.** slide subjected to 75 µg mL-1 proteinase K **B.** slide subjected to 100 µg mL-1 proteinase K **C.** slide subjected to 150 µg mL-1 proteinase K counter stained with Bismark brown Y. Hepatocyte structure changing from "plump" cells in A (lowest concentration of proteinase K) to a shrunken appearance with space between the cells in C (highest concentration of proteinase K). 400 x magnification.

No difference was seen in tissue integrity when incubating for a longer time or with an increased hybridization temperature.

3.3.2 Transmission electron microscopy

Tissue samples on TEM grids were examined for structures that had previously been described as NZ-RLO in evaluations of NZ-RLO-infected cell culture material. However, nothing consistent with these previous descriptions of NZ-RLO were observed.

Examination of sections of kidney tissue from fish W15_1255-83 and W15_494-4 revealed tubular inclusions. These inclusions measured approximately 17.5 nm in diameter and were enclosed within a membrane in the cytoplasm of macrophages or dendritic cells (Figures 3.5 and 3.6). These tubular inclusions resembled inclusions known to develop as a result of cellular degradation in salmonids (Lovy and Wadowska, 2014). These inclusions were also consistent with previous descriptions

of Birbeck-like granules (Lovy, Wright, & Speare, 2008), cells thought to be related to immune function.

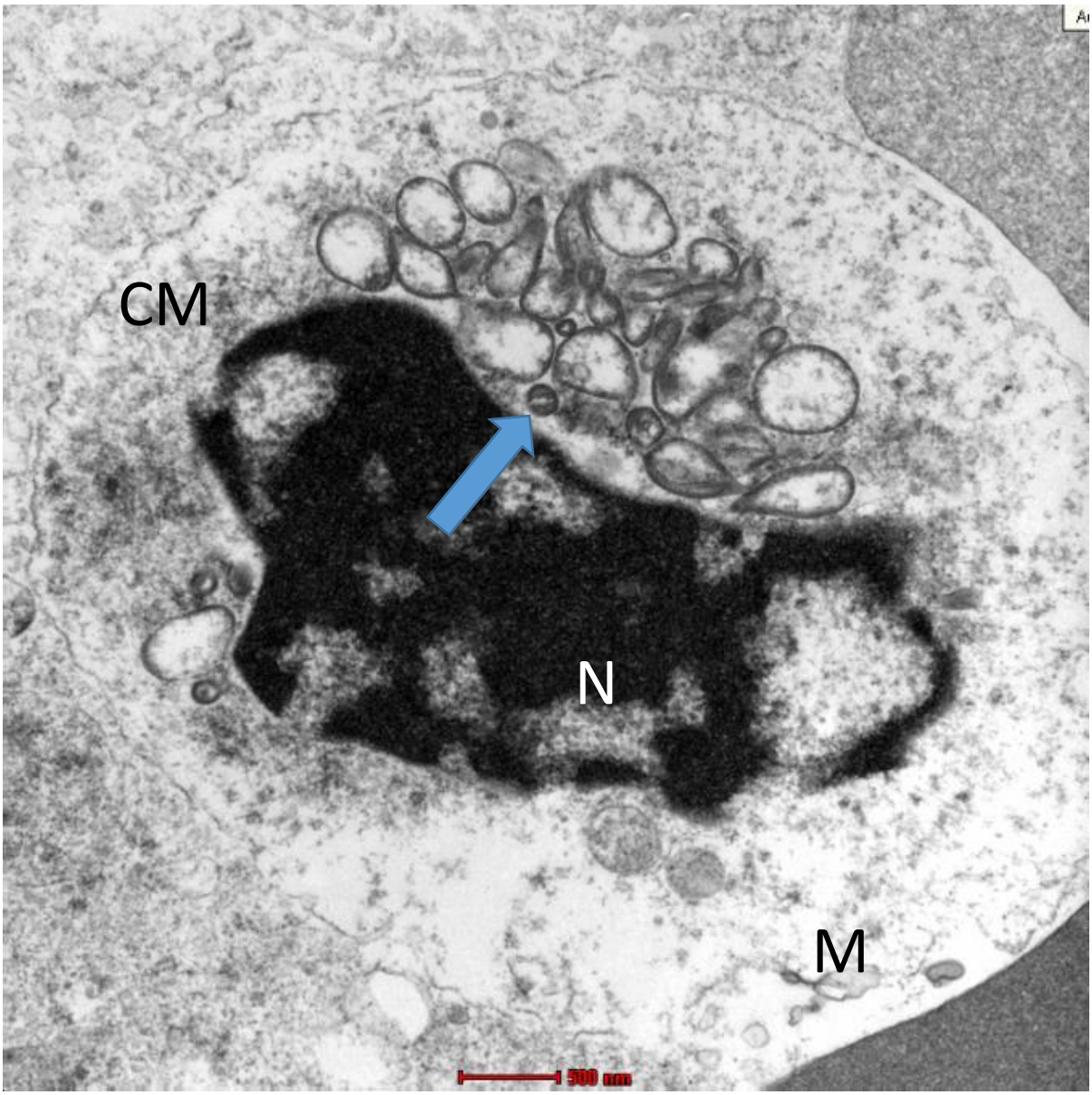


Figure 3.5. Tubular inclusions within the macrophage cell (Blue arrow). N = nucleus, M = macrophage, CM = cell membrane.

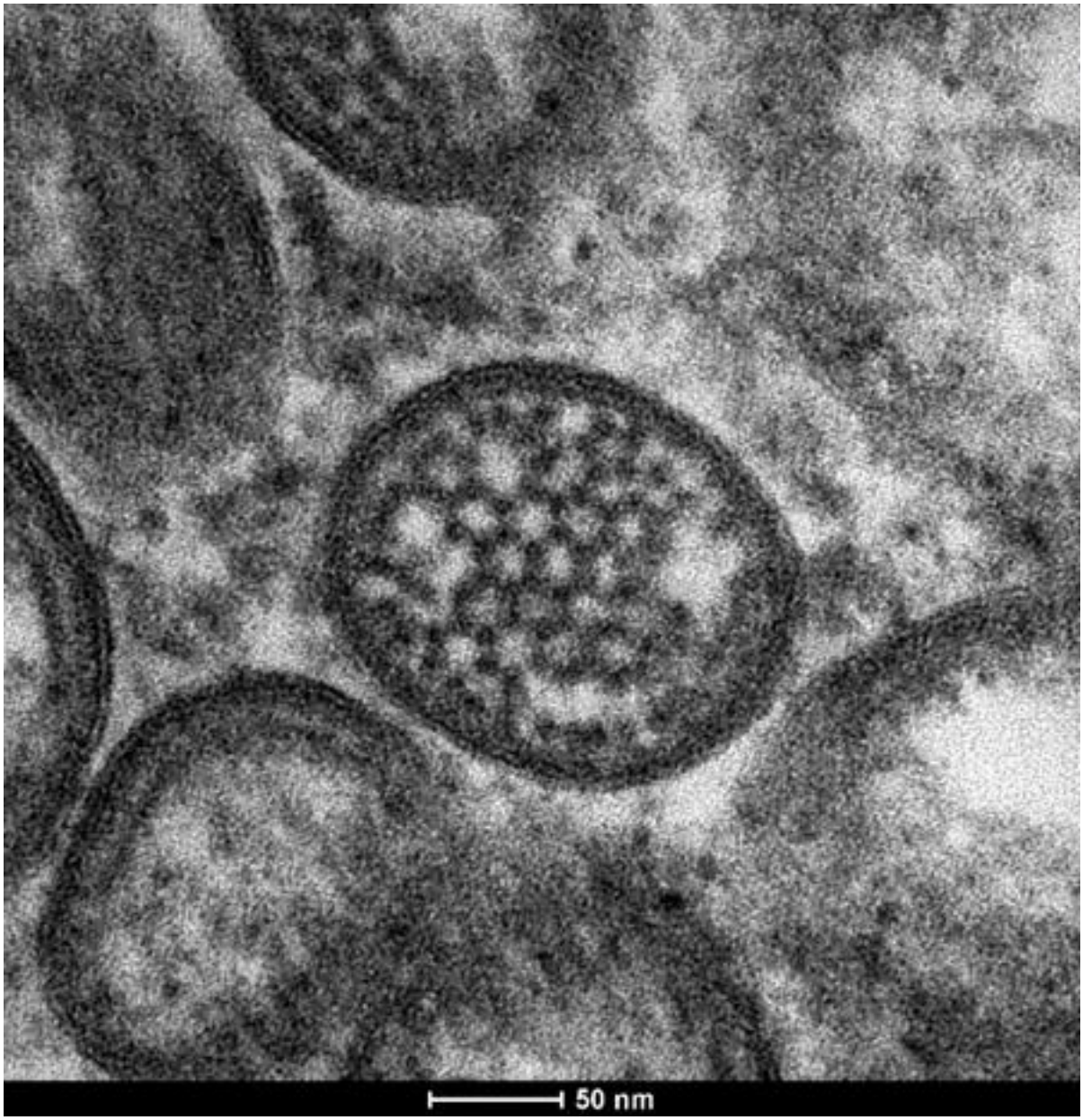


Figure 3.6. Tubular inclusion within macrophage showing a uniform lattice like cell arrangement.

3.4 Discussion

The diagnostic tools ISH and TEM were trialled on samples originating from the summer mortalities in 2015 (Chapter 2) and subsequent summer mortalities in 2016 (Ministry for Primary Industries, unpublished data). The aim of this study was to confirm the presence of NZ-RLO within the host tissue, clarify the relationship between the bacteria and disease and determine if the bacteria resembling NZ-RLO seen under light microscopy had been interpreted correctly. The potential pathogenic role of NZ-RLO in fish was demonstrated by histopathology changes. These changes were also

associated with intracellular bacteria presenting a similar microscopic appearance to RLO seen in other fish species of coccoid basophilic organisms approximately 0.8 to 1.5 µm in size in pairs or clusters within the cytoplasm of inflammatory cells (Chapter 2). Confirmation of the identity of the organisms seen was important as NZ-RLO bacteria are difficult to distinguish under light microscopy due to their small size. Additionally, it can be difficult to differentiate NZ-RLO from other small intracellular bacteria or cellular debris from within necrotic cells.

New Zealand rickettsia-like organisms were not detected by ISH in the tissues evaluated. The inability to detect NZ-RLO using ISH was considered most likely due to either the process not being fully optimised for formalin-fixed tissue, the initial inadequate fixation of the samples, or that the tissues evaluated did not contain NZ-RLO due to the small numbers of bacteria within the host. Optimisation of the ISH protocol was challenging because formalin-fixed tissue samples previously confirmed to contain NZ-RLO or closely related bacteria were not available. Cell culture positive smears were able to confirm the probe had bound to the NZ-RLO nucleic acid, nevertheless it remained possible the ISH process was not optimised for formalin-fixed tissue. Proteinase K is an important consideration for optimisation of ISH from tissue samples. Proteinase K digests proteins to reveal the DNA within cells for hybridization to occur. The concentrations of proteinase K trialled may not have been adequate to degrade the cells surrounding the NZ-RLO DNA making the DNA unavailable for hybridization. Formalin-fixed paraffin-embedded positive controls containing a high number of *Piscirickettsia salmonis* bacteria became available after the present study (Chapter 6). Evaluation of highly infected tissue samples from these later experiments showed that the protocol described here was successful on formalin-fixed paraffin-embedded tissue. The concentration of proteinase K subsequently used for the positive tissue control was 100 µg mL^{-1}, a concentration trialled in the present study. This indicated the proteinase K concentration was unlikely to be inhibiting hybridization.

Another possible reason for hybridization not occurring in the evaluated slides was due to the extended formalin fixation of tissues from the evaluated slides. Formaldehydes damage DNA by degradation, sheering, and the formation of covalent bonds (Hoffman et al., 2015), all potentially impacting the success of ISH. The amount

of damage to the DNA depends on the length of time the DNA is exposed to the formaldehyde with shorter fixation times resulting in less damage. In the present study, tissues from the initial summer mortalities (Chapter 2) were fixed in 10% formalin for at least 1 week prior to embedding. This prolonged fixation may have severely damaged the NZ-RLO DNA and prevented hybridization. This possibility was considered for Chapters 6 and 7 and the tissues used for ISH in these experiments were fixed in formalin for a maximum of two days. In the present study, formalin fixation and paraffin-embedding of the tissues could have damaged the NZ-RLO DNA to an extent where hybridization was prevented.

In-situ hybridization performed using slides made from formalin-fixed paraffin-embedded tissues fixed in formalin for 1 to 2 days were shown to be successful (Chapters 6 and 7). Differences between the present study and Chapters 6 and 7 included shorter fixation times and samples that were known to contain large numbers of NZ-RLO. It appears likely that few bacteria were present in the presently described tissues and the failure to detect NZ-RLO hybridization could have been due to the sensitivity of the test. The lowest cycle threshold value from the tissues evaluated was 29 with other samples ranging from 32 to 38 (Chapter 2). This indicates a low abundance of NZ-RLO bacteria within the samples. Therefore, it may have been due to statistical chance that none of the slides used for ISH in the present experiments contained NZ-RLO. To overcome this, performing ISH on serial sections of tissue so that the presence of NZ-RLO is more likely could have been useful. However, due to the small size of NZ-RLO, this could not have guaranteed any sample examined contained the bacterium.

Cells consistent with NZ-RLO were not visible within the tissues when examined by TEM. The failure to detect bacteria using TEM is most likely due to the infrequency of NZ-RLO within the sample or inadequate fixation. The size of tissues used for TEM is approximately 1 to 2 mm^3 and 0.1 µm thick. The area of tissue examined by TEM means many grids are required to be visualised to be able to confidently identify a pathogen in a tissue sample, particularly those containing low numbers of the target organism. In the present study either more toluidine-blue stained sections could have been evaluated to determine areas of tissue that appeared to contain bacteria, more

grids of the same tissues could have been examined or different tissue types could have been examined to increase the chance of detecting NZ-RLO.

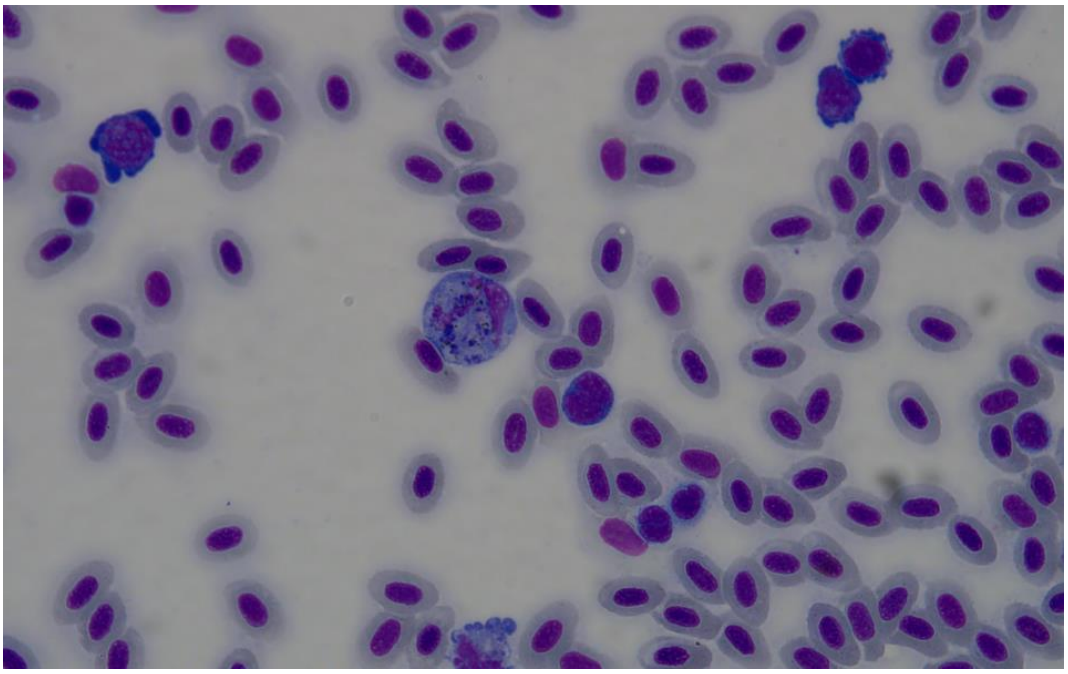
Alternatively, poor fixation of some samples prior to TEM may have contributed to the lack of detection of NZ-RLO by TEM. Optimal fixation for TEM requires immediate fixation of tissue using the correct fixative. Correct fixation is necessary to enable accurate visualisation of the fine structures. Samples from W15_494-4 were fixed in 10% formalin on necropsy. Formaldehyde is a large molecule and therefore only penetrates tissues slowly. In contrast, glutaraldehyde is the recommended fixative for TEM because the smaller molecules are able to more rapidly penetrate the tissue (Kiernan, 2000). The use of formaldehyde in the TEM samples may have resulted in excessive artefact within the tissues making NZ-RLO unrecognisable.

Although no NZ-RLO were seen by TEM, interesting tubular inclusions were observed. The inclusions visualised within the kidney from W15_1255-83 and W15_494-4 were initially suspicious of viral particles. However, a distinguishing feature to differentiate these inclusions from a virus are the size variations of the inclusions (Lovy and Wadowska, 2014). Viral aggregations present a uniform appearance whereas inclusions containing the lattice structure, as seen here, vary in size. Similar tubular inclusions to the ones detected in the present study have been reported in association with a form of cellular degeneration (Lovy and Wadowska, 2014) and are likely to be common in compromised fish. This may explain the presence of these inclusions in these unhealthy immunocompromised fish. Alternatively, these inclusions might be Birbeck-like granules. Birbeck-like granules are membrane bound and showed similarities to the inclusions seen in the present study. Birbeck-like granules have been observed in other salmonid species, although the inclusions appear to be variable depending on the species of salmonid (Lovy et al., 2008). Birbeck-like granules are thought to have a role in the immune system and in the spleen and kidney are commonly found in both healthy and immunocompromised fish. Due to this, birbeck-like granules would be expected to be in any salmonid and may not be related to the health status of these fish.

3.5 Conclusion

New Zealand rickettsia-like organisms could not be visualised by either ISH or TEM in the tissue samples evaluated from the summer mortalities. For these techniques, poor fixation or NZ-RLO being present in low abundance were the most probable causes for the failure of confirmation of NZ-RLO. This lack of visualisation of NZ-RLO *in-situ* means that the pathogenicity of NZ-RLO in the host tissues could not be confirmed. Furthermore, it could not be confirmed that the organisms seen by light microscopy were NZ-RLO (Chapter 2). Lack of visualisation of NZ-RLO in these tissue samples by ISH and TEM does not rule out NZ-RLO causing disease and being involved in the summer mortalities. It remains possible that the NZ-RLO infection had run its course prior to the fish being investigated leaving only the evidence in the tissue pathology. In the present study, a small number of samples were assessed and evaluation of different samples, different tissues, an increased number of samples, or an increased number of sections or grids of the same tissues may have yielded different results. Although ISH and TEM did not result in visualisation of NZ-RLO they did provide important protocols for future work (Chapter 6 and 7) as well as highlighting some limitations of these methods.

Chapter 4 : New Zealand rickettsia-like organism (NZ-RLO) and *Tenacibaculum maritimum*: distribution and phylogeny in farmed Chinook salmon (*Oncorhynchus tshawytscha*).



Blood smear stained with Giemsa from a fish infected with NZ-RLO. NZ-RLO within a circulating macrophage, 1000 x magnification.

This chapter has been published: Brosnahan CL, Munday J, Ha HJ, Preece M, Jones JB. (2018). New Zealand rickettsia-like organism (NZ-RLO) and *Tenacibaculum maritimum*: Distribution and phylogeny in farmed Chinook salmon (*Oncorhynchus tshawytscha*). *Journal of Fish Diseases,* 42, 85-95.

4.1 Introduction

Chinook salmon (*Oncorhynchus tshawytscha*) aquaculture in New Zealand is an economically important and evolving industry. It accounts for approximately 88% of the worldwide production of farmed Chinook salmon (Tucker, 2014) and is the second largest aquaculture industry in New Zealand (Aquaculture New Zealand, 2011b). Chinook salmon is the only salmonid species commercially produced in New Zealand and marine salmon are farmed in three areas; Canterbury, Stewart Island, and the Marlborough Sounds (Figure 4.1). These areas are geographically and genetically distinct with each growing area maintaining their own genetic stocks and in-land hatcheries (Quinn et al., 2001). The largest production area is the Marlborough Sounds which makes up for over half of the global and up to 70% of New Zealand's production of Chinook salmon (NZKS, 2017). Salmonids are non-native to New

Zealand and in addition to Chinook, other salmonid species have been introduced and exist in wild populations including brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), sockeye salmon (*Oncorhynchus nerka*), Atlantic salmon (*Salmo salar*), brook char (*Salvelinus fontinalis*), and Lake char (*Salvelinus namaycush*).

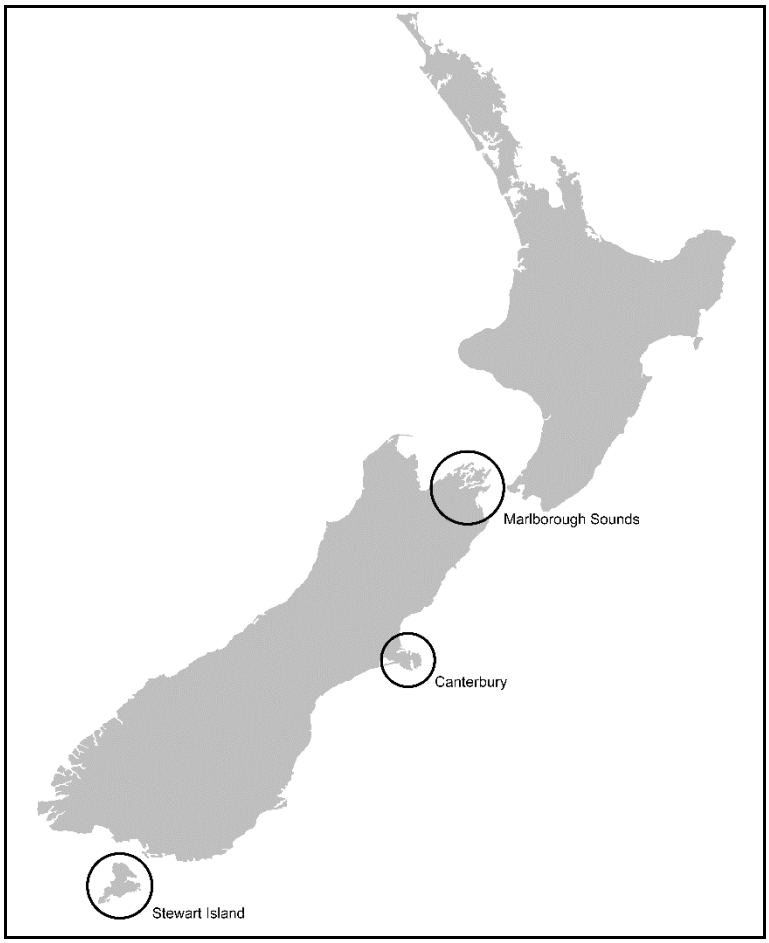


Figure 4.1. Map of New Zealand indicating the three commercial marine growing areas of Chinook salmon.

New Zealand Chinook salmon are considered to be relatively free of disease (Anderson, 1996) and no major outbreaks of disease have been reported in farmed Chinook salmon in New Zealand. However, since 2012 higher than expected mortalities have occurred at certain sites within the Marlborough Sounds. During investigations into these mortalities in 2015, two potential pathogens were identified; New Zealand rickettsia-like organism (NZ-RLO) and *Tenacibaculum maritimum* (Chapter 2). While the precise relationship between these pathogens and the mortalities was unclear, some strains of RLO and *T. maritimum* have been linked to

mortalities of salmonids in other countries (Bravo and Campos, 1989; Masumura and Wakabayashi, 1977; Corbeil et al., 2005) and experimental infection with these bacteria have been shown to cause mortalities in Atlantic salmon (Morrison, Young, Knowles, Cornish, & Carson, 2016; van Gelderen, Carson, & Nowak, 2010).

New Zealand rickettsia-like organism is a bacteria closely related to *Piscirickettsia salmonis* (Chapter 2)*. Piscirickettsia salmonis* was first described in 1989 from coho salmon (*Oncorhynchus kisutch*) in Chile (Bravo and Campos, 1989) and has not been detected in New Zealand. The disease caused by *P. salmonis* is referred to as piscirickettsiosis or salmonid rickettsial syndrome and *P. salmonis* has been responsible for high mortalities of farmed salmon particularly in Chile (Rozas and Enriquez, 2014). Since the 1980s, other RLOs or piscirickettsia-like organisms (PLOs), as well as *P. salmonis*, have been identified from a wide geographic and host range including salmonid and non-salmonid finfish (Brocklebank et al., 1993; Olsen et al., 1997; Rodger and Drinan, 1993; Grant et al., 1996; Corbeil et al., 2005; M.F., Chen et al., 2000; S.C., Chen et al., 2000; Mauel et al., 2005; Comps et al., 1996; Athanassopoulou et al., 2004; Iregui et al., 2011). *Piscirickettsia salmonis*, RLO, and PLO are not well described leading to a lack of clarity around whether the RLO and PLO detected in other studies are true *Rickettsia* species, *Piscirickettsia* species or *P. salmonis*. Additionally, the organisms are difficult to culture making it impossible to classify using phenotypic properties. However, with increasing molecular data available for these organisms, their characterisation and grouping is becoming increasingly understood.

Tenacibaculum maritimum (previously *Flexibacter maritimus* and *Cytophaga marina*) is a Gram-negative filamentous bacterium. It was first associated with increased mortalities in farmed juvenile sea bream in Japan (Masumura and Wakabayashi, 1977). *Tenacibaculum maritimum* is the causative agent of tenacibaculosis, which is mainly characterised by skin ulcers, erosions of the mouth as well as frayed fins and tail (Avendaño-Herrera et al., 2006). *Tenacibaculum maritimum* is known to affect a high number of marine fish species worldwide (Avendaño-Herrera et al., 2006). Prior to 2015, this pathogen had not been implicated in disease in New Zealand farmed salmon (Diggles et al., 2002).

There have been no previous surveys of either NZ-RLO or *T. maritimum* in farmed Chinook salmon in New Zealand. Therefore, the primary aim of this study was to determine the distribution of NZ-RLO and *T. maritimum* by PCR and to determine the phylogeny of the strains detected. Internal transcribed spacer (ITS) rRNA region and 16S rRNA gene of NZ-RLO and multi-locus sequence typing was used to compare the New Zealand *T. maritimum* isolates to strains detected globally. A second aim was to compare the rates of detection of these organisms in clinically affected fish with the rates in clinically unaffected fish. If either of these organisms were detected at a higher rate in clinically affected fish than clinically unaffected fish, this could be evidence of an association with these organisms and clinical infection of farmed salmon in New Zealand.

4.2 Methods

4.2.1 Sample collection

All samples were collected between the austral autumn 2015 and summer 2017. Fish sampled from all sites were of harvest size (approximately 3 to 4 kg). Fish showing the clinical sign of disease of skin ulcers were preferentially sampled to maximise the sensitivity of detecting the target pathogens.

4.2.1.1 Marlborough Sounds

Fish (*n* = 153) were collected from three growing sites in the Marlborough Sounds (sites 1 to 3). Site 1 was the location of initial detection of NZ-RLO and *T. maritimum* in 2015 (Chapter 2) and where the highest mortalities had been recorded. Fish were sampled twice over one year from site 1. Site 2 also experienced elevated mortalities and this site was tested five times over three years. Fish were sampled once from site three (Table 4.1).

Of the 153 fish from the Marlborough Sounds, 134 were sent whole, bagged and chilled to the Ministry for Primary Industries, Animal Health Laboratory (AHL) and necropsied within 24 hours post-mortem. For each fish, tissue samples of liver, spleen, mid-kidney, and skin ulcer (if present) were taken immediately and aseptically for DNA

extraction. The remaining fish (*n* = 19) were necropsied in the field with the same organs being aseptically sampled in the field and sent to the AHL within 24 hours post-mortem.

4.2.1.2 Canterbury

Fish (*n* = 305) were collected from one site in Canterbury (site 4). Fish were sampled three times over one year (Table 4.2). Liver, spleen, mid-kidney, and skin ulcer (if present) were aseptically removed from each fish in the field then sent chilled to the AHL.

4.2.1.3 Stewart Island

Fish (*n* = 309) were collected from one Stewart Island site (site 5) and were sampled once (Table 4.2). Liver, spleen, mid-kidney, and skin ulcer (if present) were aseptically removed from each fish in the field, frozen immediately and sent on dry ice to the AHL.

Table 4.1. Summary of numbers of fish sampled and results from all sites.

	Site				
	1	**2**	**3**	**4**	**5**
Total number of fish tested	25	98	30	305	309
Number with skin ulcers	17	50	25	4	66
Number without visible skin lesions	8	48	5	301	243
NZ-RLO positive fish	9	44	3	5	0
NZ-RLO positive fish – skin ulcers	9	44	3	0	0
NZ-RLO positive fish – no skin ulcers	0	0	0	5	0
T. maritimum positive fish	7	7	0	62	144
T. maritimum positive fish – skin ulcers	6	6	0	2	60
T. maritimum positive fish – no skin ulcers	1	1	0	60	84

4.2.2 Seawater temperature data

Seawater temperatures from sites 1, 2, 3, and 5 were retrieved from the salmon farm records and seawater temperatures from site 4 were taken from www.seatemperature.org/australia-pacific/new-zealand/christchurch-january.htm. Seawater temperature records were averaged for the month of sampling (Table 4.2).

4.2.3 DNA extraction

At the AHL, tissues were subsampled for DNA extraction by removing a small piece of tissue from each organ sampled. A 20 mg subsample of the following organs: mid-kidney, liver, and skin ulcer tissue, and 10 mg spleen tissue was macerated using a scalpel and placed into separate 1.5 mL microfuge tubes using individual scalpels and forceps for each piece of tissue. Tissue digest buffer (420 µL) and digest enzyme (4.2 µL) were added to each tube containing the tissue and lysed overnight at 56°C on a shaking platform. Nucleic acid was extracted from the lysate using the QIAXtractor automated system (Qiagen, Valencia, CA, USA) as per the manufacturers protocol.

4.2.4 PCR

4.2.4.1 Internal control PCR

The presence of amplifiable DNA within the extract was confirmed by using an internal control PCR targeting the 18S rRNA (Ribosomal 18S rRNA Endogenous Control; Life technologies, Oregen, USA).

4.2.4.2 NZ-RLO PCR

A previously published quantitative PCR (qPCR) targeting the 23S rRNA gene of *P. salmonis* was carried out on all samples as a screening test due to its reported sensitivity and specificity (Corbeil et al., 2003). The 23S rRNA gene is a conserved region and detects NZ-RLO as well as *P. salmonis.* All samples were tested in duplicate with a high and low DNA template of 1 µL and 3 µL to maximise the likelihood of detecting organisms at different concentrations within the tissue. With each PCR, DNA recovered from a pure culture of NZ-RLO grown in cell culture (W15_494 10Sp)

was run as a positive control and molecular grade water as a no template control (NTC).

A sample was considered positive if either one of the template concentrations produced a typical amplification curve, the positive control produced a typical amplification curve, and the NTC did not show any amplification. Any sample producing amplification in the qPCR was then assayed using a conventional PCR targeting the ITS rRNA region and 16S rRNA gene based on previously published methods (Marshall et al., 1998 and Mauel et al., 1996 respectively) and optimised as previously described (Chapter 2).

The ITS rRNA region and 16s rRNA gene were used to determine the strain of NZ-RLO present. Different strains can be determined by single nucleotide polymorphisms and indels within these regions. Amplicons from this PCR were purified using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, USA) and sent to EcoGene (Landcare Research, Auckland, New Zealand) for nucleotide sequencing. Sequencing was performed in both directions using the PCR primers. The resulting sequences were assembled, manually edited and trimmed using Geneious version 9 (Kearse et al., 2012) resulting in a contig of approximately 280 base pairs (bp) for the ITS region and 470 bp for the 16s rRNA gene. Consensus sequences were then compared to published sequences using the National Centre for BioTechnology Information (NCBI) nucleotide blast tool.

4.2.4.3 NZ-RLO ITS rRNA region sequence analysis

Nucleotide sequences from the ITS rRNA region of NZ-RLO1 (KU523537), NZ-RLO2 (MH378330) and NZ-RLO3 (MH378331) were used to create phylogenetic trees. The relationship of these NZ-RLO strains were compared with the following published *P. salmonis* strains; *P. salmonis* strains LF-89 (U36943), NOR-92 (U36946), ATL-4-91 (U36945), EM-90 (U36944), C1-95 (U62103), AL-10015 (EU289216), IBM-019 (KF831146), IRE-99C (AY498632), IRE-99D (AY498632) and piscirickettsia-like organism strains; SC-2004 (AY578985); and SBPLO (AY607584). All DNA sequences were imported into Geneious version 9 (Kearse et al., 2012), and aligned using Geneious align with default parameters. Alignments were then manually trimmed and

a neighbour-joining tree constructed using the Jukes Cantor substitution matrix with 100,000 bootstrap replicates. The tree selected was based on data from jmodeltest (Posada, 2008; Guindon and Gascuel 2003).

4.2.4.4 NZ-RLO 16s rRNA gene sequence analysis

Sequence information derived from the 16S rRNA gene of NZ-RLO1 (KU523536) and NZ-RLO2 were used to create phylogenetic trees. The NZ-RLO strains were compared with the following published *P. salmonis* strains; *P. salmonis* strains LF-89 (NR_025980), NOR-92 (U36946), EM-90 (U36944), Ca 19-G3-As-I (KF990236), Ca20-G3-As-I (KF990237), AL10014 (EU293855), IBM-018 (KF831181), IRE-91A (AY498633), IRE-98A (AY498634), IRE-99D (AY498637), SCO-95A (AY498636), SCO-02A (AY498635) and piscirickettsia-like organism strain; SC-2004 (AY578984). DNA sequences were aligned and trees built as above.

4.2.4.5 Tenacibaculum maritimum PCR

A qPCR targeting the 16S rRNA gene of *T. maritimum* was performed as previously described (Fringuelli et al., 2012). Any amplification < 40 cycle threshold (Ct) resulting from this PCR was considered positive based on validation work carried out at the AHL (data not shown). Samples were tested in duplicate with a high and low template volume including all PCR controls as described for the NZ-RLO PCR.

4.2.4.6 Tenacibaculum maritimum Multi-locus sequence typing (MLST)

Multi-locus sequence typing was carried out on two isolates of *T. maritimum*. Both isolates were recovered from skin ulcers; one from site 1 (W15_494#9) and one from site 2 (W15_1297 #25) on Marine Anacker & Ordal agar and verified as *T. maritimum* as described in Chapter 2. Multi-locus sequence typing was carried out as previously described (Habib et al., 2014) on 11 housekeeping genes; atpA, glyA, dnaK, gyrB, ileS, infB, rlmN, tgt, trpB, tuf, yqfO. Sequences of the 11 housekeeping genes from the two *T. maritimum* isolates (Accession numbers; MH423693, MH423695 ,MH423694, MH423696, MH423697, MH423698, MH423699, MH423702, MH423701, MH423700, MH423703) were submitted to the PubMLST *Tenacibaculum* sp. database

(http://pubmlst.org/ tenacibaculum/, Jolley and Maiden 2010, *BMC Bioinformatics*, 11:595) resulting in a sequence type (ST) based on the difference in the sequences of each housekeeping gene analysed. Sequences from the 11 housekeeping genes of the two isolates were concatenated, aligned and shown to be identical. This aligned concatenated sequence was then compared with the concatenated sequences from 73 strains of *T. maritimum* within the GenBank database. These strains were reported from salmonid and non-salmonid hosts from the following countries: Japan, Portugal, Spain, Italy, Australia, Scotland, France, Holland, Chile, Corsica, California, Malta, Norway, Croatia, South Korea, Taiwan, Philippines, and Denmark. A tree was constructed from the alignments using Geneious tree builder and the Jukes Cantor substitution matrix with 100,000 bootstrap replicates. The tree selected was based on data from jmodeltest (Posada, 2008; Guindon and Gascuel, 2003).

4.2.5 Statistical analysis

To identify if there was an association between the pathogens in fish with skin ulcers compared to fish without skin ulcers, a generalised linear model was used to test the null hypothesis that the presence of the pathogen was the same in each category of fish. The response variable in the model was a positive result using a binomial family (R package *multcomp*, Hothorn, Bretz, & Westfall, 2008). New Zealand rickettsia-like organism or *T. maritimum* detections from fish with skin ulcers and fish without skin ulcers were compared separately for each site.

Additionally, the presence of each of the pathogens (NZ-RLO or *T. maritimum*) was compared between all sites using the same generalised linear model to test the null hypothesis that there was no difference in detection of these pathogens between each site. To test for specific pair wise differences between the sites a multiple comparison procedure using Tukey contrasts was performed (R package *multcomp*, Hothorn et al., 2008). We used p values less than 0.05 ($p < 0.05$) to determine the statistical significance. NZ-RLO was not detected at site 5, hence this site was not included in any analysis including NZ-RLO. *Tenacibaculum maritimum* was not detected at site 3 hence this site was not included in any analysis including *T. maritimum*.

4.3 Results

4.3.1 Samples

Of the 153 fish sampled from Marlborough, 111 had skin ulcers. Of the fish from Canterbury and Stewart Island, four of 303 and 66 of 309 had skin ulcers, respectively.

4.3.2 Seawater temperature data

Seawater temperature was highest at sites 1 and 2 in autumn 2015 and summer 2015/2016, respectively (> 17°C) (Table 4.2).

4.3.3 PCR

All extracted DNA was shown to be amplifiable by the 18S rRNA internal control PCR. NZ-RLO DNA was amplified from four of the five sites; 1, 2, 3 and 4, and two of the three production areas; Marlborough Sounds and Canterbury (Tables 4.1 and 4.2). Three strains of NZ-RLO were identified based on differences within the ITS rRNA region and the 16S rRNA gene, tentatively named NZ-RLO1 (previously NZ-RLO), NZ-RLO2, and NZ-RLO3. New Zealand rickettsia-like organism 1 and NZ-RLO2 were only detected in the Marlborough Sounds, while NZ-RLO3 was only detected in Canterbury. Samples from site 2 were taken in different seasons over two years and showed the percent prevalence of NZ-RLO in the summer months was higher than any other months tested (Figure 4.3). A summary of the percent of fish affected with NZ-RLO1, NZ-RLO2, NZ-RLO3, and *T. maritimum* at each site and each season is shown in Table 4.2.

New Zealand rickettsia-like organism 1 was most commonly detected in the kidney (89%), NZ-RLO2 and *T. maritimum* were most commonly detected in skin ulcers (72% and 50% respectively), and NZ-RLO3 was most commonly detected in the spleen (66%). A summary of the organs each of these pathogens were detected in at all sites is shown in Table 4.3.

Table 4.2. Summary of results; percent of fish each pathogen was detected in and mean seawater temperature in the month the fish were sampled. N = number sampled.

Site	Season and year	N	NZ-RLO1 (%)	NZ-RLO2 (%)	NZ-RLO3 (%)	*Tenacibaculum maritimum* (%)	Seawater temperature (°C)
1	Autumn 2015	10	30	0	0	70	17.3
1	Spring 2015	15	0	20	0	0	13.0
2	Winter 2015	30	0	3	0	0	13.0
2	Spring 2015	16	0	29	0	0	12.5
2	Summer 2015/2016	18	12	47	0	9	17.1
2	Autumn 2016	11	9	27	0	45	16.6
2	Summer 2017	23	7	48	0	9	16.7
3	Winter 2015	30	3	10	0	0	13.5
4	Summer 2016	173	0	0	0	12	15.4^
4	Autumn 2016	117	0	0	3	30	13.7^
4	Winter 2016	13	0	0	15	54	10^
5	Summer 2016	309	0	0	0	47	14.6

^Taken from: https://www.seatemperature.org/australia-pacific/new-zealand/christchurch-January.htm average temperatures for that season.

Table 4.3. Summary of organs NZ-RLO and *Tenacibaculum maritimum* were detected in at all sites.

	NZ-RLO1 (%)	NZ-RLO2 (%)	NZ-RLO3 (%)	*Tenacibaculum maritimum* (%)
Kidney	89	28	33	32
Liver	44	11	0	44
Spleen	33	8	66	40
Skin ulcer	2	72	0	50

Often, the strain of NZ-RLO was unable to be identified (Figures 4.2 and 4.3) due to the decreased sensitivity of the conventional assay used for speciation compared with the qPCR used for screening. This result is also likely indicative of a low level of infection within these fish.

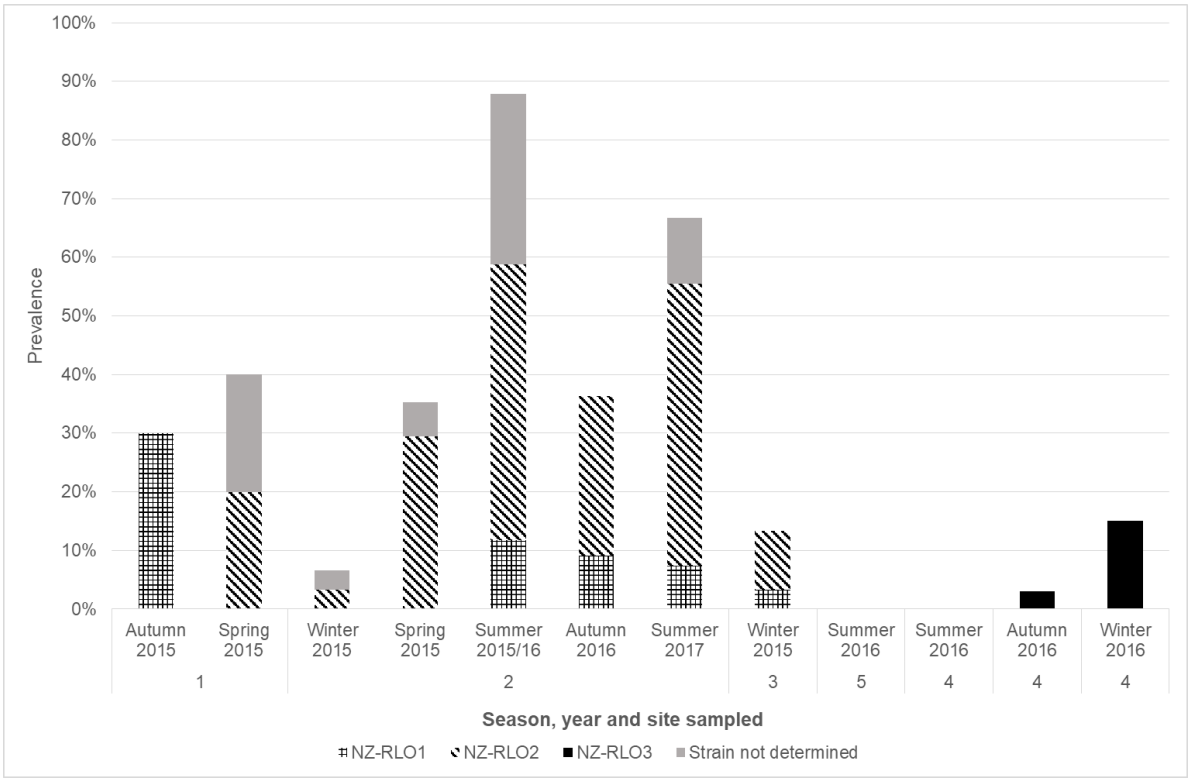


Figure 4.2. Percent prevalence of fish where NZ-RLO DNA was detected across all sites tested in each season.

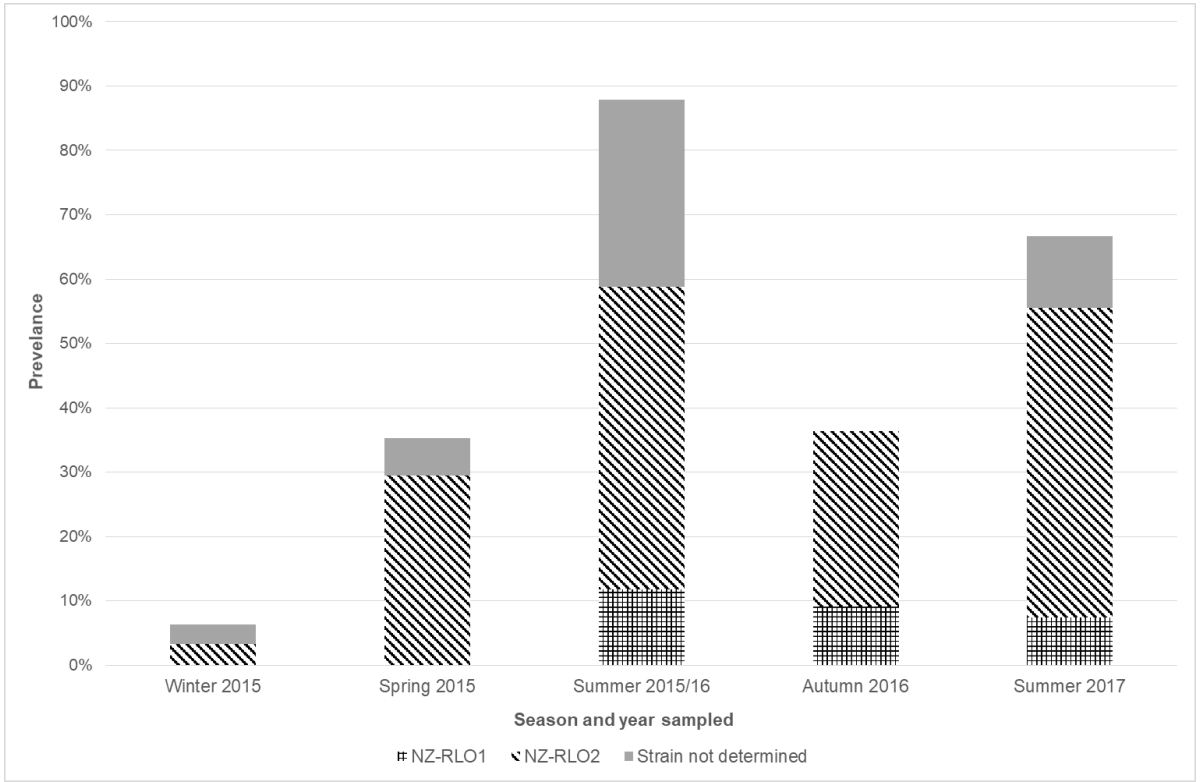


Figure 4.3. Percent prevalence of fish where NZ-RLO DNA was detected at site 2 tested in different seasons across three years.

4.3.4 Sequence analysis

4.3.4.1 NZ-RLO ITS rRNA region

Phylogenetic analysis revealed variances within the three New Zealand strains of NZ-RLO. New Zealand rickettsia-like organism 1 and NZ-RLO2 shared 85.3% homology and NZ-RLO1 and NZ-RLO3 shared 88.5% similarity. New Zealand rickettsia-like organism 2 and NZ-RLO3 were more closely related with these two isolates sharing 94.8% similarity within the ITS rRNA region.

When comparing the New Zealand strains to other published sequences, NZ-RLO1 showed highest similarity (99.6%) to Tasmanian-RLO strain SC-2004. New Zealand rickettsia-like organism 2 showed 100% similarity to *P. salmonis* strain IRE-99C from Ireland while NZ-RLO3 showed highest similarity (99.2%) to *P. salmonis* strain AL10015 from Chile (Figure 4.4).

4.3.4.2 NZ-RLO 16S rRNA gene

Phylogenetic analysis of NZ-RLO1 and NZ-RLO2 revealed variances in the 16S rRNA gene over 375 bp sharing 97% homology. Comparison of NZ-RLO strains to other published sequences revealed NZ-RLO1 showing highest similarity (100%) to Tasmanian-RLO strain and NZ-RLO2 showing highest similarity (100%) to *P. salmonis* strains from Ireland (IRE-99D) and Chile (IBM-018) (Figure 4.5).

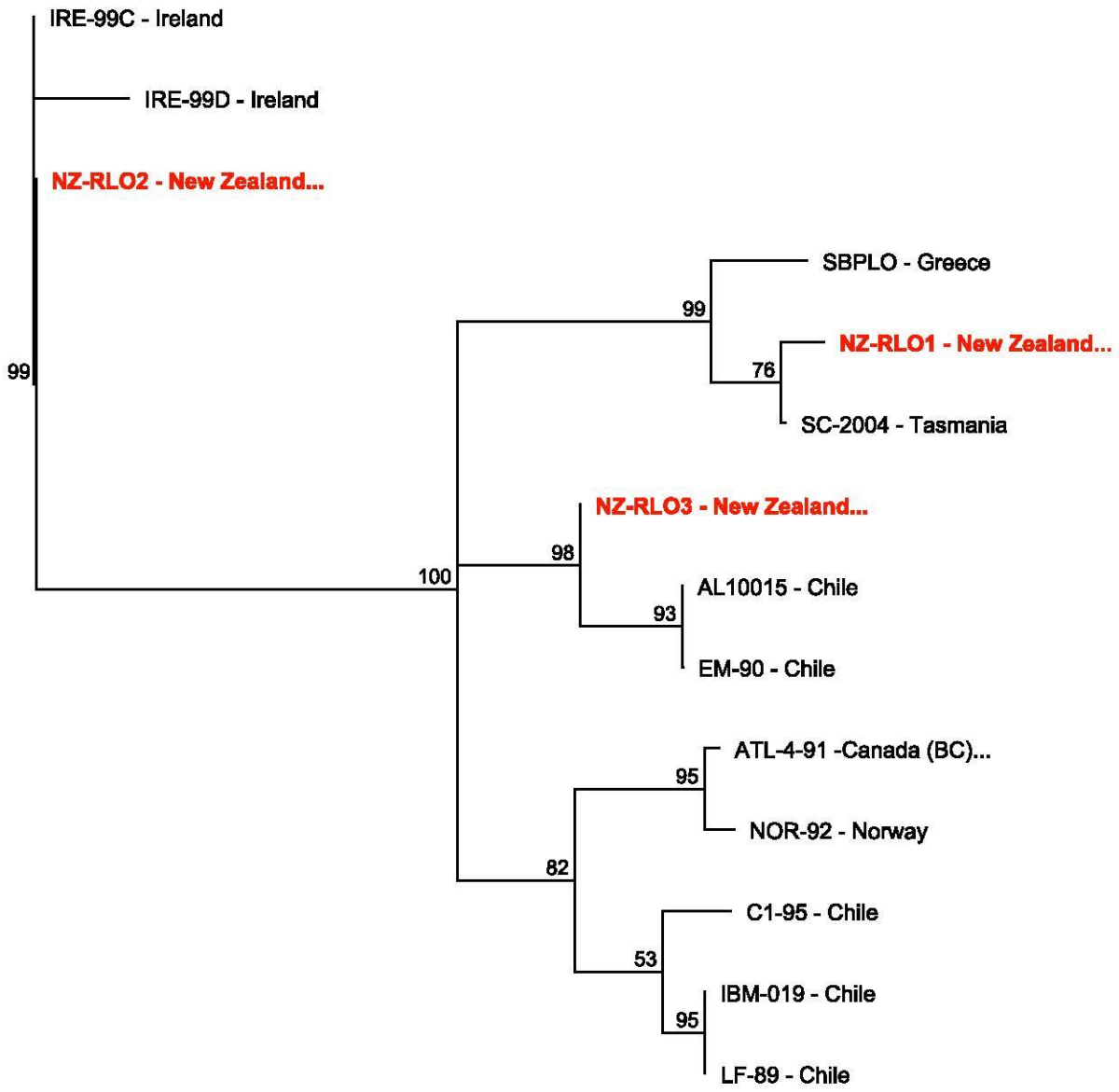


Figure 4.4. Phylogenetic tree showing genetic relatedness between the three NZ-RLO strains from Chinook salmon in New Zealand (red text) with other closely related *Piscirickettsia salmonis* and piscirickettsia-like organisms from around the world (black text). This phylogenetic tree is based on 254 bp of the internal transcribed spacer region (ITS). Numbers on the nodes indicate consensus support (%) of split.

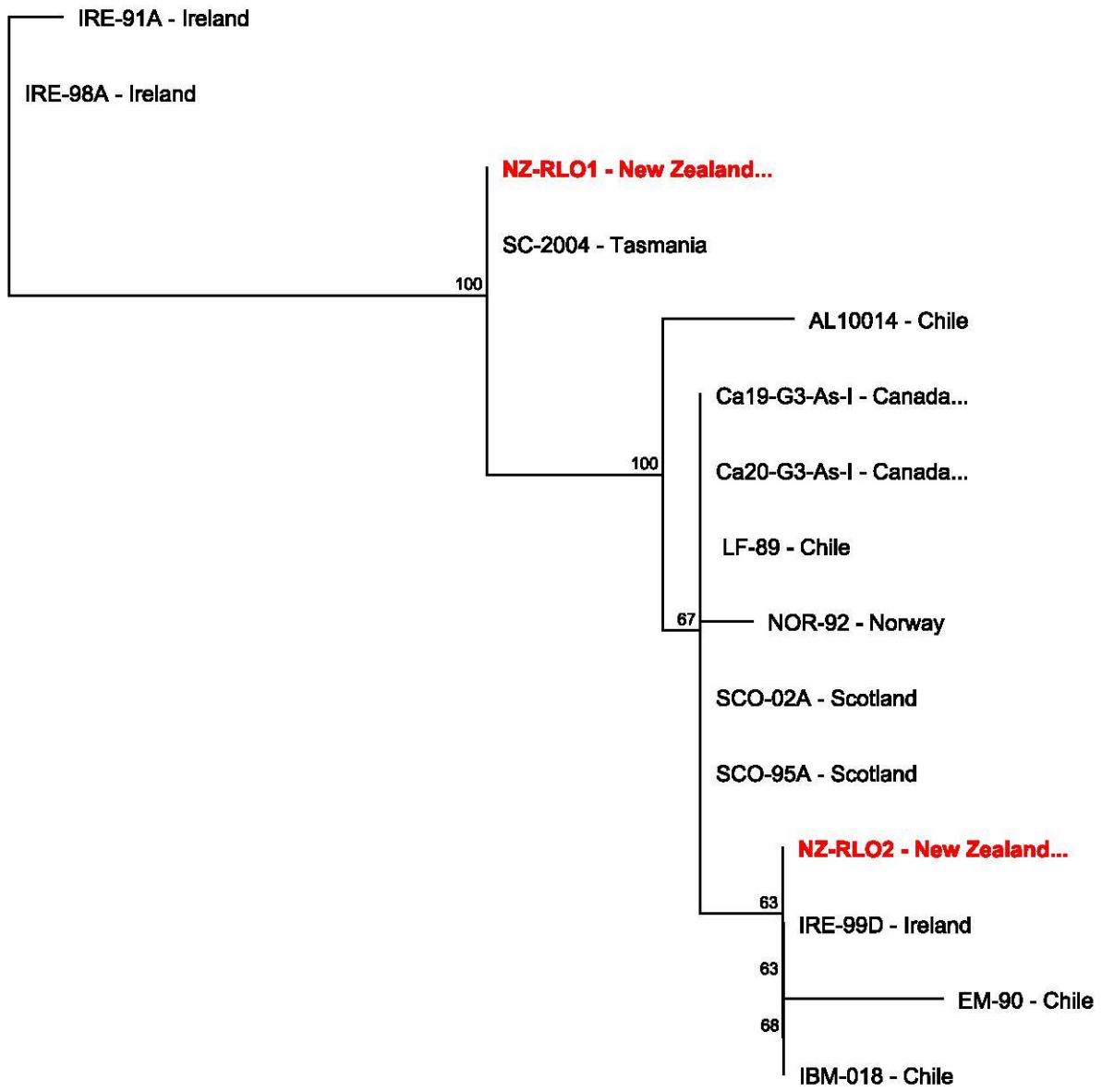


Figure 4.5. Phylogenetic tree showing genetic relatedness between the two NZ-RLO strains from Chinook salmon in New Zealand (red text) with other closely related *Piscirickettsia salmonis* and piscirickettsia-like organisms from around the world (black text). This phylogenetic tree is based on the partial 16S rRNA gene (375 bp). Numbers on the nodes indicate consensus support (%) of split.

4.3.4.3 Tenacibaculum maritimum Multi-locus sequence typing (MLST)

Both New Zealand *T. maritimum* isolates were identical and the concatenated sequences were assigned the Sequence Type (ST) 21. This ST was shared with two other isolates in the MLST database recovered from affected Atlantic salmon (DPIF_89-0528-1) and rainbow trout (DPIF_89-0235-3) in Tasmania, Australia in 1989 (Habib et al., 2014). The phylogenetic tree of the concatenated housekeeping genes

showed highest similarity (99.95%) to isolates from Tasmania, DPIF_89-0528-1 and DPIF_89-0235-3 (Figure 4.6).

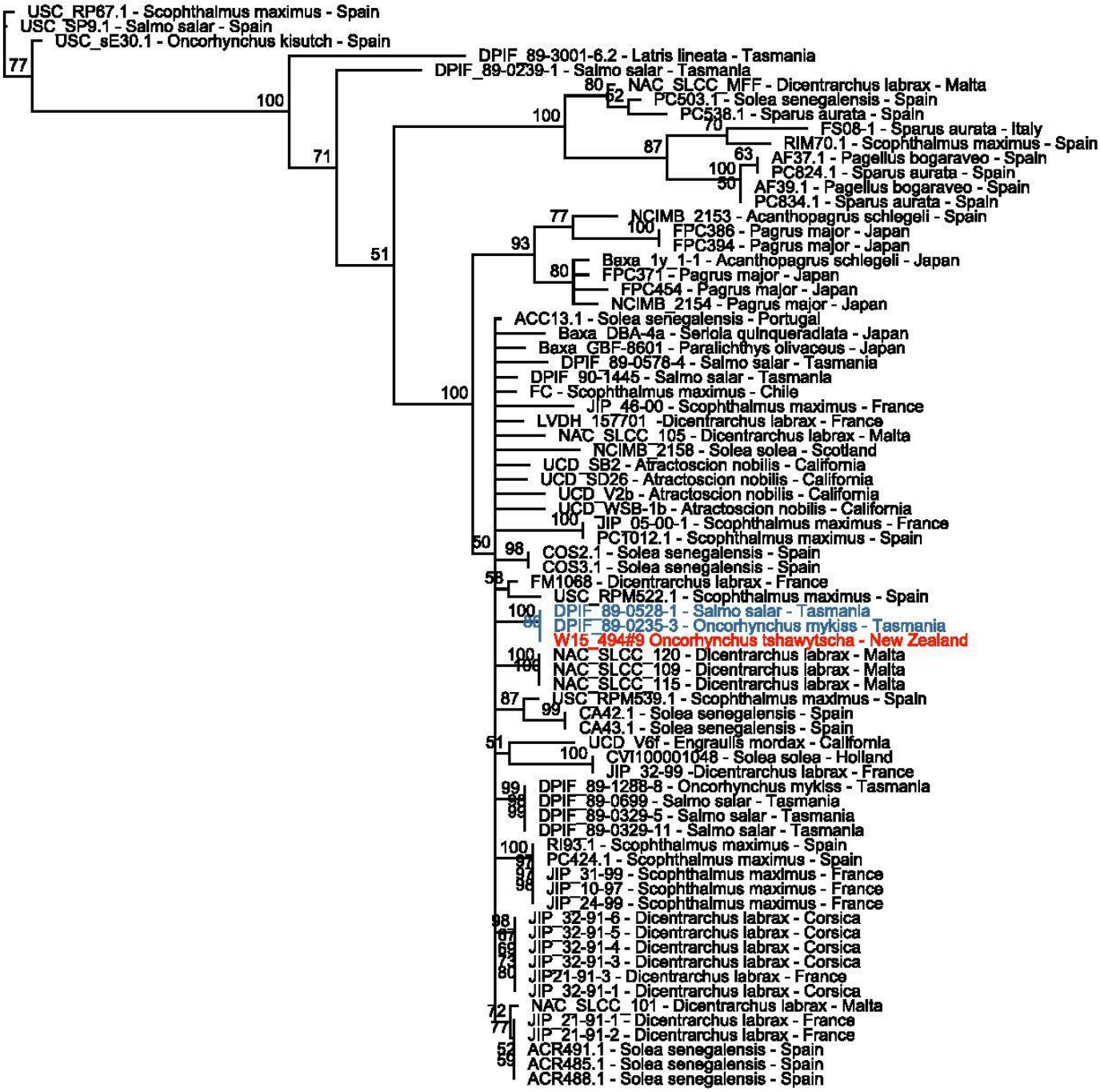


Figure 4.6. Phylogenetic tree showing genetic relatedness of the New Zealand isolate of *Tenacibaculum maritimum* from Chinook salmon (red text) with 73 *Tenacibaculum maritimum* strains from around the world (black text). The closest strains related to the New Zealand isolate are marked in blue text. This phylogenetic tree is based on MLST data from 11 housekeeping genes. Numbers on the nodes indicate consensus support (%) of split.

4.3.5 Statistical analysis

When comparing fish with skin ulcers to fish without skin ulcers that contained NZ-RLO DNA, fish with skin ulcers from sites 1 ($\chi^2 = 7.72$, $p = 0.01$) and 2 ($\chi^2 = 47.263$, $p < 0.01$) were significantly more likely to have detectible NZ-RLO than fish without skin ulcers. In contrast, NZ-RLO DNA was not detected more frequently in fish with skin ulcers than fish without skin ulcers from sites 3 ($\chi^2 = 1.16$, $p = 0.28$), or 4 ($\chi^2 = 0.134$, $p = 0.71$).

When comparing fish with skin ulcers to fish without skin ulcers that were positive for *T. maritimum*, there was no significant difference in detecting this pathogen in either fish at site 1 ($\chi^2 = 0.99$, $p = 0.32$), 2 ($\chi^2 = 1.08$, $p = 0.30$), or 4 ($\chi^2 = 1.75$, $p = 0.19$). At site 5 there was a significant difference in detecting *T. maritimum* in fish with skin ulcers over fish without skin ulcers ($\chi^2 = 73.39$, $p < 0.01$) indicating an association of *T. maritimum* with skin ulcers at this site.

When comparing the prevalence of NZ-RLO between sites, a significant difference was seen ($\chi^2 = 120.9$, $p < 0.01$). These differences were between sites 1 and 3 ($p = 0.03$), 1 and 4 ($p < 0.01$), 2 and 3 ($p < 0.01$), 2 and 4 ($p < 0.01$), and 3 and 4 ($p = 0.02$) indicating that sites 1 and 2 had a higher prevalence of NZ-RLO than site 3 and sites 1, 2 and 3 had a higher prevalence of NZ-RLO than site 4. There was no significant difference between sites 1 and 2 ($p = 0.42$) indicating these two sites had the same likelihood of detected NZ-RLO DNA.

When comparing the prevalence of *T. maritimum* between sites there was a significant difference ($\chi^2 = 76.702$, $p < 0.01$). These differences were between sites 1 and 2 ($p = 0.03$) 4 and 2 ($p = 0.03$), 5 and 2 ($p < 0.01$), and 5 and 4 ($p < 0.01$) indicating that sites 1, 4, and 5 had a higher prevalence of *T. maritimum* than site 2 and site 5 had a higher prevalence of *T. maritimum* than site 4. There were no significant differences seen between sites 4 and 1 ($p = 0.38$), and 5 and 1 ($p = 0.09$) indicating site 2 had the least likelihood of detecting *T. maritimum*.

4.4 Discussion

The distribution of NZ-RLO and *T. maritimum* found within the present study revealed a limited distribution of NZ-RLO with strains restricted to certain regions, and a widespread distribution of *T. maritimum*.

Three strains of NZ-RLO were identified within this study initially through nucleotide sequencing of the ITS rRNA region and 16S rRNA. The ITS rRNA is a non-coding locus that has a high degree of variation between closely related species due to the low evolutionary pressure that acts on it. It is commonly used to assess phylogeny and it predicts that the highest level of genetic diversity within a species will occur at its geographic origin where genetic changes have been accumulating for the longest evolutionary period (Kimura, 1983). This region has been used to clarify intraspecific variation for many pathogens including *P. salmonis* (Reid, Griffen, & Birkbeck, 2004; Casanova et al., 2003).

The ITS rRNA genetic diversity of the NZ-RLO strains found in this study suggests that NZ-RLO could have existed in New Zealand for some time (Kimura, 1983). The identical ITS rRNA sequence of NZ-RLO2 with an Irish *P. salmonis* strain however is unexpected. Although the lack of variation in the ITS rRNA region between two isolates is not uncommon (van Herwerden, Gasser, & Blair, 2000), it seems less likely that an identical ITS rRNA sequence would be seen between such geographically distinct regions such as New Zealand and Ireland had this pathogen spread gradually over time. This Irish strain was initially identified in 1999 and Reid et al., (2004), demonstrated this strain separated into its own clade or group when compared with 17 other strains of *P. salmonis* from Chile, Norway, Canada, Scotland, and Ireland. NZ-RLO1 and NZ-RLO3 are most likely Southern Hemisphere strains as the ITS rRNA region of NZ-RLO1 was almost identical to the Tasmanian RLO and NZ-RLO3 differed to any other closely related species in the database with the closest relatives being isolates from Chile. The detection of three different strains of NZ-RLO that were not present in all three regions could suggest NZ-RLO has been present in New Zealand long enough to establish regional differences spreading naturally over time, or alternatively, could have been from three separate incursions.

If the organisms spread naturally over time, this could have occurred from an origin of introduced salmonids or from the endemic wild fish stocks present in New Zealand. The introduction of salmonids into New Zealand via ova began in the mid-1880s and continued until the 1960s (McDowall, 1994). These introductions originated from Tasmania, North America, and Europe (McDowall, 1978, McDowall, 1994, Scott et al., 1978, Scott, 1984). As *P. salmonis* and RLO are not restricted to salmonids (M.F., Chen et al., 2000; S.C., Chen et al., 2000), native New Zealand fish may be the origin or a possible pathway for spread of this potential pathogen. A survey of wild fish populations for the presence of NZ-RLO could test this hypothesis.

The possibility of these organisms arriving as three separate incursions is considered less likely. Regulations exist under the importation of live fish into New Zealand including a quarantine period (Pharo, 2017) which aid in limiting the introduction of exotic diseases. The transmission of NZ-RLO is currently unknown, however transmission of *P. salmonis* is known to occur horizontally (Smith et al., 2004, Almendras et al., 1997, Cvitanich et al., 1991) and vertical transmission has only been demonstrated under experimental conditions (Larenas et al., 2003). Other pathways that could be considered for introduction of this organism include on equipment that has not been sufficiently decontaminated, in food, via a vector, or in ballast water. To confirm the significance of the diversity in the ITS rRNA region between the NZ-RLO or to give increased confidence to a source of these New Zealand isolates, further analysis would need to be carried out with multiple sequences of different strains from multiple geographic locations.

Analysis of the 16S rRNA gene of NZ-RLO1 and NZ-RLO2 allowed differences to be observed in genes that are conserved over time. Analysis of the 16S rRNA and ITS region were consistent and showed that NZ-RLO1 showed highest similarity to the Tasmanian-RLO while NZ-RLO2 was more closely related to Chilean and Irish strains of *P. salmonis*. In the present study, limited genes and strains were compared and a recent study by Gias, et al. (2018) looking at pan-genome comparison of predicted proteomes showed NZ-RLO1 and NZ-RLO2 grouped separately from the Chilean strains and were more closely related to each other. Further work is still required to understand the relationship of the NZ-RLO to strains found globally and the use of

phylogenetics with an increased number of *P. salmonis* strains from a variety of geographical locations using multiple genes may further clarify this relationship.

The *T. maritimum* isolated in this study was shown to be identical to those detected in Tasmania using MLST. MLST has commonly been used to answer questions of relatedness and bacterial typing in many species including *Flavobacterium columnare* (Ashrafi, Pulkkinen, Sundberg, Pekkala, & Ketola, 2015), *Neisseria meningitides* (Maiden et al., 1998) and *Tenacibaculum* species (Habib et al., 2014). This relatedness to the Tasmanian strains and the widespread distribution of *T. maritimum* supports the presence of *T. maritimum* in New Zealand for an extended period of time.

In this study, detection of NZ-RLO was strongly associated in fish presenting with skin ulcers from sites 1 and 2, but not at sites 3 and 4. This could indicate that NZ-RLO may be the cause of the skin ulcers at sites 1 and 2. Alternatively, the cause of the skin ulcers, or the skin ulcers themselves, lowered the resistance of the fish to NZ-RLO and therefore this pathogen could affect these fish more readily and be detected more frequently in fish with skin ulcers without this organism contributing to the development of the disease. It is also possible that NZ-RLO caused other diseases in the fish that lowered the resistance to other agents resulting in skin ulcer development or that skin ulcers are not related to infection with NZ-RLO. It is interesting to note that NZ-RLO was only associated with skin ulcers in the two sites with the highest seawater temperatures and experiencing the highest mortalities. This may suggest NZ-RLO can infect fish at colder temperatures, but is more likely to cause disease in warmer temperatures with temperature being a potential risk factor. The detection of NZ-RLO in fish not showing visible signs of disease suggests this bacteria can infect fish without causing the clinical sign of disease of skin ulcers. Fish that did not have skin ulcers but had NZ-RLO DNA present were from a site with colder temperatures and the NZ-RLO present in these five fish was a different strain than those detected from the NZ-RLO positive skin ulcers, NZ-RLO3. As well as the impact of temperature on the expression of disease, it may also indicate the differences in pathogenicity of the strains of NZ-RLO. Moreover, this study suggests that different strains of NZ-RLO may target different tissues. NZ-RLO1 was most frequently detected in the kidney, NZ-RLO2 most frequently detected in skin ulcers and NZ-RLO3 most frequently detected in the spleen. The presence of an organism most commonly in skin ulcers may indicate

this organism is an environmental contaminant and a potential secondary pathogen. This possibility cannot be excluded and additional research is required to determine if these different strains of NZ-RLO cause mortality in farmed salmon and to confirm if these NZ-RLO have an affinity for different tissues in the fish.

Tenacibaculum maritimum was only significantly associated with fish presenting with skin ulcers at site 5. The failure to detect an association between this bacteria and fish with skin ulcers at the other sites could suggest that additional environmental factors are required for infection by this bacteria to manifest as skin ulcers. As with NZ-RLO, it cannot be determined if *T. maritimum* caused the skin ulcers or the skin ulcers were indicative of other diseases that may have allowed greater infection rates by *T. maritimum*. Overall, fish presenting with skin ulcers at two sites were associated with NZ-RLO, associated with *T. maritimum* at one site, and not associated with either NZ-RLO or *T. maritimum* at two sites. This is consistent with skin ulcers being multifactorial rather than being specific for either of these infectious agents.

It is hypothesised that seawater temperature plays a key role in the presence of NZ-RLO. *In-vitro* studies into the growth characteristics of NZ-RLO1 and NZ-RLO2 have determined these organisms grow best at 15 to 18°C (Gias et al., 2018; Ministry for Primary Industries, unpublished data). New Zealand rickettsia-like organism 1 can grow in higher temperatures of 22°C whereas NZ-RLO2 will not grow above 18°C. (Gias et al., 2018). This *in-vitro* work supports the findings of increased prevalence of NZ-RLO1 and NZ-RLO2 in the fish originating from sites where the water temperatures were the highest (>17°C) and a lower prevalence or no NZ-RLO at sites with lower seawater temperatures (<15°C). The growth profile and optimal growth temperature of NZ-RLO3 is unknown as this isolate has not been cultured in the laboratory. The waters in Canterbury are below 15°C and colder than the Marlborough Sounds. This colder temperature may not be optimal for growth of NZ-RLO3 which is why it is seen in low prevalence. Then again, the colder seawater temperature may be contributing to keeping the fish immune system functioning adequately leading to the NZ-RLO3 not entering, replicating, or causing disease in the fish. When comparing the maximum summer temperature with the prevalence of NZ-RLO at all sites, the two sites with the highest temperatures; site 1 and 2, also had the highest prevalence of NZ-RLO. Additionally at site 2, samples were taken in different seasons between 2015 and

2017. This allowed an opportunistic look at NZ-RLO levels during the seasons and further supports a link between the warmer summer months and a higher prevalence of NZ-RLO DNA detected in sampled fish.

Seawater temperatures varied between the three growing regions as well as between sites within the Marlborough Sounds. Maximum summer temperatures do not exceed 16°C at sites 3, 4, and 5. At sites 1 and 2 where the highest mortalities occurred, maximum summer temperatures routinely exceeded 17°C and in 2015 were approximately 0.5 °C higher than those reported in the previous years. Additionally, it was reported by the National Oceanic and Atmospheric Administration in 2015 that there was a climate anomaly that globally impacted the sea temperature in 2015 with an average rise of 0.74°C which was the warmest year ever recorded (Saavedrea et al., 2017). Temperature is one of the most important environmental factors driving the health of fish. Temperature can affect the ability of a pathogen to grow and proliferate as well as affecting both the innate and adaptive immune response of fish. Salmon stressed by high temperatures will be less able to cope with other stressors including from pathogens (van Vleck, Deukmejian, & Kennedy, 1988). Moreover, stressed fish have also been found to be unable to repair skin wounds as well as healthy fish (Sveen et al., 2019). Temperatures of 12 to 13°C minimizes the risk of disease in both juvenile and adult Chinook salmon, 14 to 15°C is associated with an elevated risk of disease, and temperatures of 18 to 20°C are associated with a high risk of disease (Carter, 2005). The outbreak of disease is a result of the interaction between the pathogen, host, and environment. Therefore an important part of understanding the role of pathogens in mortalities are laboratory challenge trials which allow control of the environment. Challenge trials to assess the pathogenicity of NZ-RLO1 and NZ-RLO2 are described in Chapters 6 and 7.

Strain differentiation of NZ-RLO was not always possible in this study. At the time of testing, only the generic qPCR was available followed by a conventional PCR for strain identification. This conventional PCR had a reduced sensitivity which meant that when DNA from the pathogen was detected at a low level (i.e. > 35 Ct) in the qPCR the conventional assay was unable to amplify a product (Chapter 2). More sensitive and specific assays were developed for NZ-RLO1 (Chapter 5) and NZ-RLO2 (Gias et al., 2018) to overcome this problem.

Warmer seawater temperatures do not appear to have a strong correlation with increased prevalence of *T. maritimum* in this study. Experiments conducted at the AHL (data not shown) revealed that the New Zealand strain of *T. maritimum* grows at the same rate in 48 hours whether held at 15°C, 22°C, or 27°C, further supporting the hypothesis that there is no link with increased temperatures and higher prevalence of *T. maritimum*. *Tenacibaculum maritimum* has a reported temperature range of 15 to 34°C in which it can grow with an optimum growing temperature of 30°C (Avendaño - Herrera et al., 2006).

4.5 Conclusion

This study has shown that NZ-RLO has a restricted distribution in New Zealand marine farmed salmon populations with the strains limited to different regions, and a widespread distribution of *T. maritimum*.

Three strains of NZ-RLO were detected in two growing areas; the Marlborough Sounds and Canterbury. The prevalence of NZ-RLO were higher in the Marlborough Sounds and different strains were found in the Marlborough Sounds than Canterbury.

New Zealand rickettsia-like organism were detected more frequently at sites 1 and 2 where elevated mortalities occurred, and were more commonly associated with fish that had skin ulcers at these sites. *Tenacibaculum maritimum* was found more frequently at site 1 where elevated mortalities occurred but also more frequently at sites 4 and 5 where elevated mortalities had not occurred. The high prevalence of *T. maritimum* at other sites not experiencing elevated mortalities may suggest a low level infection that could be of concern for the salmon if conditions changed that compromised fish health making them more susceptible to infection.

The results from the present study suggest NZ-RLO could be the cause of skin ulcers at sites 1 and 2 and is more likely to be involved in summer mortalities in the Marlborough Sounds than *T. maritimum*.

Chapter 5 : Specific quantitative PCR to detect New Zealand rickettsia-like organism (NZ-RLO1).

Harvest time at a New Zealand marine Chinook salmon farm.

5.1 Introduction

In 2015, New Zealand rickettsia-like organism (NZ-RLO1 and NZ-RLO2) were detected in populations of farmed Chinook salmon (*Oncorhynchus tshawytscha*) experiencing elevated mortalities (Chapters 2 and 4). Due to ongoing monitoring of the health status of these populations, there is the need for sensitive, specific, reliable, and reproducible tests to detect NZ-RLO1 in Chinook salmon tissues. Additionally, this test will enable differentiation between NZ-RLO1 and the exotic *Piscirickettsia salmonis.* A specific qPCR to detect NZ-RLO2 was designed, optimised, and validated (Gias et al., 2018) and in the present study, a specific qPCR to detect NZ-RLO1 will be described.

New Zealand rickettsia-like organism 1 is a Gram-negative intracellular bacteria that is difficult to identify using traditional techniques. Such difficulties are due to its fastidious nature, requirements for specialised media or cell lines for growth, and the

potential lack of specificity with immunohistochemistry (Alday-Sanz, Rodger, Turnbull, Adams, & Richards, 1994). Therefore, PCR is the most commonly used diagnostic technique for identification (Corbeil et al., 2003). While NZ-RLO1 is closely related to the pathogen *P. salmonis* which is exotic to New Zealand, there is no rapid test to distinguish between this strain and NZ-RLO1. Currently, determining the strain of NZ-RLO requires the use of a generic qPCR for screening, which detects all NZ-RLO and *P. salmonis* strains, followed by a conventional PCR and nucleotide sequencing of the internal transcribed spacer (ITS) rRNA region. Nucleotide sequencing is required to determine the strain involved and to exclude the presence of *P. salmonis* DNA. This method is costly, time consuming and often cannot detect low levels of infection due to the reduced sensitivity of the conventional PCR in comparison with the generic qPCR (Chapter 2).

The aim of the present study was to develop and validate a qPCR assay to detect and identify NZ-RLO1 in Chinook salmon tissue, to enable confirmation of clinical cases and the ability to detect sub-clinical infections.

5.2 Methods

5.2.1 Design of primers and probe

The ITS rRNA region was selected as the target for this qPCR assay. This region was analysed using Geneious version R9 (Kearse et al., 2012). A forward primer (PS_ITS_RTF) was designed and the reverse primer (PS_ITS_4RT) from a previously published conventional assay (Marshall et al., 1998) was used. A probe (NZ-RLO1_Pr) located between the forward and reverse primer was designed to specifically amplify a 79 base pair (bp) product for NZ-RLO1 (Table 5.1; Figure 5.1). *In silico* specificity of primers and probe was examined by performing a basic local alignment search in the National Centre for Biotechnology information database (Madden, 2013).

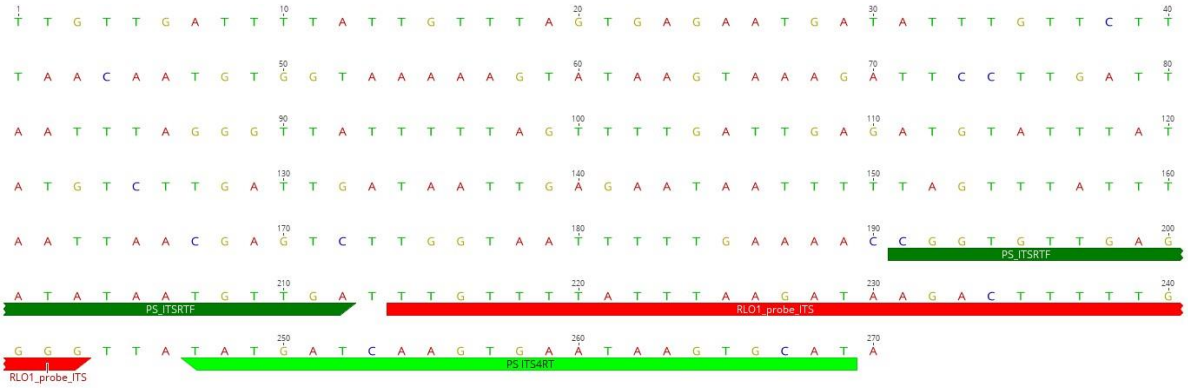


Figure 5.1. Location of primers and probe in the ITS rRNA region of NZ-RLO1.

Table 5.1. Primers and probe sequence for NZ-RLO1 qPCR assay.

Primer name	Nucleotide sequence (5' – 3')
PS_ITS1_RTF	CGGTGTTGAGATATAATGTTGA
PS_ITS4_RT	ATGCACTTATTCACTTGATCATA
NZ_RLO1_Pr	FAM-TTGTTTTATTTAAGATAAGACTTTTTGGGG-BHQ-1

5.2.2 Bacterial and parasite isolates and DNA preparation

Renibacterium salmoninarum and *Myxobolus cerebralis* DNA was extracted from infected tissues, and all other bacterial DNA was extracted from pure cultures (Table 5.2). DNA from both infected tissues and pure culture were extracted using Qiagen QIAamp mini kit (Qiagen, CA, USA) following the manufacturer's protocol for tissue. All DNA was stored at -20°C in low bind DNA microfuge tubes until used.

Table 5.2. Bacterial strains and parasites available for specificity testing.

Pathogen	Reference/Source	PCR result
NZ-RLO1	IDC W15_494 10Sp	+
NZ-RLO2	IDC W16_237	-
Piscirickettsia salmonis	LF-89, ATCC VR-1361	-
Tenacibaculum maritimum	IDC W15_494#9	-
*Renibacterium salmoninarum**	Pacific Biological Station, Canada	-
Mycobacterium fortuitum	IDC T3-F40	-
Flavobacterium psychrophilum	ATCC 49511	-
Aeromonas salmonicida ssp. *salmonicida*	ATCC 33658	-
Yersinia ruckeri	ATCC 29473	-
*Myxobolus cerebralis**	University of Veterinary Medicine, Germany	-
Lactococcus garvieae	ATCC 49321	-
Streptococcus iniae	ATC C 29178	-

*DNA extracted from infected tissue. ATCC = American type culture collection, IDC = Investigation and Diagnostic Centre.

5.2.3 PCR amplification

Amplification by qPCR was carried out on a CFX 96 real-time PCR detection system (Bio-Rad, Hercules, USA). For pure cultures, the reaction mixture consisted of 1 µL of DNA (1 – 10 ng), 10 µL 2 X SsoAdvanced Universal probes supermix (Bio-rad), 0.25 µM of each primer, 0.3 µM probe and molecular grade water to a final volume of 20 µL. For DNA derived from tissue, 1 µL and 3 µL of DNA was used as a template to account for potential varying levels of infection of NZ-RLO1 within the tissue sample. The same amount of primers, probes, and master mix were used as for pure culture with the molecular grade water volume adjusted accordingly. The cycling conditions were as follows: initial denaturation at 95˚C for 2 min then 40 cycles of denaturation at 95˚C for 15 sec and annealing/extension at 60˚C for 30 sec.

The presence of amplifiable DNA from material derived from tissue was confirmed by using an internal control PCR targeting the 18S rRNA gene (Ribosomal 18S rRNA Endogenous Control; Life technologies, Oregon, USA).

DNA extracted from a pure culture of NZ-RLO1 grown in cell culture was used as a positive control in each assay. To assess environmental contamination during qPCR set up, molecular grade water was used as no template controls (NTC) in each assay performed. A qPCR run was valid if there was no amplification observed in the NTC and a sigmoidal curve was observed in the positive control. The threshold was set automatically by the CFX manager software and was approximately 60 relative fluorescence units during this study.

5.2.4 Analytical specificity

A range of bacterial species were used to determine the analytical specificity of the assay (*n* = 11). These included strains that are both closely related and un-related to NZ-RLO1 (Table 5.2). To confirm cross reactivity did not occur from host tissue or related strains of NZ-RLO, genomic DNA from NZ-RLO negative Chinook salmon tissue was spiked with DNA from pure cultures of NZRLO1, NZRLO2 and *P. salmonis* LF-89 (ATCC VR-1361) at varying concentrations (Table 5.3). Assays were conducted in triplicate.

Table 5.3. Matrix of salmon DNA spiked with varying concentrations of NZ-RLO1, NZ-RLO2 and *Piscirickettsia salmonis* LF-89 to assess cross-reactivity with similar organisms as well as inhibition issues in host DNA.

Sample	NZ-RLO1	NZ-RLO-2	*Piscirickettsia salmonis* LF-89
1	0.2 ng μL^{-1}	0.2 ng μL^{-1}	0.2 ng μL^{-1}
2	0.2 ng μL^{-1}	0.04 ng μL^{-1}	0.04 ng μL^{-1}
3	0.04 ng μL^{-1}	0.2 ng μL^{-1}	0.04 ng μL^{-1}
4	0.04 ng μL^{-1}	0.04 ng μL^{-1}	0.2 ng μL^{-1}

5.2.5 Analytical sensitivity

Analytical sensitivity, or limit of detection (LOD), was determined based on the lowest dilution detected in all of the replicates.

DNA extracted from a pure culture of NZ-RLO1 was diluted in two solutions; 1) DNA extracted from salmon kidney tissue that was negative for NZ-RLO, 2) molecular grade water. This was performed to assess any PCR inhibitors present in the salmon DNA

and to ensure no cross-reactivity occurred in the presence of Chinook salmon DNA. DNA extracted from NZ-RLO1 grown in cell culture was quantified using Qubit flourometer (Life technologies) and a titration was performed to determine the tissue culture infectious dose in 50% of the cells inoculated ($TCID_{50}$) following the Spearman-Kärber method (Spearman, 1908; Kärber, 1931). Ten-fold serial dilutions were prepared in both molecular grade water and negative Chinook salmon DNA (neat to 10^{-7}) and used as a template for the qPCR. Analytical sensitivity was run in triplicate for each dilution by one user. The log_{10} of the mean DNA concentration was plotted against the corresponding cycle threshold (Ct) value to determine the R^2 value.

5.2.6 Assay repeatability

Assay repeatability for different NZ-RLO1 concentrations was determined by assessing the intra-run and inter-run percentage co-efficient of variation (% CV) of the Ct values in the qPCR assay. For the intra-run % CV, the assay was performed on 10 fold serial dilutions (neat to 10^{-6}) of NZ-RLO1 DNA by one user and was performed in triplicate for each dilution. The inter-run % CV was determined on seven 10 fold dilutions of NZ-RLO1 diluted in both molecular water and salmon DNA and carried out in triplicate for each dilution by three different users. The % CV was expressed as a mean for each of the dilutions. The log_{10} of the DNA concentration was plotted against the corresponding Ct value to determine the R^2 value and amplification efficiency was determined as above.

5.2.7 Assay reproducibility

Reproducibility of the assay was tested by two different users. A blind panel of 20 DNA samples was provided and tested in duplicate by each user. The % CV was determined for each sample between the users with a value of < 10% determine acceptable.

5.2.8 Diagnostic sensitivity

Diagnostic sensitivity (DSe) was determined using multiple tissue samples from Chinook salmon tissue (kidney, liver, spleen) previously confirmed to contain NZ-RLO1 based on cell culture, histology, generic qPCR, nucleotide sequencing and

clinical signs of disease (*n* = 42). Twenty four samples were taken from fish of harvest size from sea-pens at one location during an investigation into elevated mortalities. On necropsy, all of these fish presented with skin ulcers on the body surface, one presented with a pale liver, two with mottled kidneys and two with reddening of the intestine. Eighteen samples were taken from an infection trial of fish injected with NZ-RLO1 that were positive for NZ-RLO1 by cell culture or histology (Chapter 7).

All samples were tested in the NZ-RLO1 qPCR in duplicate with a template volume of 1 µL and 3 µL respectively. The sample numbers tested were based on available confirmed positive material. Based on the sample size available, an estimated 98% DSe with a 5% error and 95% confidence was established (OIE, 2017).

5.2.9 Diagnostic specificity

Diagnostic specificity (DSp) was determined using tissue samples from 60 Chinook salmon of a population known to be negative for NZ-RLO based on previous testing by a generic qPCR for *P. salmonis* (Corbeil et al., 2003) and absence of clinical signs (*n* = 160). The samples were taken from fish of harvest size from sea-pens at one location during routine surveillance (Chapter 4, site 5).

All samples were tested in the NZ-RLO1 qPCR with a template volume of 1 µL and 3 µL respectively. Based on the numbers of tissues available an estimated 92% DSp with a 5% error and 95% confidence was established (OIE, 2017).

5.3 Results

5.3.1 Analytical specificity and sensitivity

All non-target bacteria and parasites (*n* = 11) as well as uninfected Chinook salmon DNA resulted in no amplification in the NZ-RLO1 qPCR and 100% analytical specificity (Table 5.2).

The concentration of the cell culture used in the dilution series was determined to be 1.2 x 10^5 $TCID_{50}$. The LOD revealed that 2 fg μL^{-1}, equivalent to 10 $TCID_{50}$, was

consistently detected. A linear relationship between the Ct values and the concentration of DNA for the qPCR assay was observed. The amplification efficiency was 88% (Figure 5.2).

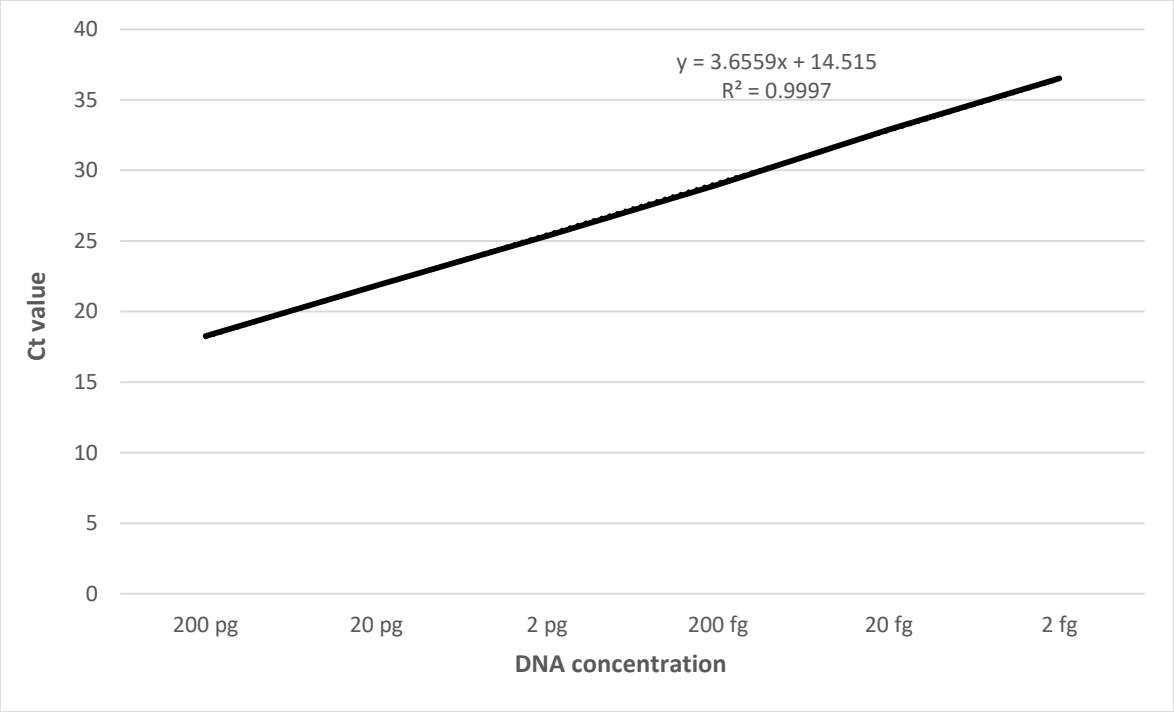


Figure 5.2. Correlation between DNA concentration and Ct values for triplicates of a six step log10 dilution of NZ-RLO1 DNA extracted from a pure cell culture.

5.3.2 Repeatability

The intra-run % CV ranged from 0.07 to 1.74 for DNA diluted in water with an R^2 value of 99.8% and 0.33 to 4.87 for DNA diluted in salmon DNA with an R^2 value of 99.9% (Table 5.4).

Table 5.4. Intra-run repeatability presented as co-efficient of variation for NZ-RLO1 DNA dilution series using water and salmon DNA as a diluent.

Dilutions using nuclease free water		Dilutions using salmon DNA	
DNA ($TCID_{50}$)	**% CV**	**DNA ($TCID_{50}$)**	**% CV**
1.2×10^5	1.74	1.2×10^5	1.14
1.2×10^4	0.07	1.2×10^4	0.48
1.2×10^3	0.52	1.2×10^3	0.41
1.2×10^2	0.18	1.2×10^2	0.33
1.2×10^1	1.02	1.2×10^1	0.49
1.2 x 10	1.11	1.2 x 10	4.87

The inter-run % CV ranged from 2.19 to 3.26 for DNA diluted in water and 1.03 to 2.51 for DNA diluted in salmon DNA (Table 5.5). For each user, the R^2 value and amplification efficiency was determined in water and salmon DNA. The R^2 value for each user in water was ≥ 99.8% and in salmon DNA was ≥ 99.7%. The amplification efficiency of the qPCR for each user in water and salmon DNA ranged from 81-88.57% (Table 5.6).

Table 5.5. Inter-run repeatability presented as co-efficient of variation for NZ-RLO1 DNA dilution series using water and salmon DNA as a diluent. (3 replicates for each concentration of DNA, repeated by 3 different users).

Dilutions using nuclease free water		Dilutions using salmon DNA	
DNA ($TCID_{50}$)	**% CV**	**DNA ($TCID_{50}$)**	**% CV**
1.2×10^5	2.87	1.2×10^5	2.50
1.2×10^4	2.59	1.2×10^4	2.51
1.2×10^3	2.53	1.2×10^3	1.84
1.2×10^2	2.19	1.2×10^2	2.11
1.2×10^1	3.26	1.2×10^1	2.24
1.2 x 10	2.90	1.2 x 10	1.03

Table 5.6. Repeatability of three users. NZ-RLO1 DNA dilution series diluted in nuclease free water and salmon DNA.

	DNA diluted in water			DNA diluted in salmon DNA		
	User 1	**User 2**	**User 3**	**User 1**	**User 2**	**User 3**
Slope	-3.69	-3.67	-3.65	-3.87	-3.63	-3.65
Coefficient of determination (R^2)	0.9993	0.9976	0.9997	0.9969	0.9987	0.9993
Efficiency (%)	87	87	88	81	89	88

5.3.3 Reproducibility

The blind panel of samples returned similar Ct values for each sample containing NZ-RLO1. Samples containing no NZ-RLO1 DNA resulted in no amplification for both users. The % CV of the average Ct values between each user was < 10% for all samples (Table 5.7).

5.3.4 Diagnostic sensitivity and specificity

All samples from the positive reference population showed amplification in the NZ-RLO1 qPCR with Ct values ranging from 13.26 to 39.37. Based on the sample size tested, this resulted in a DSe of 98% for the qPCR.

Samples from the non-infected or negative population showed no amplification in the NZ-RLO1 by qPCR. Based on the sample size tested, this resulted in a DSp of 92%.

Table 5.7. Panel of 20 samples for reproducibility of the NZ-RLO1 qPCR.

Sample	Negative salmon DNA (µL)	NZ-RLO1 (µL)	NZ-RLO2 (µL)	*T. maritimum* (µL)	User 1, rep. 1 (Ct value)	User 1, rep. 2 (Ct value)	Average (Ct value)	User 2, rep. 1 (Ct value)	User 2, rep. 2 (Ct value)	Average (Ct value)	SD between averages (Ct value)	% CV
1	5	5	0	0	19.40	19.58	19.49	19.66	19.69	19.68	0.13	0.66
2	10	0	0	0	N	N	-	N	N	-	-	-
3	5	2	2	1	21.02	21.08	21.05	20.72	20.95	20.84	0.15	0.73
4	5	3	0	2	20.45	20.34	20.40	20.59	20.76	20.68	0.20	0.96
5	5	5	0	0	19.91	19.85	19.88	19.76	19.76	19.76	0.08	0.43
6	10	0	0	0	N	N	-	N	N	-	-	-
7	7	1	1	1	22.16	22.20	22.18	22.00	22.10	22.05	0.09	0.42
8	7	1	2	0	22.08	22.05	22.07	20.38	20.07	20.23	1.30	6.43
9	5	1	4	0	21.97	21.91	21.94	20.15	20.08	20.12	1.29	6.42
10	5	1	0	4	21.98	22.02	22.00	21.67	21.84	21.76	0.17	0.80
11	9	1	0	0	21.63	21.65	21.64	21.60	22.00	21.80	0.11	0.52
12	7	0	2	1	N	N	-	N	N	-	-	-
13	7	3	0	0	20.44	20.37	20.41	20.58	20.22	20.40	0.00	0.02
14	5	5	0	0	19.66	19.76	19.71	19.73	19.76	19.75	0.02	0.13
15	5	2	3	0	21.15	21.03	21.09	21.06	20.87	20.97	0.09	0.42
16	10	0	0	0	N	N	-	N	N	-	-	-
17	5	3	0	2	19.48	19.45	19.47	19.58	19.70	19.64	0.12	0.63
18	10	0	0	0	N	N	-	N	N	-	-	-
19	9	0	1	0	N	N	-	N	N	-	-	-
20	9	1	0	0	21.78	21.87	21.83	21.97	22.31	22.14	0.22	1.01

N = no amplification in qPCR. SD = Standard deviation. % CV = Percent co-efficient of variation of the average Ct values between each user.

5.4 Discussion

New Zealand rickettsia-like organism 1 is closely related to *P. salmonis*, one of the most serious disease issues for salmon aquaculture in Chile (Bravo and Midtlyng, 2007). Having robust tests available to be able to efficiently differentiate between the exotic *P. salmonis* and NZ-RLO strains present in New Zealand is therefore paramount for the salmon industry as well as biosecurity decision makers.

The intended purpose of this study was to develop and validate an assay to determine the presence of NZ-RLO1 in both clinically and sub-clinically affected salmon. Due to the intracellular and fastidious nature of this organism, traditional techniques for identification can be problematic, leading to the need for reliable and sensitive molecular tools. This qPCR assay targets the ITS rRNA region based on the DNA variation in this area between strains of *P. salmonis* and NZ-RLO (Chapter 2). New Zealand rickettsia-like organism 1 has a 19 base pair (bp) deletion in the ITS rRNA region that is not present in other known NZ-RLO and *P. salmonis* strains. This variation of 19 bp deletion is also present in the Tasmanian-RLO (Corbeil et al., 2005) and the *piscirickettsia*-like organism isolated from white seabass (SBPLO; M.F., Chen et al., 2000). Due to this, it is expected this assay will also be able to be used to detect these strains. The ability for this assay to detect Tasmanian-RLO may be of use to the Tasmanian salmon industry and we don't anticipate any issues with the adoption to tissue from other host species, for example Atlantic salmon (*Salmo salar*). There are currently no tests to differentiate the Tasmanian-RLO from NZ-RLO1 using molecular tools and there is not enough information publically available on either organism to determine if biochemical tests or growth characteristics would allow a distinction. Furthermore, there is not enough genetic data to compare both of these isolates to determine differentiations at a genetic level.

The infectious dose of NZ-RLO1 in clinically affected fish and the concentration of NZ-RLO1 in sub-clinically affected fish remains unknown. However, the analytical sensitivity of this qPCR is such that very low levels of NZ-RLO1 DNA (2 fg μL^{-1}, 10 $TCID_{50}$) were able to be detected. Based on information from the challenge trial (Chapter 7), this is lower than the levels within clinically affected fish and may be at levels low enough to detect sub-clinically affected fish. This rational for sub-clinical

detection is also based on findings from infection studies carried out in Atlantic salmon with the Tasmanian-RLO strain where concentrations of 10^3 CFU mL^{-1} and below did not induce mortalities under experimental conditions (Morrison et al., 2016).

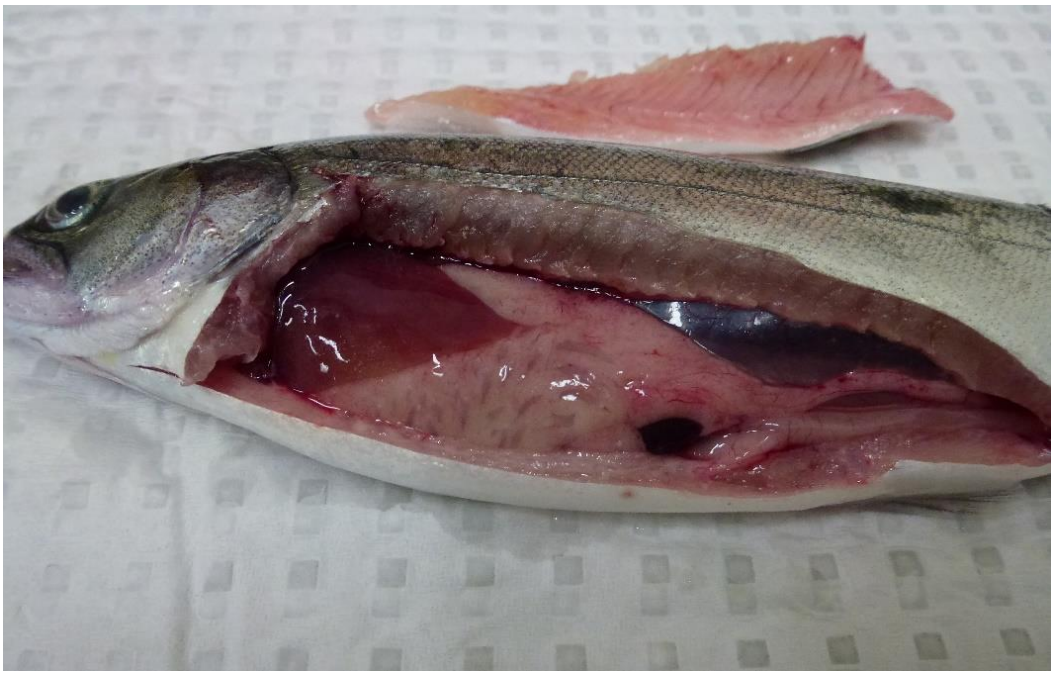
The qPCR presented in the present study demonstrates a high level of repeatability. This is based on the low % CV values obtained (intra run, inter run and the blind panel results), the R^2 values, and the amplification efficiency. However, the amplification efficiency for samples diluted in salmon DNA was lower for one user. This result is most likely due to pipetting error when using small volumes of 1 µL, due to incorrect mixing of the dilutions prior to sample transfer. Alternatively, it could be due to inhibitors in the sample when salmon DNA is present.

Diagnostic specificity (DSp) and sensitivity (DSe) are often dependent on available samples at the time of validation of a new test. Sufficient numbers of true positive and true negative samples are often problematic to source, resulting in lower than desired DSp and DSe. To increase the confidence in a new test, confirmatory tests; such as nucleotide sequencing, should be obtained from samples when the new test is implemented with DSp and DSe estimates being updated over time (Jacobson, 1998). Adding to this DSp and DSe data over time will increase confidence in the results obtained and should be carried out for this qPCR assay.

5.5 Conclusion

This study has demonstrated a specific, sensitive, and repeatable qPCR assay for the detection of NZ-RLO1 in Chinook salmon tissues. This assay can be readily adopted to be used alongside the current qPCR tests (*P. salmonis* and NZ-RLO2) for a variety of purposes including health screening, surveillance, response programmes and diagnostic testing. This qPCR will be used in Chapter 7.

Chapter 6 : Pathogenicity of the bacterium New Zealand rickettsia-like organism (NZ-RLO2) in Chinook salmon (*Oncorhynchus tshawytscha*, Walbaum) smolt.



A Chinook salmon smolt inoculated with a high dose of NZ-RLO2 that died during the study showing petechial haemorrhage of the internal adipose tissue.

This chapter has been published: Brosnahan CL, Davie PS, Munday JS, Kennedy L, Preece M, Barnes S, Jones JB, McDonald WL. (2019) Pathogenicity of the bacterium New Zealand rickettsia-like organism (NZ-RLO2) in Chinook salmon (*Oncorhynchus tshawytscha*, Walbaum) smolt. *Diseases of Aquatic Organisms*, 134(3), 175-187.

6.1 Introduction

Chinook, or king salmon (*Oncorhynchus tshawytscha*) have been farmed in New Zealand since the 1980s after first being introduced from North America in the late 1800s (McDowall, 1994). Chinook salmon are the only salmonid species farmed in New Zealand. As the majority of farmed salmon worldwide are Atlantic salmon (*Salmo salar*), there has been little published research on disease in Chinook salmon under commercial conditions.

New Zealand Chinook salmon are considered to be relatively free of disease (Anderson, 1996) with several major salmonid pathogens such as infectious salmon anaemia virus, viral haemorrhagic septicaemia virus, infectious haematopoietic

necrosis virus, salmonid alphavirus, and *Renibacterium salmoninarum* exotic to New Zealand (Diggles, 2016). However, since 2012 higher than expected mortalities have occurred at some farmed sites within the Marlborough Sounds (Norman et al., 2013). As these mortalities have occurred in the summer months they have been termed “summer mortalities”. In 2015 during a summer mortality event, an investigation was carried out which led to the identification of two rickettsia-like organisms (RLOs) in farmed Chinook salmon that were subsequently named NZ-RLO1 and NZ-RLO2 (Chapter 2; Gias et al., 2018). These organisms are closely related to *Piscirickettsia salmonis*, a major cause of disease in salmon farms throughout the world, particularly in Chile where it is the most common infectious disease in farmed salmonids (Rozas and Enriquez, 2014; Price, Ibarra, Sanchez, & St-Hilaire, 2017). While NZ-RLO are closely related to *Piscirickettsia salmonis*, the pathogenicity of NZ-RLO is unknown. Epidemiological studies have suggested a likely association with NZ-RLO1 and NZ-RLO2 and disease (Chapter 4). The pathogenicity of RLOs is dependent on the strain, host and the environment (House et al., 1999). The pathogenicity of *P. salmonis* and some closely related RLO strains have been evaluated in Atlantic salmon (House et al., 1999; Garces, Larenas, Smith, Sandino, & Lannan, 1991; Smith et al., 2004; Morrison et al., 2016) however, the pathogenicity of the newly identified NZ-RLOs have not yet been evaluated in any fish species, including Chinook salmon.

The aim of the present study was to investigate the pathogenicity of NZ-RLO2 in Chinook salmon via intra-peritoneal injection. If inoculating salmon with this bacteria resulted in disease, it would suggest NZ-RLO2 may have been a contributor to the summer mortalities in New Zealand Chinook salmon.

6.2 Materials and methods

6.2.1 Ethics statement

All experimental procedures involving the use of live fish were approved by the AgResearch Grasslands Animal Ethics Committee (Approval number AEC14122) under the New Zealand Animal Welfare Act 1999. Experiments were conducted in a PC2+ transitional containment facility at the Ministry for Primary Industries, Animal Health Laboratory (AHL), Wallaceville, New Zealand.

6.2.2 NZ-RLO2 isolate origin

Initial identification and isolation of the NZ-RLO1 and NZ-RLO2 bacteria were carried out in an enhanced PC3 laboratory from which viable bacterial isolates were unable to be removed. In order to determine if the bacteria caused mortality by *in-vivo* methods, it was necessary to re-isolate these strains in a lower containment laboratory which was successful for NZ-RLO2 only. New Zealand rickettsia-like organism 2 was recovered from a skin ulcer of a moribund farmed Chinook salmon of harvest size (approximately 3 kg) originating from the South Island of New Zealand as previously described (Gias et al., 2018).

The NZ-RLO2 isolate was cultured in a monolayer of Epithelioma papulosum cyprini (EPC, ECACC-93120820) cell line grown in Hank's minimal essential medium (MEM) (Gibco, Life Technologies, NY, USA) supplemented with 10% foetal bovine serum (FBS, Utah, USA) (MEM + 10% FBS) and passage number three was used for inoculation. The bacteria were incubated for seven days at 15°C until approximately 85% cytopathic effect (CPE) was observed. The remaining monolayer was lifted by cell scraping and a titration was performed to determine the tissue culture infectious dose in 50% of the cells inoculated ($TCID_{50}$) following the Spearman-Kärber method (Spearman, 1908, Kärber, 1931). The concentration of the inoculum administered to fish in the high dose group was calculated to be 8×10^5 $TCID_{50}$ $50\mu L^{-1}$. This was then diluted 1/10 and 1/100 with MEM + 10% FBS to obtain the inoculums administered to the fish in the medium (8×10^4 $TCID_{50}$ $50\mu L^{-1}$) and low (8×10^3 $TCID_{50}$ $50\mu L^{-1}$) doses.

6.2.3 Fish used in study and tank set up

Chinook salmon smolt (*n* = 128) were obtained from a commercial freshwater hatchery and conditioned from freshwater to seawater immediately prior to the study. Fish were approximately 185 days post hatch and had an average size (±SD) of 87 ± 18 g. To confirm the fish were naïve to NZ-RLOs, smolt (*n* = 20) were randomly selected and euthanised by iso-eugenol (AQUI-S, Lower Hutt, New Zealand) at a rate of 175 mg L^{-1} x 20 min. Fish were necropsied and the kidney, liver, and spleen were aseptically removed and screened for the presence of NZ-RLOs using a generic qPCR (Corbeil et al., 2003). These fish were also examined for any other pathogens using histology.

Remaining fish were divided between 18 individual 100 L tanks containing aerated artificial seawater, 33 ppt (salt) (AquaOne, Sydney, Australia), at 15 ± 1°C. Fish were provided with a 12:12 hour photoperiod. Each tank possessed independent mechanical sponge filtration, chemical filtration and biological filtration and were held at a stocking density of approximately 5 kg m^{-3} (six fish per tank). Fish were acclimatised to these tank conditions for two weeks before being inoculated with NZ-RLO2. Throughout acclimatisation and the experiment, fish were fed once a day (Skretting, Stavanger, Norway; 51% protein, 21% lipid, 3 mm pellets) with any uneaten food being removed from each tank daily.

6.2.4 Challenge with NZ-RLO2

The number of fish used in each of the treatments were determined through a power analysis (calculations not shown) based on conservative predictions that were lower than the mortality of reported overseas strains of RLO (Birkbeck, Rennie, Hunter, Laidler, & Wadsworth, 2004; Valenzuela-Mirando and Gallardo-Escarate, 2016; Morrison et al., 2016). These predictions were that fish infected with a high, medium, or low dose of NZ-RLO2 would have mortalities of at least 70%, 50%, or 30% respectively by the end of the 30 day study. This analysis allowed a minimum number of animals to be used to ensure suitable power to detect statistically significant differences between the inoculated groups when compared to the controls. However, these numbers did not allow for a statistically significant difference to be detected between the dosed groups.

All fish were sedated using iso-eugenol (AQUI-S) at 25 mg L^{-1} and tagged (VI alpha tags, Northwest marine tech, WA, USA) while under sedation. All injections were administered as an intraperitoneal injection (i.p) with 23 gauge x 5 mm needles (Eurovet, Germany). All fish, control and challenged, were kept at a density of six per tank. Control fish (n = 60) were subdivided evenly into two groups (i.p and no-i.p control fish) over 10 tanks with the i.p control fish receiving a 0.1 mL i.p injection of cell culture media (MEM + 10% FBS). Based on sample size calculations, the number of tanks per dose group were one, two, and five for the high, medium, and low dosed group respectively with fish receiving a 0.1 mL i.p injection of the required dose of NZ-

RLO2. This equated to 6 fish in the high dose group, 12 fish in the medium dose group and 30 fish in the low dose group.

During the study, fish were checked at least three times a day with mortalities being removed immediately. Fish showing overt signs of disease such as darkening of skin, loss of equilibrium or not responding to stimuli were euthanised using iso-eugenol (AQUI-S). Euthanised fish and mortalities were processed for diagnostic testing as stated below. All fish were euthanised at the end of the study at 30 days post inoculation (dpi) and processed for diagnostic testing.

6.2.5 Pathology

6.2.5.1 Blood smear preparation and gross necropsy

Following euthanasia or death, each fish was measured (fork length), weighed and visually assessed for any abnormalities. A blood sample was taken by caudal venous puncture. A blood smear was created and stained with modified Giemsa stain (Sigma) for 30 min prior to microscopic examination for the presence of NZ-RLO2.

6.2.5.2 Histology

Tissue samples were taken from the gills, skin/skeletal muscle at the lateral line, skin ulcers where present, heart, liver, pyloric caeca, spleen, anterior and mid-kidney, brain, and mid-intestine from all fish and fixed in 10% buffered formalin for one to two days. Samples were then transferred to 70% ethanol, prior to being embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E) for histological examination.

6.2.5.3 In-situ hybridization

The liver, kidney, spleen, gills, and pancreas from one fish inoculated with a high dose and one fish inoculated with a medium dose of NZ-RLO2 were used for chromogenic *in situ* hybridization (ISH) and performed as previously described (Chapter 3). All experiments included a positive control that comprised of tissue from an Atlantic

salmon (*Salmo salar*) that was known to be infected with *P. salmonis* and a negative control (*Crassostrea gigas* tissue). Cover slipped ISH slides were examined under an Olympus BX51 light microscope.

6.2.6 Recovery of NZ-RLO2 from fish tissue in cell culture

A representative number of fish were sampled for cell culture from each inoculation dose; six from the high dose, eight from the medium dose, 27 from the low dose and six from the control groups. Kidney, spleen and liver were aseptically removed from each fish and combined into a sterile vial (~500 mg in total). Tissue samples were diluted 1/10 in MEM+10% FBS with the addition of penicillin (100 µg mL^{-1}), homogenised then diluted further to 1/100 and 1/1000 in MEM +10% FBS. An aliquot (100 µL) of each dilution was inoculated into separate wells of a 24 well plate seeded with a monolayer of EPC. Following adsorption for 30 min at RT, 1 mL of MEM + 10% FBS was carefully added to each well and cultures were incubated at 15°C for 14 days. Cultures were observed under light microscopy for the presence of cytopathic effect (CPE). Cultures displaying no CPE after 14 days were then passaged once by transferring 100 µL of the first culture into new wells of a 24 well plate seeded with EPC cells at the same cell rate and re-incubated for a total of 28 days.

DNA was extracted from two samples at each dose displaying CPE; high, medium and low dose, to confirm replication of NZ-RLO2 in the cell culture. For each of these samples, an aliquot of 200 µL of the culture showing CPE after 14 or 28 days (P1) and from the initial homogenate (P0) was taken. DNA was then extracted and subjected to NZ-RLO2 qPCR as described below. If the CPE was due to replication of NZ-RLO2 a decrease in the Ct value was expected in the P1 samples compared with the P0 samples.

6.2.7 Molecular tests

6.2.7.1 DNA extraction from tissues

Fish on arrival. Following euthanasia, ~200 mg of the kidney, liver, and spleen was aseptically removed from each fish and combined into a MagNA lyser green bead tube

(Roche, Penzberg, Germany). Phosphate buffered saline was then added (500 µL) and the tissue homogenised in the MagNA lyser (Roche) at 6,500 rpm for 30 sec. A subsample of this homogenate (80 µL) was then used for DNA extraction using the QIAamp mini kit (Qiagen, Hilden, Germany) as per the manufacturer's protocol.

Experimental fish. Following euthanasia, individual samples of approximately 20 mg of each kidney, liver, digestive tract (mid-intestine), skin ulcer (when present), and 10 mg of spleen were aseptically collected from each fish. DNA was extracted from each tissue sample separately on the QIAcube high throughput automated extraction robot using the QIAamp HT kit as per the manufacturer's protocol (Qiagen)

All extracted DNA was assessed for suitability for qPCR by performing an internal control 18S rRNA qPCR following the manufacturer's protocol (Ribosomal 18S rRNA Endogenous Control; Life technologies).

6.2.7.2 Generic qPCR to detect NZ-RLO in fish on arrival to the facility

The presence of NZ-RLO DNA in these fish was evaluated using a previously published qPCR targeting the 23S rRNA gene of *P. salmonis* (Corbeil et al., 2003). Per reaction, ~ 150 ng genomic DNA (i.e. 2 µL) was used and all samples were tested in duplicate. DNA extracted from pure cell culture of NZ-RLO2 was run as a positive control and molecular grade water was used as a no template control to assess environmental contamination with each qPCR.

6.2.7.3 Specific qPCR to detect NZ-RLO2 in experimental fish

DNA extracted from samples of kidney, liver, spleen, and digestive tract from all 108 challenged fish were analysed by qPCR for the presence of NZ-RLO2 (n = 432) including skin ulcers where present (n = 12).

The NZ-RLO2 qPCR was performed as previously described (Gias et al., 2018). Briefly, each reaction consisted of 2 µL of DNA (~150 ng genomic DNA), 10 µL 2 X SsoAdvanced Universal Probes Supermix (Bio-rad, Hercules, USA), 0.5 µM of each primer, 0.2 µM of probe and water to a final volume of 20 µL. The PCR was carried

out on a CFX 96 real-time PCR detection system (Bio-Rad, Hercules, USA) with the following cycling conditions: initial denaturation at 95°C for 2 min, 50 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 30 sec.

6.2.8 Statistical analysis

Statistical analysis was performed in R version 3.5.2 (R Core Team, 2015). The weight and lengths of fish were compared using a one-way ANOVA to examine differences between the control and dosed fish. A generalised linear model (GLM) with weight or lengths of fish as the response variable and treatment type (dosed and control) as the explanatory variable was fitted to the data with a Gaussian distribution (R package *multcomp*, Hothorn et al., 2008).

Cumulative mortality rates of each dosed fish group were compared to both control groups in a GLM with a binomial error distribution. The two control groups were also compared to each other using this model. Specific pair wise differences between the groups were tested using Tukey contrasts and *p* values were adjusted using the Benjamini and Hochberg method (R package multcomp, Hothorn et al., 2008). Analysis was carried out to compare mortalities between the control and dosed groups from 1 to 22 days post inoculation (dpi) and 1 to 30 dpi. For the low dose group, analysis was also carried out to compare mortalities to the control groups from 23 to 30 dpi.

Two analyses of the Ct values deriving from the NZ-RLO2 qPCR were carried out: 1) Ct values from all organs (kidney, liver, spleen, and digestive tract) were compared between the inoculated groups (high, medium, and low); 2) Ct values from all organs were compared within the inoculated groups. Analysis 1 was carried out to determine if the load of bacteria detected in the three inoculated groups differed significantly. Analysis 2 was carried out to determine if any organ within each dosed group had a significantly different load of bacteria. The response variable in the model was the Ct value, which was log transformed to meet the assumptions of normality. Specific pair-wise differences between groups was carried out as above.

The significance of the explanatory variables in all models were assessed using likelihood ratio tests. We used *p* values < 0.05 to determine statistical significance.

6.3 Results

6.3.1 Cumulative mortality

Death of fish inoculated with a high dose of NZ-RLO2 first occurred at 6 days post inoculation (dpi) and all fish had died by 8 dpi. Fish inoculated with a medium dose first died at 7 dpi and all fish had died by 23 dpi. The first deaths of fish inoculated with a low dose occurred at 11 dpi and reached 63% by the end of the 30 day study. By the end of the study, the cumulative mortalities of the fish in the control groups, no-i.p and i.p, reached 17% and 10% respectively. At 23 dpi, a mechanical failure occurred in the laboratory air conditioning unit which resulted in a 2°C rise in water temperature for a 24 hour period. A spike in mortality in all remaining groups followed this temperature increase with mortalities in the control groups plateauing out before the end of the trial (Figure 6.1).

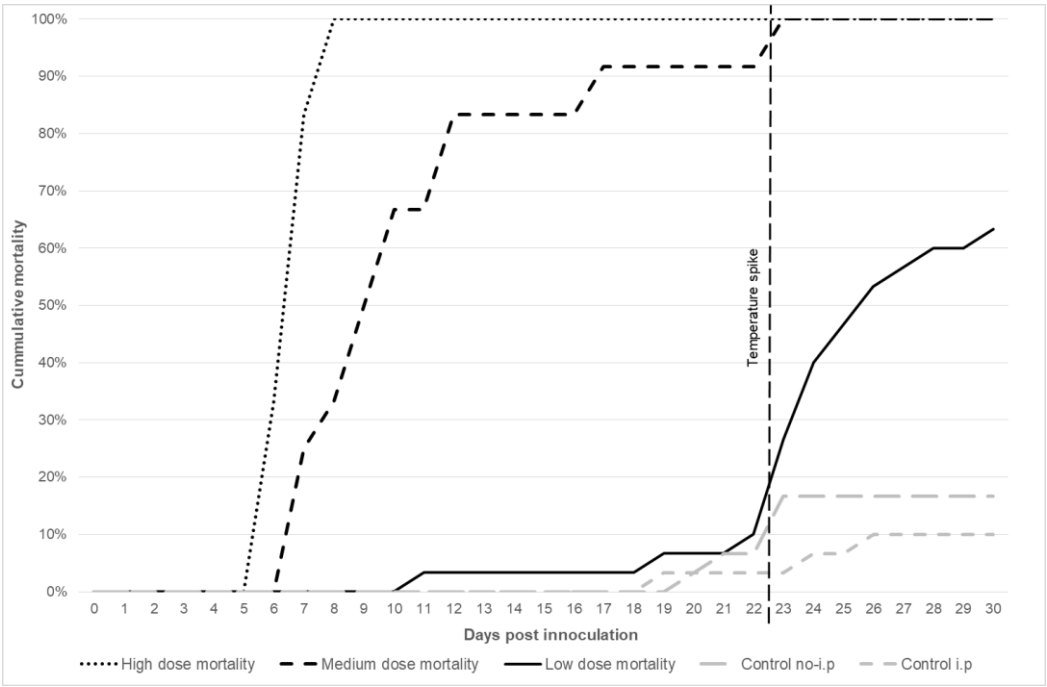


Figure 6.1. Cumulative mortalities of fish in the NZ-RLO2 study. High dose indicates that fish were inoculated with a high dose of NZ-RLO2, medium dose indicates fish were inoculated with a medium dose of NZ-RLO2, low dose indicates fish were inoculated with a low dose of NZ-RLO2. Fish in the control no-i.p group did not receive any intraperitoneal injection while fish in the control i.p group received an intraperitoneal injection that did not contain NZ-RLO2. Dashed line = day of temperature increase.

Mortality of fish in the high dose group was significantly higher than the mortality of both control groups at 22 dpi ($\chi^2 = 11.64$, $p < 0.01$) and 30 dpi ($\chi^2 = 7.98$, $p = 0.02$). Mortalities of fish in the medium dose group were significantly higher than in both control groups at 22 dpi and 30 dpi ($\chi^2 = 8.85$, $p = 0.01$, $\chi^2 = 6.77$, $p = 0.03$, respectively). The mortality rates in the low dose group were compared to the controls at 22 and 30 dpi as well as comparing the difference in low dose mortalities to the controls from the temperature spike onwards (23 to 30 dpi). At 22 dpi there was no significant difference ($\chi^2 = 1.11$, $p = 0.57$) between the low dose group and the control groups. At 30 dpi the differences were not significant ($p = 0.06$). When comparing the low dose group to the controls from the temperature spike onwards (23 - 30 dpi) there was a significant difference ($\chi^2 = 8.3$, $p = 0.02$).

There were no significant differences in the cumulative mortalities between the two control groups ($p = 1$).

6.3.2 Gross pathology

No significant differences were seen when comparing the average weights ($\chi^2 = 4.97$, $p = 0.29$) and lengths ($\chi^2 = 7.47$, $p = 0.11$) of fish between all experimental groups.

New Zealand rickettsia-like organism 2 were visible within white blood cells obtained from the caudal vein (Figure 6.2) in two fish from each of the high and medium dose groups and from six fish in the low dose group.

Necropsy examination of fish in the high dose group revealed 83% had petechial haemorrhage in the adipose tissue surrounding the pyloric caeca (Figure 6.3). Other common findings were pale liver (33%) and an increase of clear fluid in the coelomic cavity (33%). Abnormalities were not observed in any other organs.

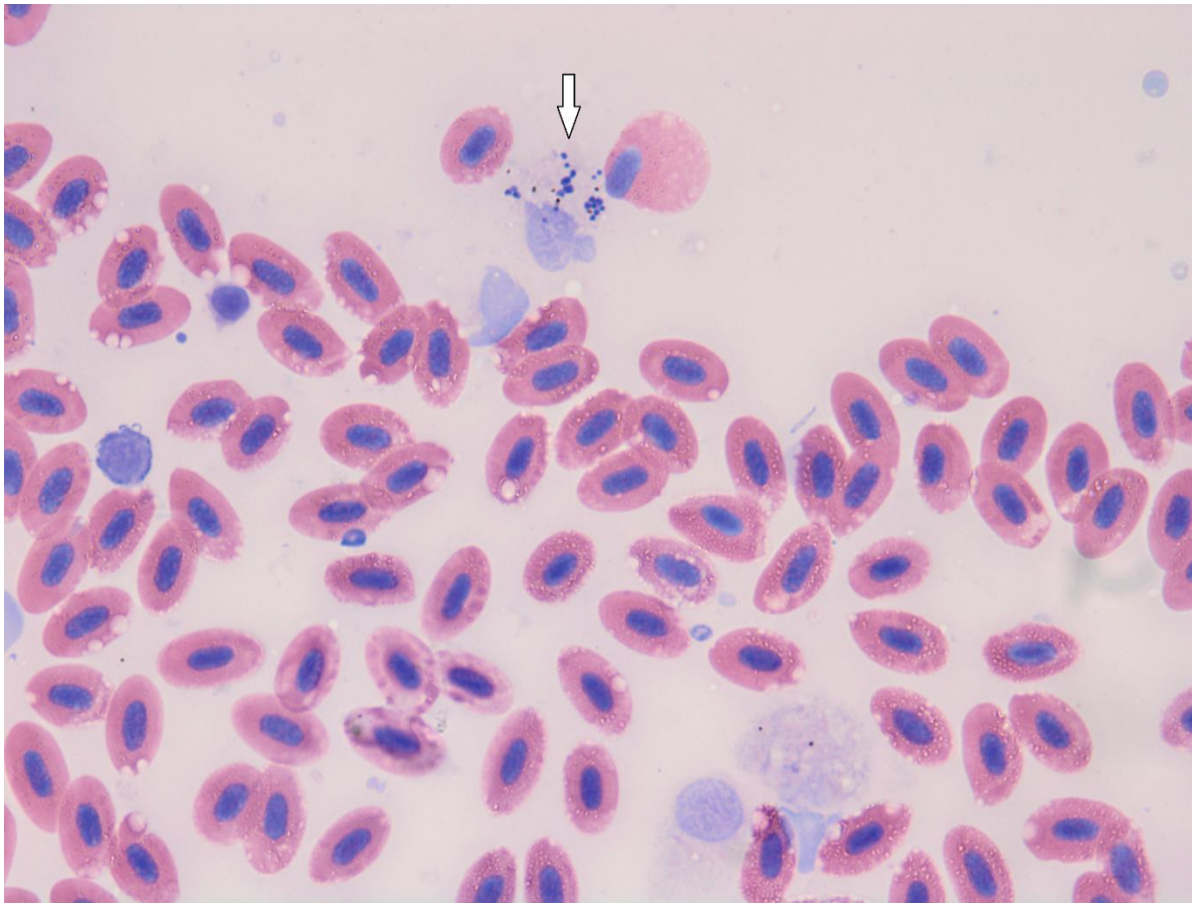


Figure 6.2. Blood smear of fish inoculated with NZ-RLO2. Blue stained coccoid cells ~ 1 µm resembling NZ-RLO2 were observed within circulating white blood cells (arrow). Giemsa stain, 1000 X magnification.

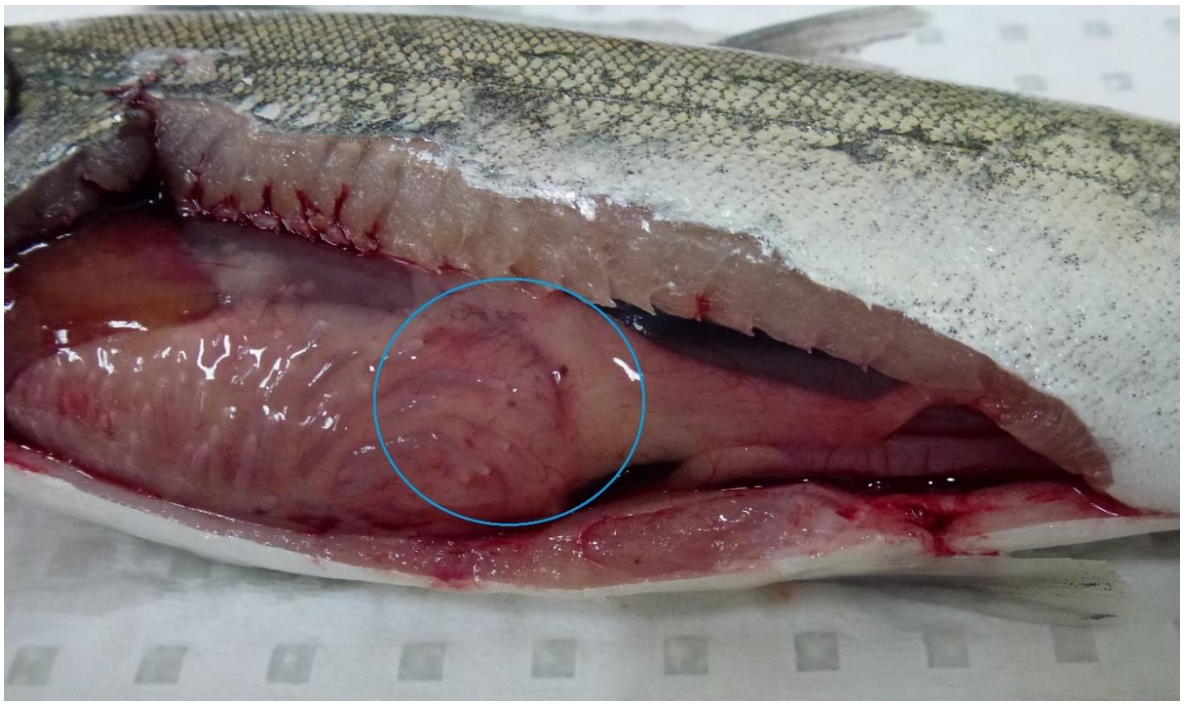


Figure 6.3. Petechial haemorrhage in the adipose surrounding the pyloric ceca (circle) were commonly seen on necropsy examination of fish that died after being inoculated with NZ-RLO2.

Similarly to fish in the high dose group, petechial haemorrhage in the adipose tissue surrounding the pyloric caeca was the most frequent lesion seen in fish necropsied following exposure to a medium dose of NZ-RLO2 (86%). Additionally, splenomegaly (33%) and pale liver were observed (17%). Ascites was seen in one fish necropsied (8%). No abnormalities were detected in any other organs.

In fish inoculated with a low dose of NZ-RLO2, petechial haemorrhage in the adipose tissue surrounding the pyloric caeca was observed in 47% of fish necropsied. Of these 47%, 74% had died during the study with the remaining being euthanised at the end of the study. Fish inoculated with a low dose of NZ-RLO2 were the only group where skin ulcers were observed. Skin ulcers were observed in 40% of fish from 23 dpi onwards. Of these fish, 33% were fish that died during the study and 67% were euthanised at the end of the study. Skin ulcers presented as ulcers extending into the musculature of approximately 2 to 3 mm diameter in nine fish and as lesions with scale loss not extending into the musculature in three fish (Figures 6.4 and 6.5). Splenomegaly was observed in 10% of fish, two fish that died during the study and one that was euthanised at the end of the study. Pale liver was observed in 7% of fish that died during the study. No abnormalities were detected in any other organs.

Of the control fish that died during the study, five had a fluid filled stomach, one had reddening in the intestine and two had no abnormalities detected. Necropsy examination of the control fish that were euthanised at the end of the study did not reveal any significant gross abnormalities.

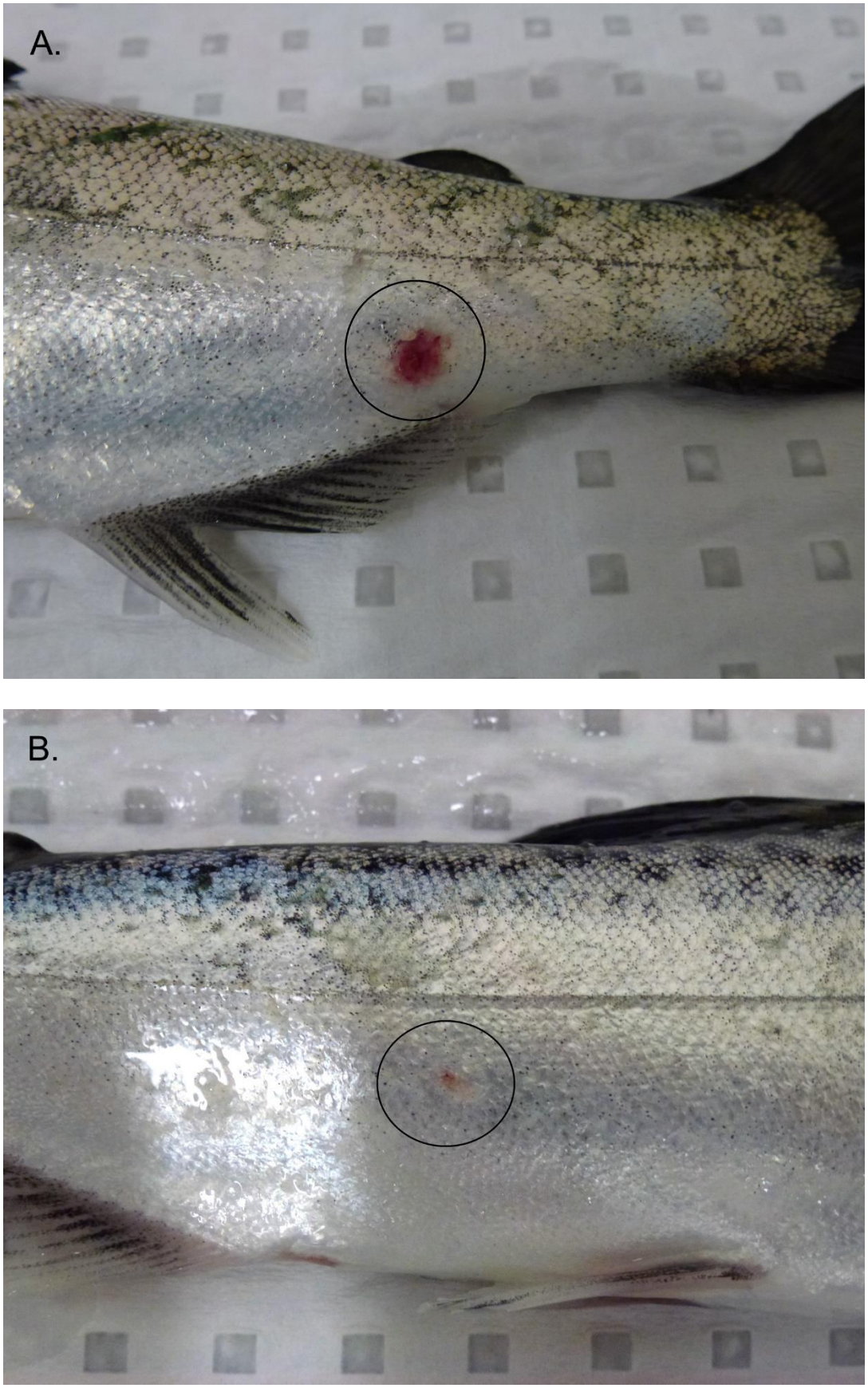


Figure 6.4. A and **B**. Examples of skin ulcers extending into the musculature (black circles) from fish inoculated with a low dose of NZ-RLO2. All samples were positive for NZ-RLO2 by qPCR.

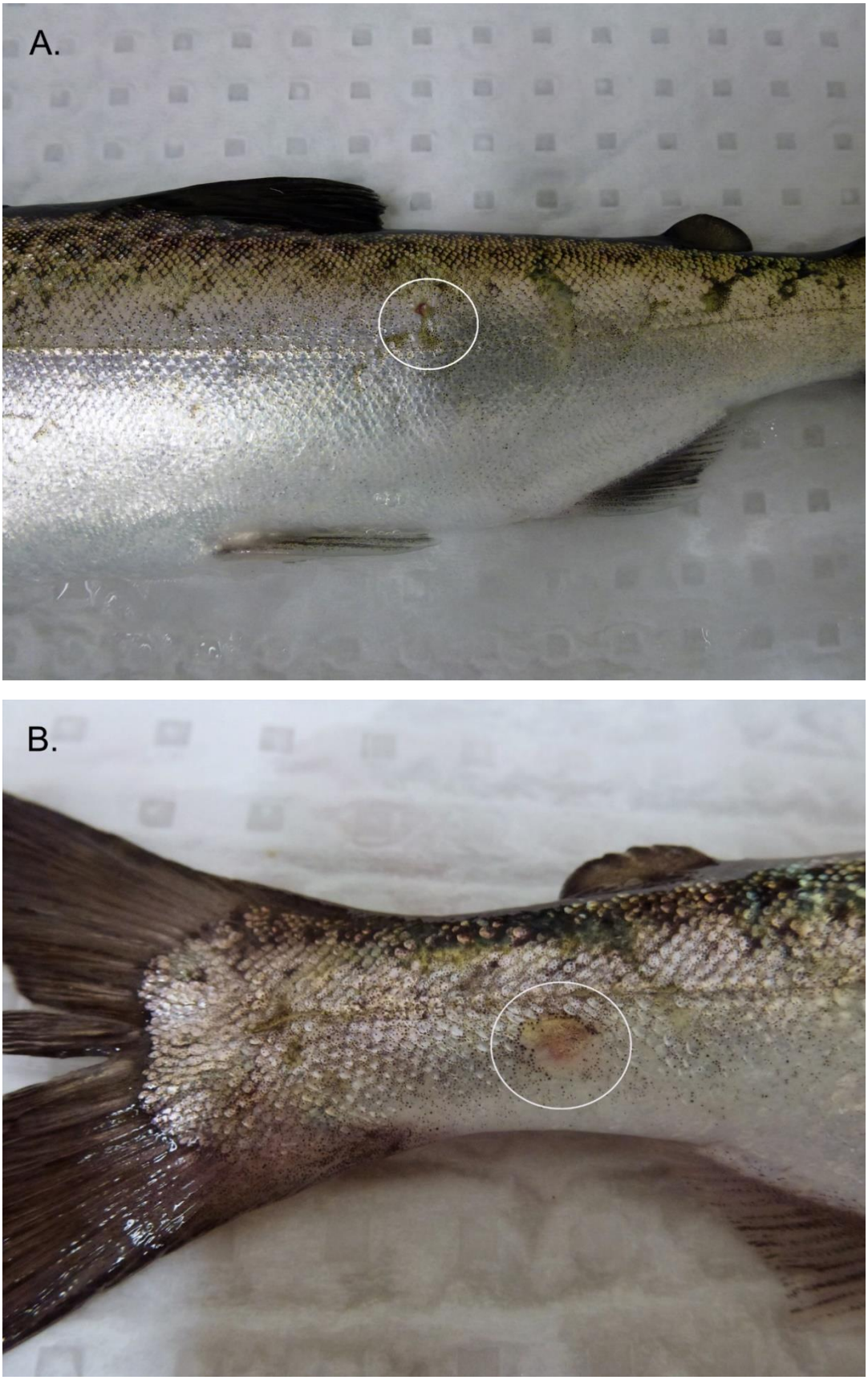
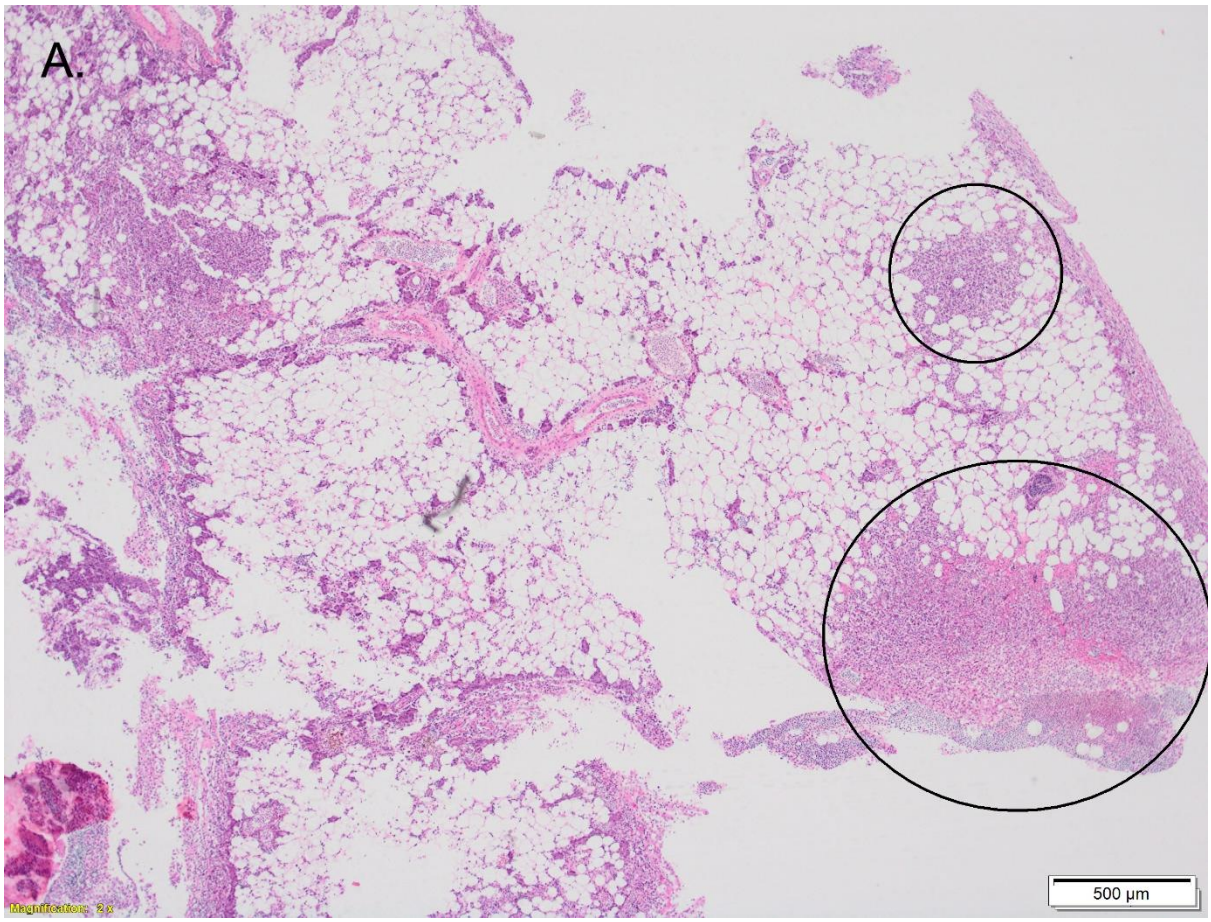


Figure 6.5. Examples of scale loss and reddening. **A.** ulcer not extending into the musculature (circle) **B.** skin ulcers extending into the musculature (circle). Fish inoculated with a low dose of NZ-RLO2. Both samples were negative for NZ-RLO2 by qPCR.

6.3.3 Histopathology

The most frequent histological lesion in fish that were inoculated with NZ-RLO2 was the presence of necrosis and moderate to severe neutrophilic inflammation of the exocrine pancreas and peri-pancreatic adipose tissue (Figure 6.6 A). These lesions were observed in 100%, 92%, and 37% of fish in the high, medium, or low dose groups respectively (Figure 6.9). New Zealand rickettsia-like organism 2 were frequently visible as 0.8 to 1.2 µm basophilic cocci as pairs or clusters within the cytoplasm of infiltrating inflammatory cells (Figure 6.6 B).



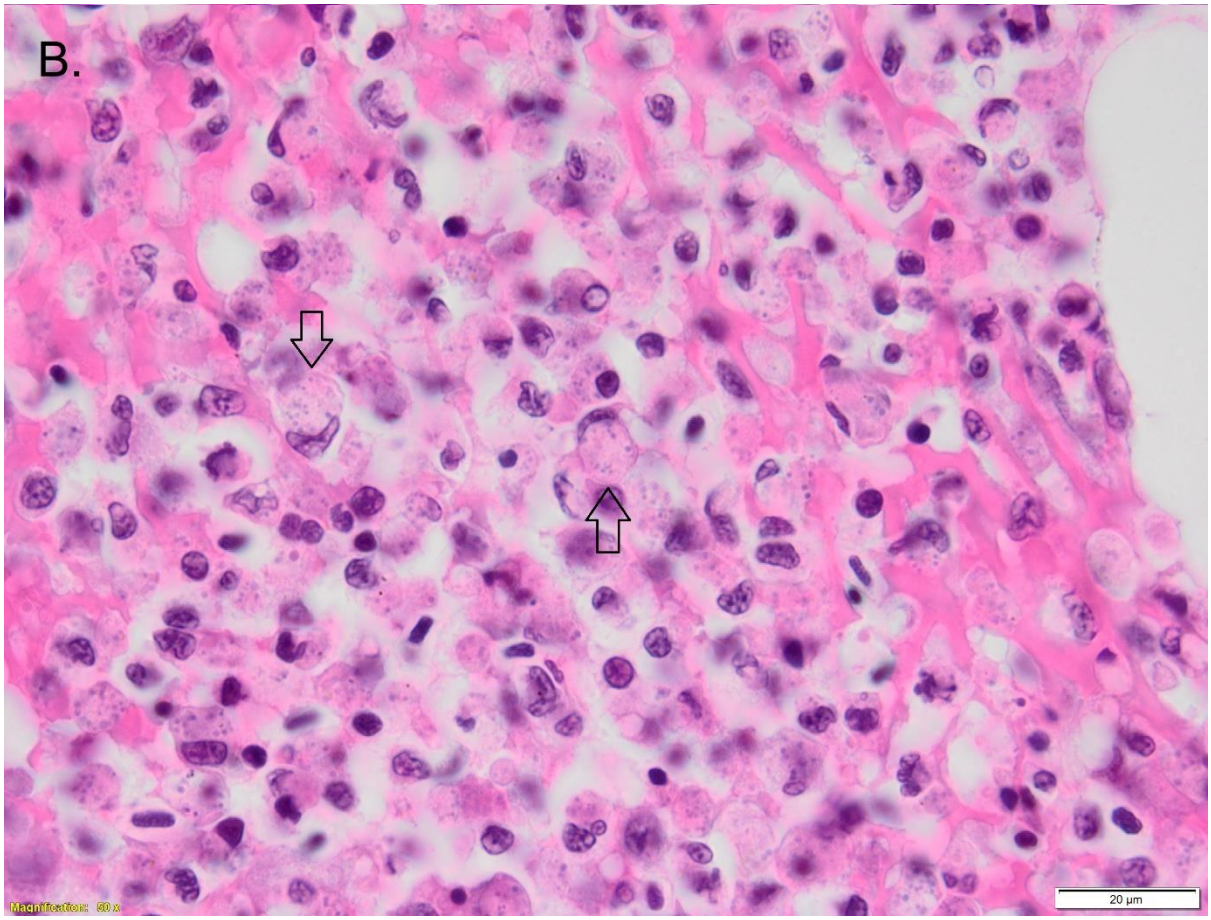


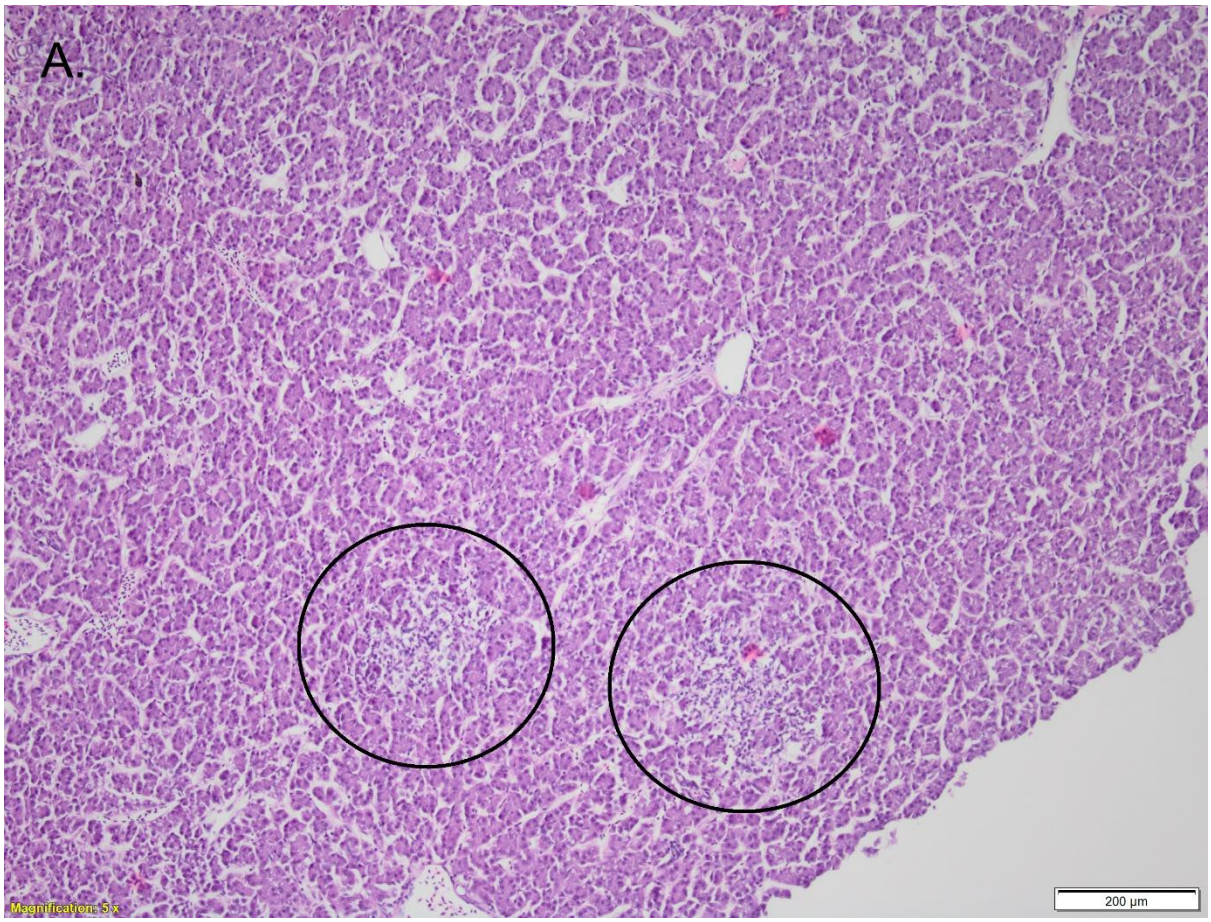
Figure 6.6. A. Pancreas from a fish that died after inoculation with NZ-RLO2. Note the severe infiltrate of inflammatory cells within the surrounding adipose tissue (circles), 400 x magnification. **B**. Pancreas from a fish that died after inoculation with NZ-RLO2. Note the presence of cocci within the cytoplasm of the inflammatory cells (arrows), 1000 x magnification.

Examination of the liver of fish inoculated with NZ-RLO2 revealed multifocal areas of hepatocellular necrosis (Figure 6 7 A). These foci appeared as hepatocytes showing nuclear pyknosis. The foci of necrosis were associated with mild to moderate predominantly mononuclear infiltrates. Hepatocellular necrosis was seen in 83%, 67%, and 54% of fish in the high, medium, or low dose groups respectively. Intracytoplasmic pairs or clusters of rounded basophilic single cells organisms, interpreted as NZ-RLO2, were associated with the foci of hepatocellular necrosis (Figure 6.7 B). Examination of the kidneys revealed moderate diffuse individual cell karyorrhexis and pyknosis with apparent depletion of haematopoietic cells. There was occasional or complete loss of tubular epithelium in areas. Renal necrosis was seen in 100%, 67%, and 33% of fish in the high, medium, or low dose respectively. The spleen showed a depletion of lymphatic and haematopoietic cells which was observed in 100%, 83%, and 57% of fish in the high, medium, or low dose groups respectively. Pericarditis that was associated with the presence of bacteria resembling NZ-RLO2

was observed in 17%, 25%, and 13% of fish in the high, medium, or low dose groups respectively.

On examination of the brain, congestion in the meningeal blood vessels was observed in 50% of the fish for both high and medium doses and 10% in the low dose group. NZ-RLO2 were not observed in these affected areas.

Skeletal muscle of fish inoculated with NZ-RLO2 showed areas of inflammation underneath the stratum compactum both between the red and the white layers of muscle, and within the red layer of muscle sometimes tracking down vessels and myosepta. Thickening of artery walls with associated inflammation (Figure 6.8) was observed in 33%, 50%, and 23% of fish inoculated with high, medium, or low dose of NZ-RLO2 respectively. Inflammation was also noted occasionally in control fish (12%, Figure 6.9).



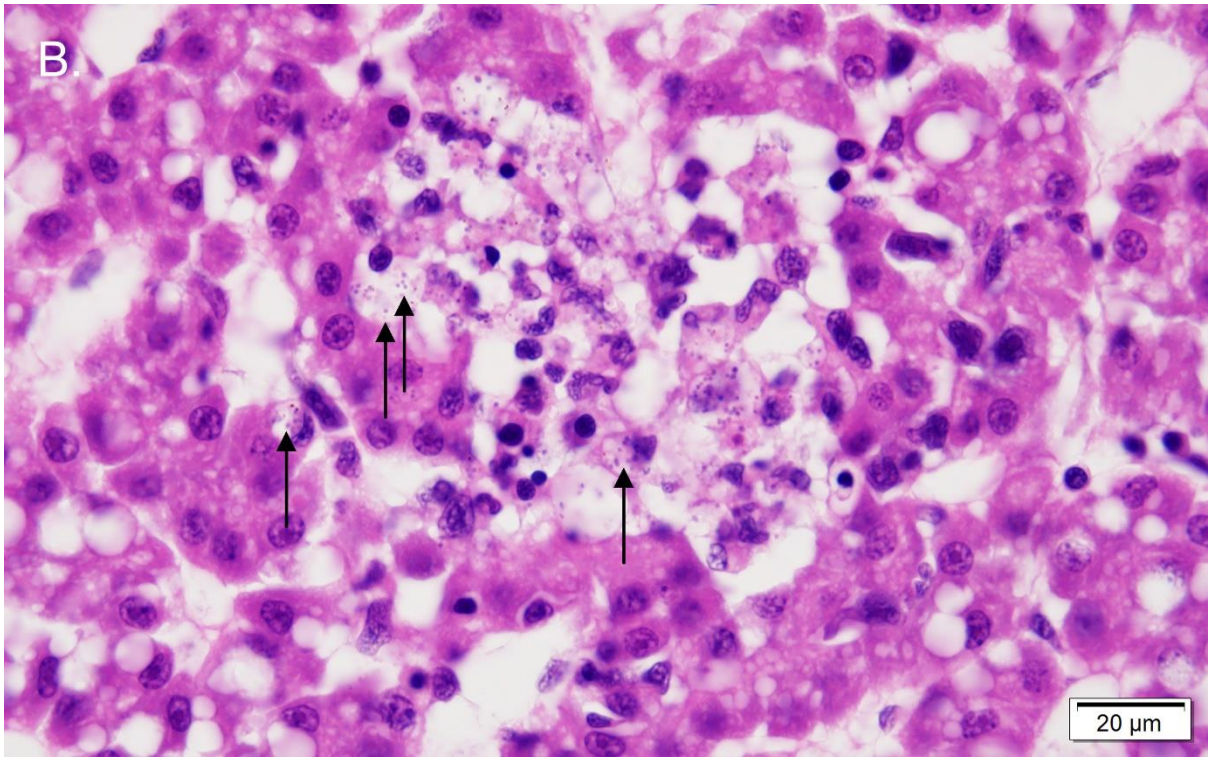


Figure 6.7. Liver from a fish that died after inoculation with NZ-RLO2. **A**. Note the multi-focal areas of necrosis (arrows), 100 x magnification. **B.** Note the presence of cocci within the cytoplasm of inflammatory cells within areas of necrosis (arrows), 1000 x magnification.

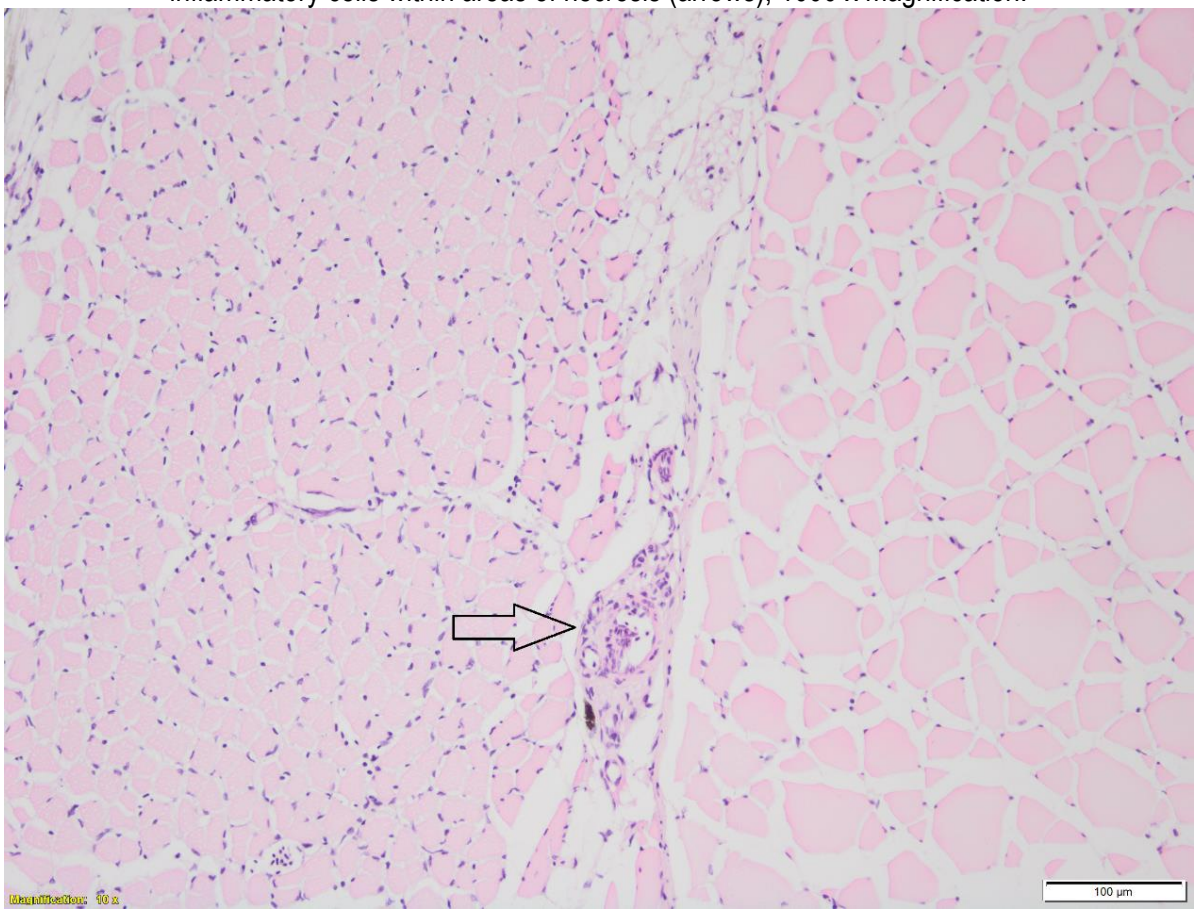


Figure 6.8. Skeletal muscle from a fish inoculated with NZ-RLO2. Note the thickening of the artery walls with associated inflammation (arrow), 200 x magnification.

Bacteria resembling NZ-RLO2 were visible in at least one of the tissues in 83%, 50%, and 3% of fish in the high, medium, or low dose group respectively (Figure 6.10).

No significant histological lesions were observed in sections of the gill or intestine from any of the fish inoculated with NZ-RLO2.No significant lesions were observed in the control groups analysed or the fish assessed for general health on arrival.

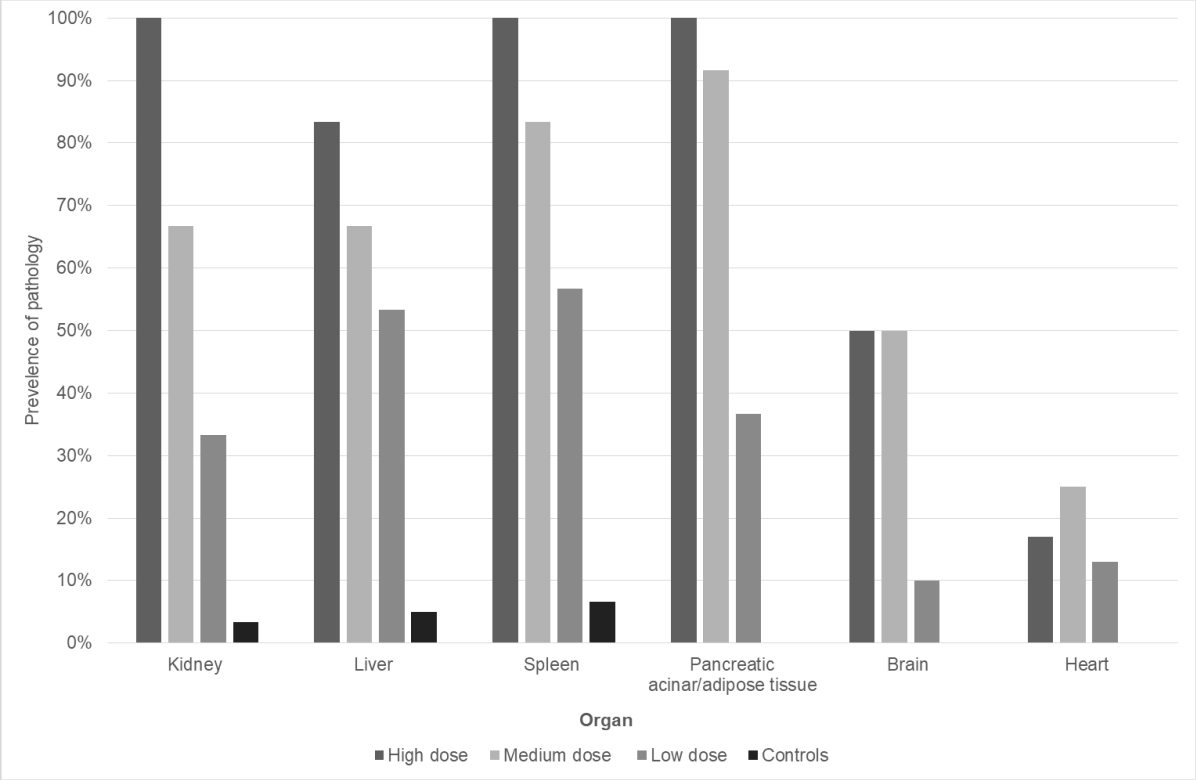


Figure 6.9. Prevalence of pathology (%) noted in the different organs between treatment groups of fish inoculated with NZ-RLO2 as well as control groups. High dose indicates that fish were inoculated with a high dose of NZ-RLO2, Medium dose indicates that fish were inoculated with a medium dose of NZ-RLO2, Low dose indicates that fish were inoculated with a low dose of NZ-RLO2, Controls indicates that fish were not inoculated with NZ-RLO2.

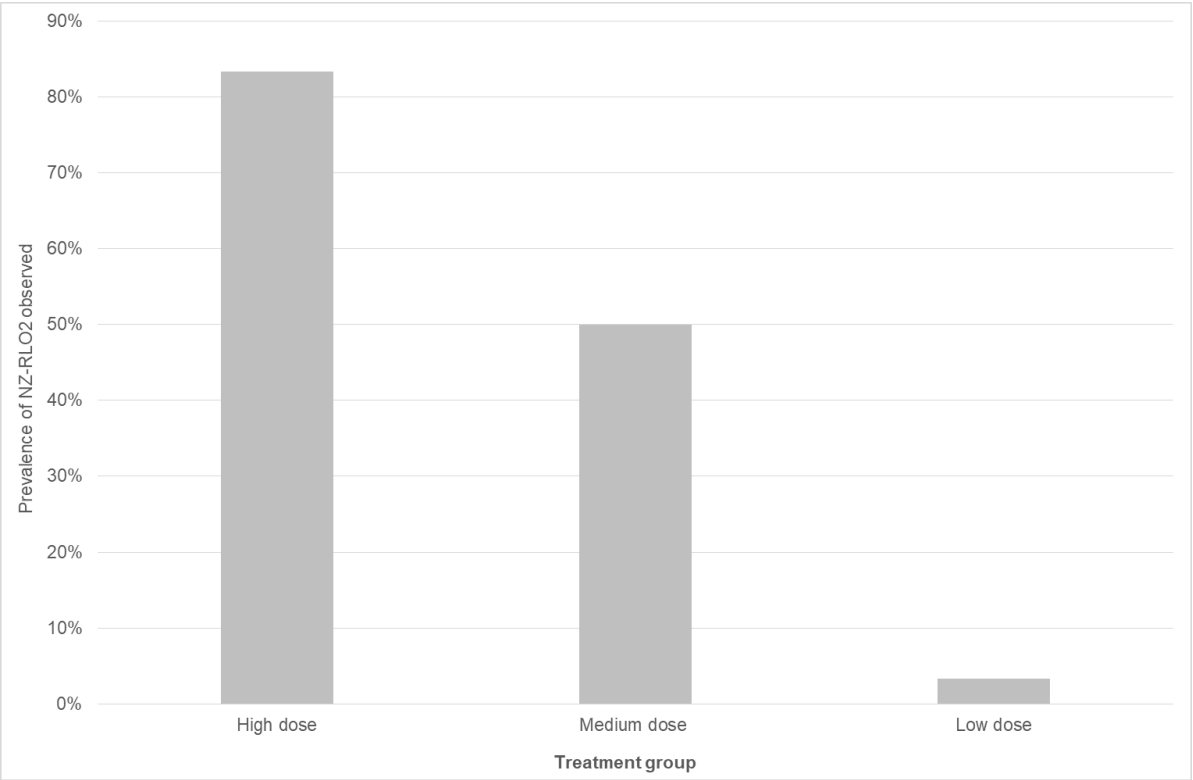


Figure 6.10. Prevalence of NZ-RLO2 observed in tissues evaluated using histology from fish inoculated with NZ-RLO2. High dose indicates that fish were inoculated with a high dose of NZ-RLO2, Medium dose indicates that fish were inoculated with a medium dose of NZ-RLO2, Low dose indicates that fish were inoculated with a low dose of NZ-RLO2.

6.3.4 *In situ* hybridization

In-situ hybridization was performed on sections from two fish inoculated with NZ-RLO2 that died during the study, one from a high dose and one from a medium dose. Hybridization to the ISH probe was visible in the liver, spleen (Figure 6.11 A), and pancreatic tissue (Figure 6.11 B) from both fish. Probe hybridization was not observed in the gills or kidney of the samples evaluated. Probe hybridization was observed within areas of necrosis and inflammation.

Positive signals were observed in the positive control tissue (Figure 6.12). No signals were observed from the negative control tissue.

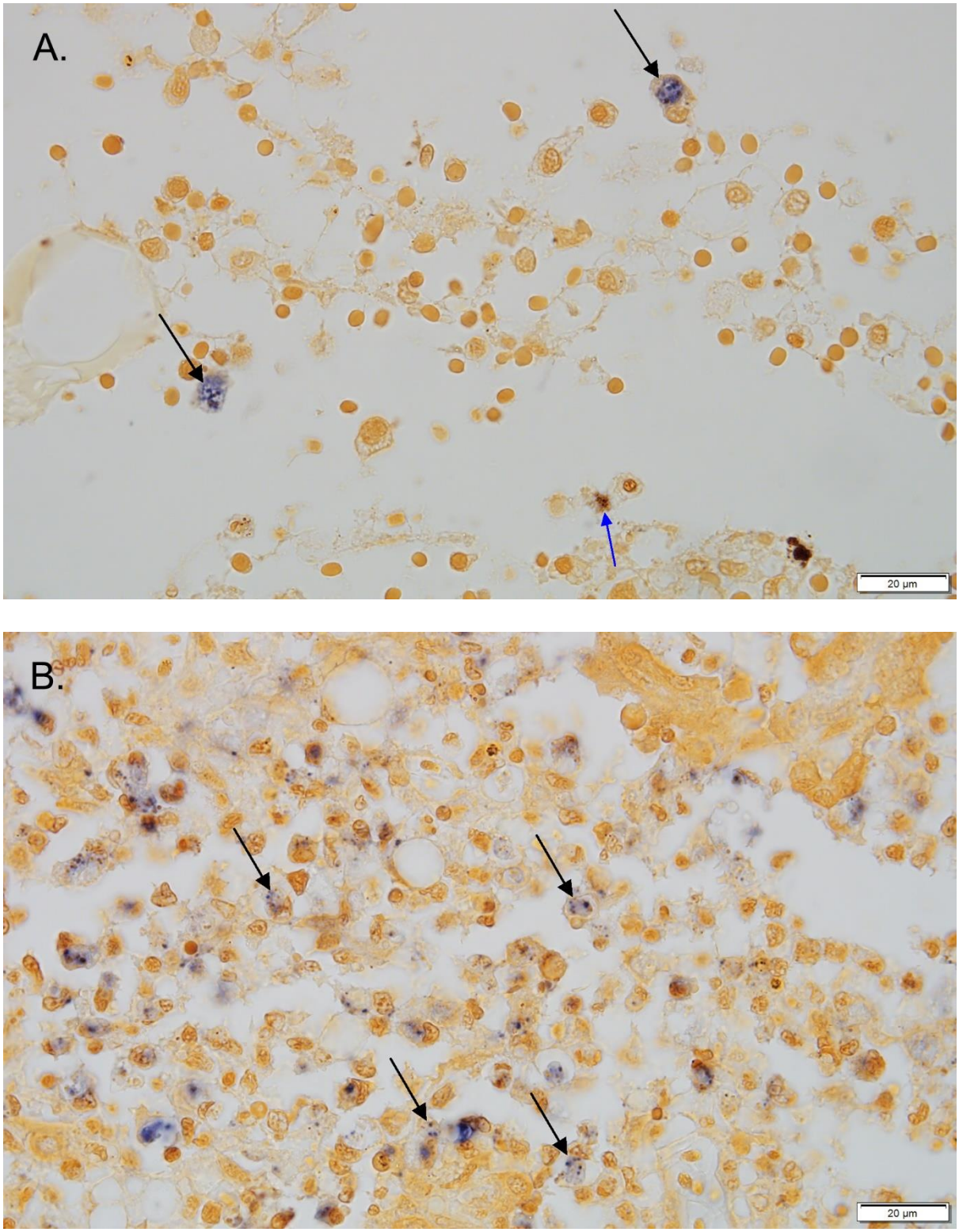


Figure 6.11. Fish inoculated with a high dose of NZ-RLO2 subjected to *in-situ* hybridization. Note the hybridization of the labelled probe displayed as a dark blue signal. **A**. Spleen (Black arrows = NZ-RLO2, Blue arrow = melanin). **B**. Pancreas and surrounding fat (arrows indicating some cells with hybridization). 1000 x magnification.

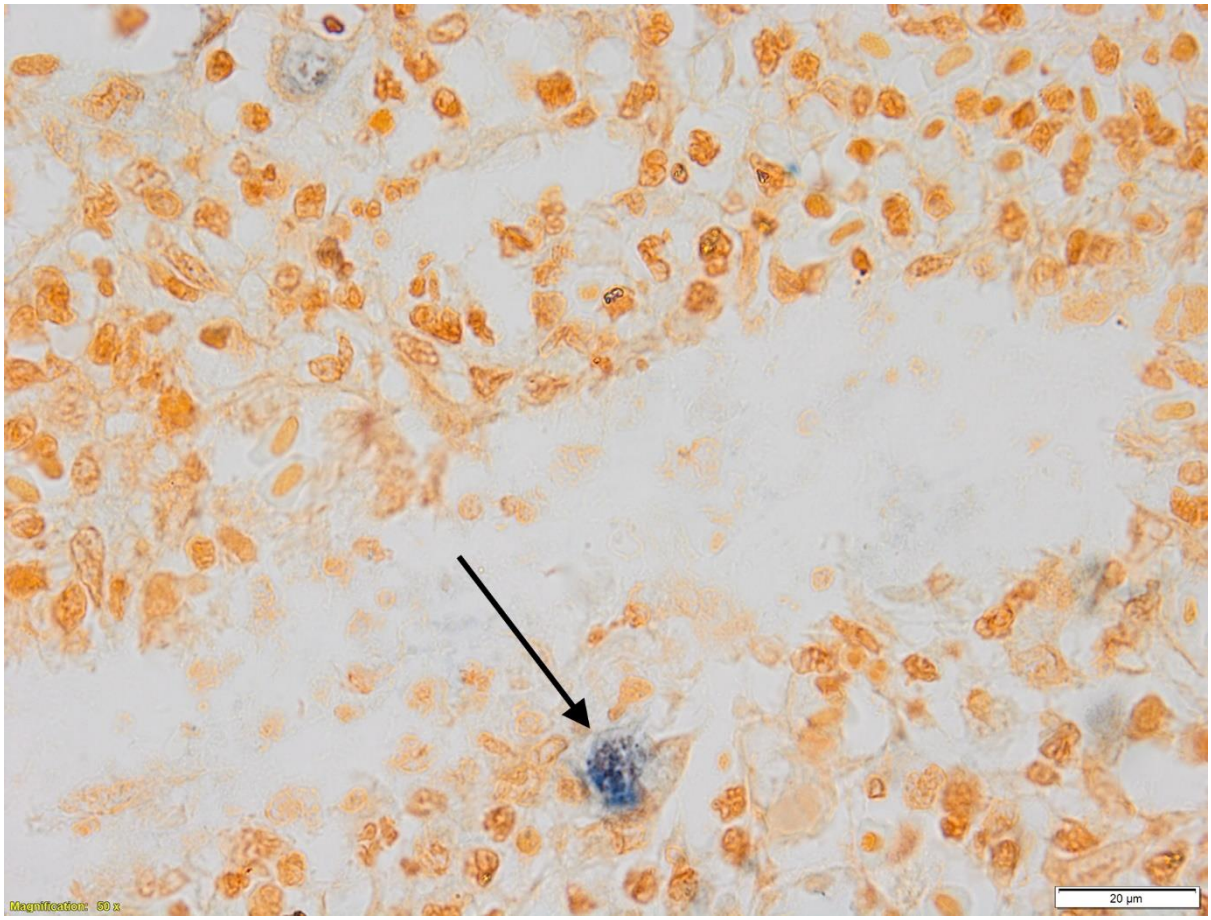


Figure 6.12. Positive control material showing hybridization to the labelled probe in spleen (arrow). 1000 x magnification.

6.3.5 Recovery of NZ-RLO2 from tissue in cell culture

In cell culture, NZ-RLO2 was recovered from all fish inoculated with a high dose of NZ-RLO2, six of the eight fish inoculated with a medium dose of NZ-RLO2 and 10 of the 27 fish inoculated with a low dose of NZ-RLO2 by the observation of CPE after 14 or 28 days incubation (Table 6.1; Figure 6.13). None of the six control fish tested displayed CPE in cell culture after 28 days incubation.

The presence of replicating NZ-RLO2 in cell culture was confirmed using qPCR with the P1 material resulting in Ct values ranging from 21.31 to 24.59, equating to $\geq 10^4$ $TCID_{50}$, an increase from the original P0 material which resulted in Ct values ranging from 31.90 to 39.03 equating to $\leq 10^2$ $TCID_{50}$ (Table 6.2).

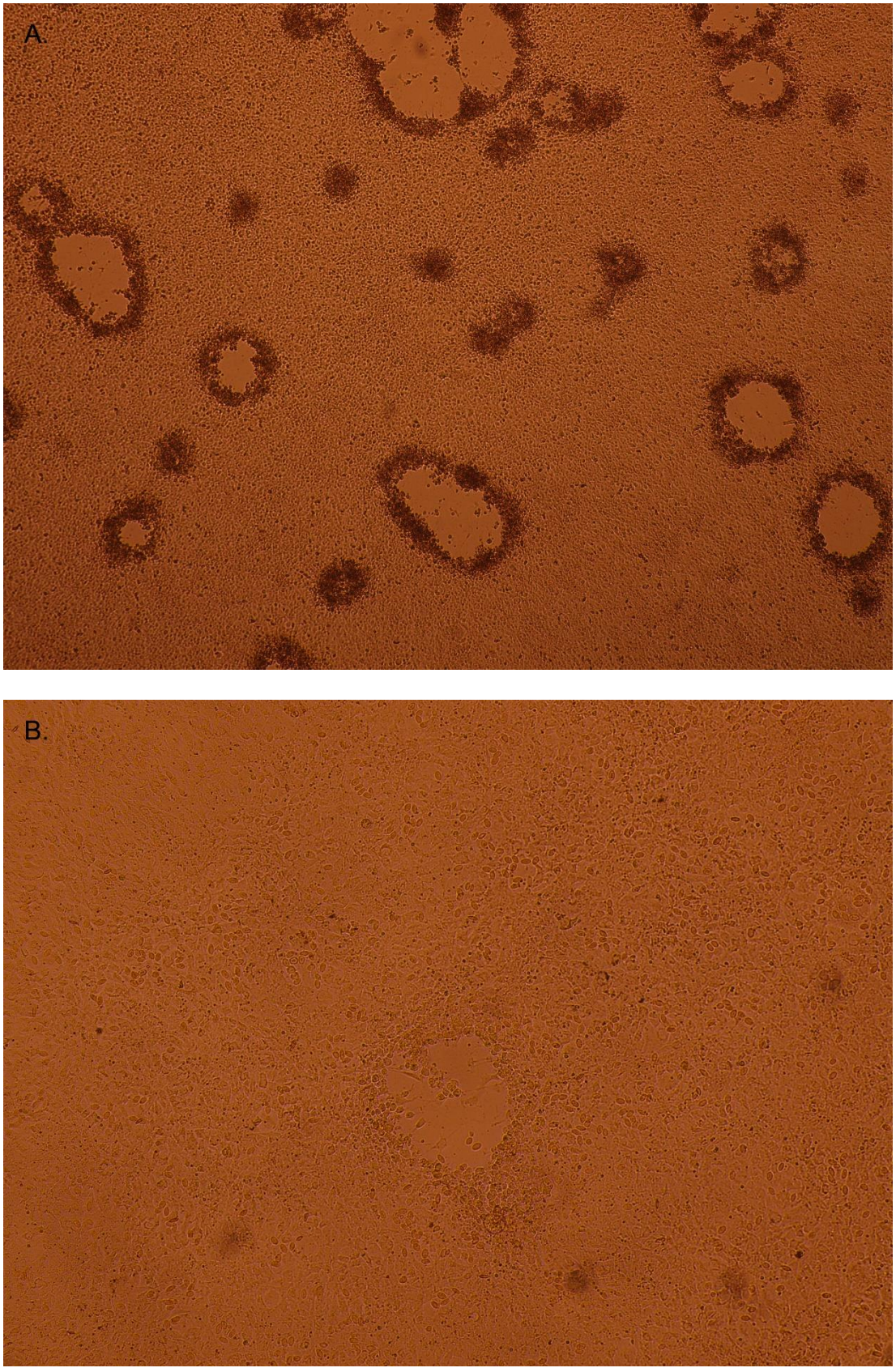


Figure 6.13. A. Cytopathic effect (CPE) observed from liver, kidney and spleen of fish inoculated with NZ-RLO2 incubated on Epithelioma papulosum cyprini cell lines at day seven. **B.** Around the areas of cytopathic effect, rounding of the cells is seen which is typical of NZ-RLO CPE.

Table 6.1. NZ-RLO2 qPCR cycle threshold (Ct) values of all tissues at all inoculation doses; high dose (H; *n* = 6) indicates salmon were inoculated with a high dose of NZ-RLO2, medium dose (M; *n* = 12) indicates that salmon were inoculated with a medium dose of NZ-RLO2, and low dose (L; *n* = 30) indicates that salmon were inoculated with a low dose of NZ-RLO2.

Infectious dose	Ct value				
	Kidney	Liver	Spleen	Digestive tract	Skin ulcer
H	26.37	28.54	26.71	29.01	NA
H	33.14	29.1	30.08	27.81	NA
H	28.63	27.59	27.26	26.31	NA
H	28.34	29.26	29.1	28.02	NA
H[I]	25.99	28.26	24.54	24.31	NA
H	37.87	37.65	35.69	41.43	NA
M	34.85	32.51	32.55	38.36	NA
M	29.18	30.24	29.88	30.08	NA
M	31.41	28.99	30.23	30.12	NA
M	34.67	31.54	32.73	30.44	NA
M	30.7	31.24	28.43	30.41	NA
M	45	39.07	38.74	45	NA
M	26.41	27.57	27.28	23.89	NA
M[I]	29.34	29.38	N	32.71	NA
M	N	38.6	N	N	NA
M	N	N	N	N	NA
M	36.51	N	42.98	N	NA
M	40.76	N	44.92	N	NA
L	N	N	N	N	NA
L	N	N	N	N	NA
L	N	N	N	N	N
L	N	N	N	N	NA
L	N	N	35.81	33.65	32.67
L	N	N	N	39.15	NA
L	N	N	N	N	NA
L	N	N	N	N	NA
L	N	N	N	N	NA
L	39.59	N	N	N	NA
L	N	N	N	39.76	NA
L	40.62	N	N	N	NA
L	N	N	38.61	42.25	31.02
L	N	N	N	N	NA
L	42.96	43.8	40.01	N	33.25
L	N	N	N	N	N
L	37.51	41.97	40.39	38.33	38.74

L	35.05	N	N	N	NA
L	40.98	37.25	34.93	32.85	NA
L	44.04	N	N	N	27.82
L	N	N	37.64	N	N
L	38.39	N	N	N	N
L	N	N	N	N	NA
L	N	N	N	N	NA
L	N	N	N	N	41.65
L	N	N	N	N	NA
L	N	N	N	N	N
L	N	N	N	N	NA
L	40.98	N	43.79	N	NA
L	N	40.76	40.24	N	26.7

Red cells = growth in cell culture; Blue cells = no growth in cell culture; no colour = sample not tested for cell culture; N = negative qPCR result; NA = no lesion present; [I] = fish tissues that were subjected to *in-situ* hybridization.

Table 6.2. NZ-RLO2 qPCR cycle threshold (Ct) values and equivalent $TCID_{50}$ of cell-cultures with CPE to confirm growth of NZ-RLO2. P0 = original tissue homogenate, P1 = growth in cell culture material after 14 days incubation.

Infectious dose	P0 Ct	$TCID_{50}$ equivalent	P1 Ct	$TCID_{50}$ equivalent
High	32.36	10^2	21.40	10^5
High	31.90	10^2	22.02	10^5
Medium	35.51	10^1	21.31	10^5
Medium	39.03	10^1	21.875	10^5
Low	36.96	10^1	23.14	10^5
Low	37.12	10^1	24.59	10^4

6.3.6 Evaluation of tissues using qPCR

All DNA recovered from tissue samples were shown to be appropriate for PCR by amplification in the internal control 18S rRNA PCR.

Tissue samples from the 20 fish analysed at the beginning of the study showed no amplification of DNA in the generic *Piscirickettsia salmonis* qPCR.

Use of the specific NZ-RLO2 qPCR revealed all tissue samples from all fish in the high dose group had NZ-RLO2 DNA present. Fish inoculated with a medium dose of NZ-RLO2 showed DNA was amplified from at least one tissue from 92% of fish. In the low dose group, NZ-RLO2 DNA was amplified from at least one tissue of 43% of fish. Of the 12 skin ulcers tested, NZ-RLO2 DNA was amplified from seven using the NZ-RLO2

qPCR (Table 6.1).The NZ-RLO2 qPCR did not amplify DNA from any tissues of the 60 control fish.

There was a significant difference in the Ct values derived from the NZ-RLO2 qPCR when comparing all organs between the high, medium, or low dosed groups ($\chi^2 = 16.9$, $p < 0.01$). Differences were significant between the high and medium dose groups compared to the low dose (both $p < 0.01$). There was no significant difference seen in Ct values between the medium and high dose groups ($p = 0.34$). There was no significant difference between the Ct values from the NZ-RLO2 qPCR between any of the organs within each dosed group; high, medium, or low ($\chi^2 = 2.94$, $p = 0.4$; $\chi^2 = 2.03$, $p = 0.57$; $\chi^2 = 3.66$, $p = 0.3$).

The average Ct values of NZ-RLO2 DNA from fish tissues were compared to the cumulative mortality rates for the high, medium, or low dose groups (Figure 6.14). This revealed that increased mortality rates coincided with increased levels of NZ-RLO2 DNA, as noted by a decrease in the average Ct value.

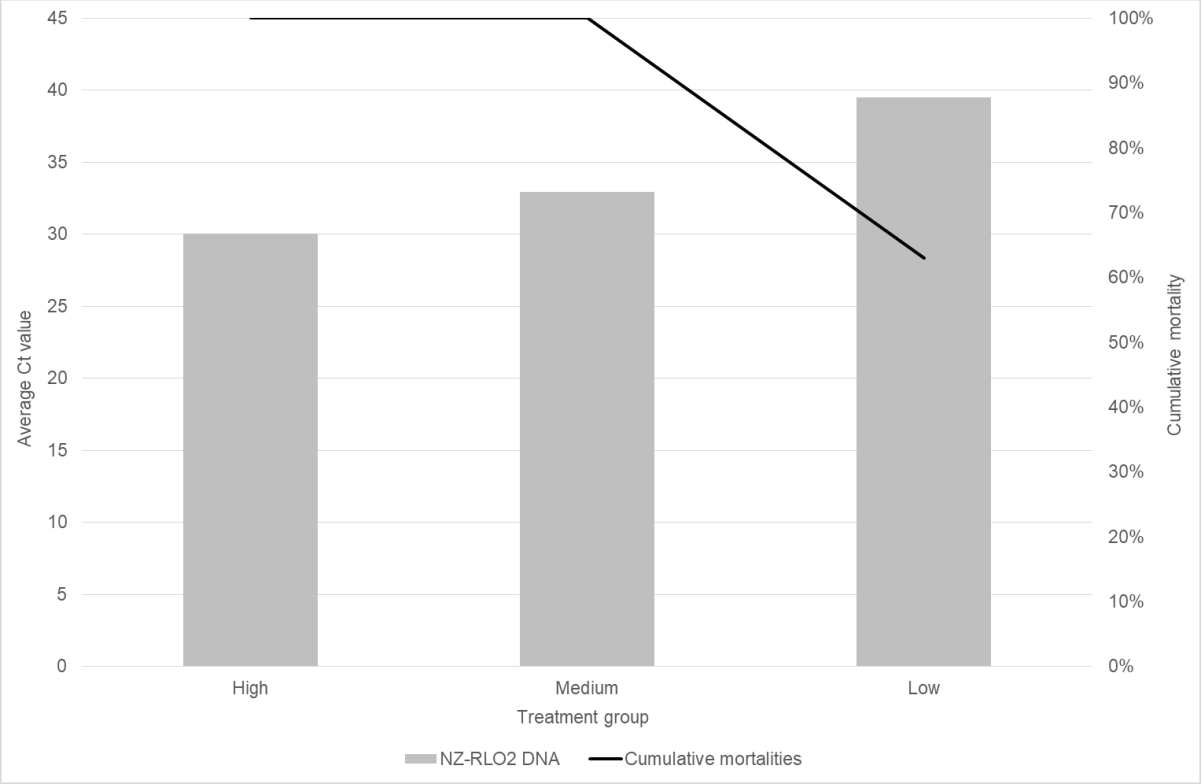


Figure 6.14.Summary of the average Ct values derived from the specific NZ-RLO2 qPCR from all internal organs of fish inoculated with either a high, medium or low dose of NZ-RLO2 with the cumulative mortalities for each inoculation dose. High dose indicates that fish were inoculated with a high dose of NZ-RLO2, Medium dose indicates that fish were inoculated with a medium dose of NZ-RLO2, and Low dose indicates that fish were inoculated with a low dose of NZ-RLO2.

6.4 Discussion

Elevated mortalities of Chinook salmon at farmed sites within the Marlborough Sounds have occurred since 2012 (Norman et al., 2013) and two strains of NZ-RLO have been identified from fish during these summer mortalities (Chapter 2; Gias et al., 2018). However, the exact relationship between NZ-RLO strains and mortalities remained unknown. This study was performed to determine the pathogenicity of NZ-RLO2 in Chinook salmon smolt. The study demonstrated that NZ-RLO2 can cause disease and mortality in salmon experimentally inoculated. The mortalities induced by NZ-RLO2 were not unexpected as this bacteria was originally isolated from moribund fish. However, it was considered possible that NZ-RLO2 could either be of low pathogenicity or a secondary pathogen due to it not being detected in the fish investigated in Chapter 2 and that it was most commonly found in skin ulcers from summer mortalities (Chapter 4). The results of the present study confirms that NZ-

RLO2 can cause disease and death in salmon and, therefore, suggest this organism could contribute to summer mortalities.

While the gross lesions of summer mortalities in Chinook salmon have not been fully defined, a significant proportion of fish from these mortality events have been observed to have petechial haemorrhage within the abdominal fat and skin ulcers. In the present study, fish infected with NZ-RLO2 were observed to have similar petechial haemorrhage as well as skin ulcers. While many different disease processes can result in these gross lesions, it is possible that NZ-RLO2 was the cause of these lesions observed during the summer mortalities. Additionally, petechial haemorrhage within the abdominal adipose tissue has been described in Atlantic salmon that were experimentally infected with Tasmanian-RLO (Morrison et al., 2016) suggesting this lesion frequently develops due to RLO infection in salmon. However, gross lesions described from Atlantic salmon infected with *Piscirickettsia salmonis,* such as the presence of creamy coloured circular nodules in the liver and swollen kidneys (Birrell, Mitchell, & Bruno, 2003; Freyer and Mauel, 1997; Rozas and Enriquez, 2014), were not observed in the present study nor have the authors observed them during the summer mortalities.

It is possible that infection with NZ-RLO2 caused the skin ulcers observed in the inoculated fish. Skin ulcers were only observed in fish in the low dose group from 23 dpi onwards following a temperature spike. This suggests that the infection has to be present for a longer time or an increase in water temperature needs to occur for this clinical sign to develop. Perhaps, the longer NZ-RLO2 is present in the fish, the more it is likely to replicate and shed into the water column via faeces or mucus leading to infection via the skin. As well as the temperature increase during this study, possibly leading to increased replication of NZ-RLO2 it may have also impacted on the mucosal barriers of the skin (Jensen et al., 2015) leading to an increased likelihood of infection by bacteria.

New Zealand rickettsia-like organism 2 was not detected by qPCR in every skin ulcer analysed during the present study. It is therefore possible that in the skin ulcers that did not have NZ-RLO2 DNA, developed due to secondary infection by a different organism in a fish stressed by NZ-RLO2 and an increased temperature. Alternatively,

NZ-RLO2 could have directly caused the skin ulcers, but the bacteria was missed within the skin ulcer due to sampling as the entire ulcer was not assessed. In future, homogenisation of the entire skin ulcer with a sub-sample for DNA extraction may help to eliminate false negative results. Whether or not NZ-RLO2 can cause skin ulcers is important and the presence of NZ-RLO2 on the skin of fish suggests likely horizontal transmission from close contact with infected fish or from NZ-RLO2 in the water column. Such transmission is important for the spread of *P. salmonis* and piscirickettsia-like organisms in other fish species (Cvitanich et al., 1991; Fryer and Mauel, 1997; Smith et al., 1999; M.F., Chen et al., 2000). Immersion or cohabitation studies would be valuable to confirm this route of transmission for NZ-RLO2.

In the present study, histology of the skeletal muscle revealed inflammation in a proportion of fish infected with NZ-RLO2. However, as a proportion of sections from control fish showed similar inflammation, it cannot be confirmed this was due to NZ-RLO2. In the present study, the fish had been handled as part of the experimental manipulation and it is possible the histological changes could have been due to this.

Histology of fish from the summer mortalities have predominantly revealed focal areas of necrosis in the liver and depletion of haemopoetic tissue in both the kidney and spleen. Similar lesions were observed in fish inoculated with NZ-RLO2 in the present study. However, in the fish deliberately inoculated with NZ-RLO2 neutrophilic inflammation and necrosis of the exocrine pancreas and peri-pancreatic adipose tissue was most consistently seen. This lesion was not commonly observed in fish from the summer mortalities. The differences in the histological lesions could suggest that NZ-RLO2 does not cause summer mortalities or the infection level seen in the fish analysed from the summer mortalities were not high enough to present with this lesion. Alternatively, the differences in the lesions could simply be due to the use of i.p injection to experimentally inoculate the fish in the present study. Inoculation by i.p injection allowed the dose of bacteria administered to be controlled. As this is not the natural route of infection, immersion or co-habitation trials should be performed to study the pathogenicity and resulting disease manifestations when infection occurs by this more natural transmission route.

New Zealand rickettsia-like organism 2 were observed in the lesions of many of the fish using histology from the high or medium dose groups and were confirmed using ISH. This is in contrast to fish from the summer mortalities where NZ-RLO could not be confirmed using *in-situ* hybridization (ISH). The lack of visible NZ-RLO using histology in the fish from the summer mortalities could suggest a different cause of disease. However, as NZ-RLO2 were rarely seen in fish in the low dose groups that had lesions, this could be due to the infection level of NZ-RLO2. In the present study, pairs or clusters of NZ-RLO2 were visible within the inflammatory cells in tissues that showed evidence of necrosis in fish from the high and medium dose groups. The presence of NZ-RLO2 in association within the lesions adds evidence that these bacteria were causative of the lesions rather than being present incidentally in the tissues.

No NZ-RLO2 were observed within sections of the brain or heart. This is in contrast to infections by RLOs in other fish species in which the brain and heart consistently show histological evidence of infection, often including the presence of visible organisms (Skarmeta, Henríquez, Zahr, Orrego, & Marsha, 2000). The brain and heart are sites that *P. salmonis* and Tasmanian-RLO have been observed in from tissue smears, immunohistochemistry, or histology (Cvitanich et al., 1991; McCarthy et al., 2005; Morrison et al., 2016). In the present study, salmon infected with NZ-RLO2 were found to have pericarditis but NZ-RLO2 organisms were rarely observed in association with this lesion. In contrast, changes in the brain tended to be less consistent in the experimentally infected fish and NZ-RLO2 were not detected in sections of the brain. It is possible that evaluation of these organs by molecular testing, immunohistochemistry, or ISH may allow more frequent detection of NZ-RLO2. However, brain pathology has been reportedly linked to later stages of disease progression i.e. > 35 dpi (Rozas-Serri et al., 2017) and the shorter length of this study may be a reason it was not observed.

Intra-cytoplasmic organisms were visible in blood smears from some fish experimentally infected with NZ-RLO2. This suggests NZ-RLO2 are able to spread systemically in the body through the blood. Such dissemination in the body has been well documented in the literature for RLOs in Atlantic salmon and other finfish (Birell et al., 2003; Morrison et al., 2016; Marcos-López et al., 2017; Rozas-Serri et al., 2017).

New Zealand rickettsia-like organism 2 was most commonly observed in fish from the high dose group and lowest in fish from the low dose group. In fish infected with a low dose of NZ-RLO2, these organisms were not abundant and if visualisation of organisms is required in these lower levels of infection, serial sections of tissue for histology, immunohistochemistry, or ISH may be necessary. The focal nature of the organism within the tissue at low levels could also help when understanding the cell culture results in comparison to the qPCR results. In the high and medium dosed fish, organs with lower Ct values all resulted in a positive cell culture result, however in fish inoculated with a low dose of NZ-RLO2, two of the 27 samples were cell culture negative but qPCR positive and three of the 27 samples were cell culture positive but qPCR negative. This suggests at a low level infection with NZ-RLO2, different diagnostic tests may provide variable results and may be an explanation for the variability seen in Chapter 2 where three fish were positive for NZ-RLO using PCR and seven showed signs of NZ-RLO infection using histology.

The qPCR testing gave an indication of the infection level in the tissues tested. The number of copies of NZ-RLO2 detected in fish from the summer mortalities were similar to the copy numbers detected in fish inoculated with a low dose of NZ-RLO2 (Ministry for Primary Industries, unpublished). This comparison further suggests the involvement of NZ-RLO2 in the summer mortalities as infection with this level of NZ-RLO2 may result in mortalities. Furthermore, other diagnostic avenues carried out during the summer mortalities did not clearly reveal involvement from any other significant pathogens (Chapter 2).

Results from the present study suggest a dose dependent relationship between NZ-RLO2 and pathogenicity in Chinook salmon and that some fish may survive a low level infection under experimental laboratory conditions. Statistical analysis carried out on cumulative mortalities gave further confidence to this hypothesis as there was no significant difference in the mortalities when comparing the low dose mortalities with the control fish prior to the unexpected temperature spike. Once the seawater temperature was accidently increased there was a significant difference in the mortality rate of fish in the low dose group compared to the control group. This suggests that Chinook salmon could be infected with a low level of NZ-RLO2, but may not cause significant mortality until a stressor is introduce i.e. a rise in the seawater

temperature. It is possible then that the mortality events associated with NZ-RLO2 only develop in summer due to an interaction between increased seawater temperature and increased pathogenicity of the organism. Increased seawater temperature is a well-established risk factor for many diseases of aquatic animals and increased water temperature has been associated with outbreaks of disease due to *P. salmonis* (Branson and Diaz-Munoz, 1991; Gaggero et al., 1995; Stene, Bang Jensen, Knutsen, Olsen, & Viljugrein, 2014; Rees et al., 2014). Furthermore, NZ-RLO were detected at a higher prevalence in the summer months (Chapter 4). During the summer mortalities, seawater temperatures were consistently above 17°C (New Zealand King Salmon, pers. comm.) and out of the optimal temperature range for Chinook salmon, which is between 12 to 17°C. While a combination of NZ-RLO2 infection and warmer waters is an attractive hypothesis to explain the development of summer mortalities in New Zealand Chinook salmon, further controlled studies assessing temperature, as well as other possible stress factors, would need to be carried out to confirm the relationship between environmental stressors and mortality in NZ-RLO2 infected fish.

There were no significant differences in the abundance of NZ-RLO2 DNA between the four organs collected for analysis from inoculated fish. This suggests NZ-RLO2 are able to survive and replicate in multiple organs within the body. This observation is also useful when planning survey strategies to detect this organism in infected fish. A widespread distribution of the organism in the body has also been reported for *P. salmonis* and Tasmanian-RLO (Almendras et al., 2000; Morrison et al., 2016).

The fish used during this study were post-smolt and replicated what occurs in the natural farming environment where fish of this size are transferred to seawater. Post-smolt were used because studies of other RLO suggest that NZ-RLO2 is likely to be rapidly inactivated in freshwater (Morrison et al., 2016; Fryer and Mauel, 1997). If NZ-RLO2 cannot survive in freshwater, fish are most likely to be first exposed to, and subsequently infected, as they enter seawater at the post-smolt stage. Additionally, smoltification is a particularly stressful event in the production cycle of salmon due to the dramatic physiological and anatomical changes making this age group more susceptible to any further environmental changes and disease outbreaks (Roberts and Pearson, 2005). Therefore, using fish that were stressed by smoltification at the time of inoculation would be expected to maximize the chances of inducing disease and

mortalities due to inoculation by the bacteria. By carrying out the experiment in saltwater, the likelihood that additional infections from shedding and re-infection would occur was also maximised. However, exposure of fish of different ages to this pathogen would also be valuable to see if fish of different ages vary in their susceptibility to NZ-RLO2-induced disease.

6.5 Conclusion

The present study demonstrated NZ-RLO2 can cause disease and mortalities in Chinook salmon smolt when administered by i.p injection in a laboratory environment. Fish inoculated with a low dose of NZ-RLO2 showed similar gross and histological lesions to those fish analysed in the summer mortalities.

The mortality rate due to inoculation was dose dependent with lesions in the pancreas, adipose, and liver developing most consistently in infected fish. New Zealand rickettsia-like organism 2 was recoverable in pure culture from infected fish at all inoculation doses. This study suggests NZ-RLO2 may be involved in the summer mortalities seen in Chinook salmon in New Zealand.

Chapter 7 : Pathogenicity of the bacterium New Zealand rickettsia-like organism (NZ-RLO1) in Chinook salmon (*Oncorhynchus tshawytscha*, Walbaum) smolt.

A Chinook salmon smolt inoculated with a high dose of NZ-RLO1 that died during the study with red circular lesions present on the liver.

7.1 Introduction

Chinook salmon (*Oncorhynchus tshawytscha*) are the only salmonids farmed in New Zealand and New Zealand produces approximately 88% of the farmed Chinook salmon globally (Tuker, 2014). New Zealand salmonids are free of all the major pathogens that affect salmonids globally such as infectious salmon anaemia virus, viral haemorrhagic septicaemia virus, infectious haematopoietic necrosis virus, salmonid alphavirus, and *Renibacterium salmoninarum* (Diggles, 2016). However, since 2012 higher than expected mortalities have occurred at certain marine farmed salmon sites in the Marlborough Sounds. These mortality events often occurred within the summer months and were termed 'summer mortalities'. In 2015 New Zealand rickettsia-like organisms (NZ-RLO) were identified in affected farmed Chinook salmon. The two NZ-RLOs identified were termed NZ-RLO1 and NZ-RLO2 (Chapter 2; Gias et al., 2018). New Zealand rickettsia-like organism 1 and NZ-RLO2 are closely related to *Piscirickettsia salmonis*, the cause of the most common infectious disease of farmed salmonids in Chile (Price et al., 2017). The pathogenicity of different strains of *P.*

salmonis can vary with mortalities levels being reported from 8% to 90% (Brocklebank et al., 1992; Branson and Diaz-Munoz, 1991). Variation in mortality levels is dependent on the strain of the bacteria, the host species and the environment (House et al., 1999). Pathogenicity of NZ-RLO2 in Chinook salmon smolt was previously evaluated (Chapter 6) and resulted in cumulative mortalities of 100% in fish inoculated with a high or medium dose of NZ-RLO2, and 63% in fish inoculated with a low dose of NZ-RLO2. Fish inoculated with NZ-RLO2 that died during the experiment often had petechial haemorrhage within the internal adipose. Histological lesions in these fish consisted of neutrophilic and necrotizing pancreatitis and steatitis with intra-cytoplasmic organisms often visible within inflammatory cells in these necrotic foci. Additionally, hepatic necrosis, haematopoietic cell necrosis, and splenic and renal lymphoid depletion were observed.

The aim of the present study was to investigate the pathogenicity of NZ-RLO1 in Chinook salmon. If inoculating salmon with this bacteria resulted in disease, it would suggest NZ-RLO1 may have also contributed to summer mortalities in New Zealand Chinook salmon. Although direct comparison between the pathogenicity of NZ-RLO1 and NZ-RLO2 was not possible as the experiments were not run concurrently, the two experiments investigating the NZ-RLO strains may suggest which of these bacteria are most likely to contribute to summer mortalities in Chinook salmon.

7.2 Materials and methods

7.2.1 Ethics statement

This study was performed under the same ethics approval as the NZ-RLO2 study (Chapter 6).

7.2.2 NZ-RLO1 isolate origin

New Zealand rickettsia-like organism 1 was isolated from the spleen of a moribund farmed Chinook salmon of harvest size (~3 kg) originating from the South Island during a 2015 summer mortality event (Chapter 2).

The NZ-RLO1 isolate was cultured in a monolayer of Epithelioma papulosum cyprini (EPC, ECACC-93120820) cell line grown in Hank's minimal essential medium (MEM) (Gibco, Life Technologies, NY, USA) supplemented with 10% foetal bovine serum (FBS; HyClone, Utah, USA) (MEM + 10% FBS). New Zealand rickettsia-like organism 1 was incubated for seven days at 15°C until approximately 90% cytopathic effect was observed. Passage nine was used for this study and a titration was performed to determine the tissue culture infectious dose in 50% of the cells inoculated ($TCID_{50}$) following the Spearman-Kärber method (Spearman, 1908; Kärber, 1931). The concentration of the inoculum administered to fish in the high dose group was calculated as 5.6×10^4 $TCID_{50}$ $50\mu L^{-1}$. The inoculum was then diluted 1/10 and 1/100 with MEM + 10% FBS to obtain the dose administered to the fish in the medium (5.6×10^3 $TCID_{50}$ $50\mu L^{-1}$) and low (5.6×10^2 $TCID_{50}$ $50\mu L^{-1}$) dose groups.

7.2.3 Fish used in study and tank set up

Salmon smolt were approximately 185 days post hatch and had an average weight (± SD) of 82 ± 12 g (*n* = 20). Tank set up, water temperature, feeding and monitoring of the fish during the study were identical to the NZ-RLO2 study (Chapter 6).

7.2.4 Challenge with NZ-RLO1

The number of fish used in each of the treatments were based on the same sample size calculations as for the NZ-RLO2 study (Chapter 6). The number of fish used in the study were based on conservative predictions that were lower than the mortality caused by other *P. salmonis* strains (Birkbeck et al., 2004; Valenzuela-Mirando and Gallardo-Escarate, 2016; Morrison et al., 2016). These conservative predictions were that fish infected with a high, medium, or low dose of NZ-RLO1 would have mortalities of at least 70%, 50%, or 30% respectively by the end of the 30 day study. These numbers allowed for a statistically significant difference to be detected between each of the dosed groups compared to the control groups. All fish were sedated using iso-eugenol (AQUI-S) at 25 mg L^{-1} and tagged (VI alpha tags, Northwest marine tech, WA, USA) while under sedation as for the NZ-RLO2 study (Chapter 6). All injections were administered via an intraperitoneal (i.p) injection with 23 gauge x 5 mm needles (Eurovet, Germany). All fish, control and challenged, were kept at a density of six per

tank. Control fish (*n* = 60) were subdivided evenly into two groups (i.p and no-i.p control fish) with the i.p control fish receiving a 0.1 mL i.p injection of cell culture media (MEM + 10% FBS). Based on sample size calculations, the number of tanks per dose were one, two, or five for the high, medium, or low dose respectively with fish receiving a 0.1 mL i.p injection of the required dose of NZ-RLO1. This equated to 6 fish in the high dose group, 12 fish in the medium dose group and 30 fish in the low dose group. During the study, fish were monitored at least three times a day with mortalities removed and sampled immediately. Fish showing overt signs of disease such as darkening of the skin, loss of equilibrium or a lack of response to stimuli were euthanised using iso- eugenol (AQUI-S, Lower Hutt, New Zealand) at a rate of 175 mg L^{-1} for 20 min. On completion of the study, 30 days after inoculation, all surviving fish were euthanised. Mortalities and euthanised fish were processed for diagnostic testing in the same way.

7.2.5 Pathology

7.2.5.1 Blood smear preparation and gross necropsy

Following euthanasia or death, each fish was measured (fork length), weighed and visually assessed for any abnormalities. A blood sample was taken from each fish by caudal venous puncture. A blood smear was created and stained with modified Giemsa stain (Sigma, Missouri, USA) for 30 min prior to microscopic examination for the presence of NZ-RLO1.

7.2.5.2 Histology

Tissue samples were taken from the gills, skin and skeletal muscle at the lateral line, skin ulcers where present, heart, liver, pyloric caeca, spleen, anterior and mid-kidney, brain, and mid-intestine from all fish and fixed in 10% buffered formalin for one to two hays then transferred to 70% ethanol. Samples were then embedded in paraffin and sectioned and stained with haematoxylin and eosin (H&E) for histological examination.

7.2.5.3. In-situ hybridization

Tissue samples from four fish that had been inoculated with NZ-RLO1 were evaluated by *in situ* hybridization (ISH). The tissues evaluated were: kidney, liver, spleen, skin, gills, brain, heart, pyloric caeca, adipose fat, and mid-intestine. Two fish evaluated had been inoculated with a high dose of NZ-RLO1 and died during the study while the other two fish had been inoculated with a low dose of NZ-RLO1 and had survived the study. *In-situ* hybridization was carried out as previously described (Chapter 3). All experiments included a positive control that comprised of tissue from an Atlantic salmon (*Salmo salar*) that was known to be infected with *P. salmonis* and a negative control (*Crassostrea gigas* tissue). Cover-slipped slides were examined under an Olympus BX51 light microscope.

7.2.6 Recovery of NZ-RLO1 from fish tissue in cell culture and agar

7.2.6.1 Cell culture

A representative number of fish were selected for cell culture from each inoculation dose; six from the high dose group, 10 from the medium dose group, 12 from the low dose group, and 11 from the control groups. Kidney, spleen, and liver were aseptically removed from each fish and combined into a sterile vial (~500 mg in total). Tissues were diluted 1/10 in MEM+10% FBS with the addition of penicillin (100 µg/mL), homogenised then diluted further to 1/100 and 1/1000 in MEM +10% FBS. An aliquot (100 µL) of each dilution was inoculated into separate wells of a 24 well plate seeded with a monolayer of EPC. Following adsorption for 30 min at RT, 1 mL of MEM + 10% FBS was added to each well and cultures were incubated at 15°C for 14 days. Cultures were observed under light microscopy for the presence of cytopathic effect (CPE). Cultures displaying no CPE after 14 days were passaged once by transferring 100 µL of the initial culture into new well of a 24 well plate seeded with EPC cells and re-incubated for a total of 28 days.

Following incubation, DNA was extracted from two samples from fish that had been inoculated with a high dose of NZ-RLO1, one sample from a fish inoculated with a medium dose of NZ-RLO1 and two samples from fish inoculated with a low dose of

NZ-RLO1 all displaying CPE. Cell cultures that contained tissues from fish in the control groups did not display CPE and were not evaluated by qPCR. DNA extraction was carried out as follows: for each sample, an aliquot of 200 µL of the culture showing CPE (P1) and from the initial homogenate (P0) was taken. DNA was then extracted and subjected to NZ-RLO1 qPCR. If the CPE was due to growth of NZ-RLO1 a decrease or the same Ct value was expected in the P1 samples compared with the P0 samples.

7.2.6.2 Agar culture

Fish were evaluated to determine if NZ-RLO1 could be recovered directly from infected tissues onto agar without the need for cell culture. The kidney, spleen, and liver of fish that had died during the study after being inoculated with NZ-RLO1 were evaluated; three from fish inoculated with a high dose of NZ-RLO1, one from fish inoculated with a medium dose of NZ-RLO1, and three from fish inoculated with a low dose of NZ-RLO1. Sterile 10 µL loops were inserted into the tissue then plated directly onto cysteine heart agar with 5% sheep blood (CHAB + 5%; Fort Richard, Auckland, New Zealand). Plates were incubated for 28 days at 15°C. Bacterial colonies that grew were confirmed to be NZ-RLO1 by using a specific NZ-RLO1 qPCR.

7.2.7 Molecular tests

7.2.7.1 DNA extraction from tissues

Fish on arrival. Following euthanasia, ~200 mg total of the kidney, liver, and spleen were aseptically removed from each fish and combined into a MagNA lyser green bead tube (Roche, Penzberg, Germany). Phosphate buffered saline was added (500 µL) and the tissue was homogenised in the MagNA lyser (Roche) at 6,500 rpm for 30 sec. A subsample of this homogenate (80 µL) was used for DNA extraction using the QIAamp mini kit (Qiagen, Hilden, Germany) as per the manufacturer's protocol for tissue.

Experimental fish. Following euthanasia or death, samples of approximately 20 mg of each kidney, liver, mid-intestine, skin ulcer (when present), and 10 mg of spleen

were aseptically collected from each fish. DNA was extracted from each tissue sample separately on the QIAcube high throughput automated extraction robot using the QIAamp HT kit as per the manufacturer's protocol (Qiagen).

All extracted DNA was assessed for suitability for qPCR by performing an internal control PCR targeting the 18S rRNA gene following the manufacturer's protocol (Ribosomal 18S rRNA Endogenous Control; Life technologies, Oregon, USA).

7.2.7.2 Generic NZ-RLO qPCR to detect NZ-RLO in fish on arrival to the facility

To ensure fish were not infected with NZ-RLO at the start of the study, a generic qPCR was carried out targeting the 23S rRNA gene of *P. salmonis* (Corbeil et al., 2003) used to detect all NZ-RLO strains. The methods used to perform this qPCR were as described in the NZ-RLO2 study (Chapter 6).

7.2.7.3 Specific qPCR to detect NZ-RLO1 in experimental fish

DNA extracted from tissue samples of kidney, liver, spleen, and mid-intestine from all 108 experimental fish were analysed by qPCR to detect the presence of NZ-RLO1 DNA. Skin ulcers were also analysed; one from a fish in the high dose group, two from fish in the medium dose group, seven from fish in the low dose group, and two from fish in the control groups.

The NZ-RLO1 qPCR was performed as previously described (Chapter 5). Each reaction consisted of 2 µL of DNA (~150 ng genomic DNA), 10 µL 2 X SsoAdvanced Universal Probes Supermix (Bio-rad, Hercules, USA), 0.25 µM of each primer, 0.3 µM of probe and water to a final volume of 20 µL. The PCR was carried out on a CFX 96 real-time PCR detection system (Bio-Rad, Hercules, USA) with the following cycling conditions; initial denaturation at 95°C for 2 min, 50 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 30 sec. With each PCR, molecular grade water was run as a no template control (NTC) and a positive control using DNA extracted from a pure cell culture of NZ-RLO (W15_494 10Sp) at 0.1 ng was used.

7.2.8 Statistical analysis

Statistical analysis was performed in R version 3.5.2 (R Core Team, 2015) to investigate potential differences between weights and lengths of the fish in the groups inoculated with NZ-RLO1 compared to the control groups. The weight and lengths of fish were compared using a one-way ANOVA to examine differences between the control and dosed fish. A generalised linear model (GLM) with weight or lengths of fish as the response variable and treatment type (dosed and control) as the explanatory variable was fitted to the data with a Gaussian distribution (R package *multcomp*, Hothorn et al., 2008).

Cumulative mortality rates in each inoculation dose group and control groups were compared to the control groups in a GLM with a binomial error distribution. The two control groups were also compared to each other using this model.

Two analyses of Ct values from the NZ-RLO1 qPCR were carried out: 1) Ct values detected from all organs (kidney, liver, spleen, and mid-intestine) were compared between the high, medium, and low dose groups; 2) Ct values detected from all organs were compared within each of the high, medium, and low dose groups. Analysis 1 looked to determine if the concentration of bacteria detected between the three dosed groups differed significantly. Analysis 2 looked to determine if any organ within each dosed group had a significantly different concentration of bacteria. The response variable in the model was the Ct value, which was log transformed to meet the assumptions of normality. Specific pair wise differences between the groups were tested using Tukey contrasts and *p* values were adjusted using the Benjamini and Hochberg method (R package multcomp, Hothorn et al., 2008).
The significance of the explanatory variables in all models were assessed using likelihood ratio tests. We used p values < 0.05 to determine statistical significance.

7.3 Results

7.3.1 Cumulative mortality

Fish that had been inoculated with a high dose of NZ-RLO1 first died at 12 days post inoculation (dpi) and four of six fish (67%) died prior to the completion of the study at 30 dpi. Fish that had been inoculated with a medium dose of NZ-RLO1 first died at 16 dpi and by 30 dpi, three of twelve fish (25%) had died. Fish that had been inoculated with a low dose of NZ-RLO1 first died at 14 dpi and five of 30 fish (17%) had died by the completion of the study. Although no fish in the intraperitoneal injection (i.p) control group died during the study, two of 30 fish (7%) in the no-i.p control group died within the 30 day study (Figure 7.1).

Mortality rates in the groups of fish inoculated with NZ-RLO1 were not significantly higher than the mortalities of fish in the control groups (p = 0.08, 0.22 and 0.06 for the high, medium and low dose groups respectively). There was no significant difference in the rate of mortality between the two control groups (p = 0.54).

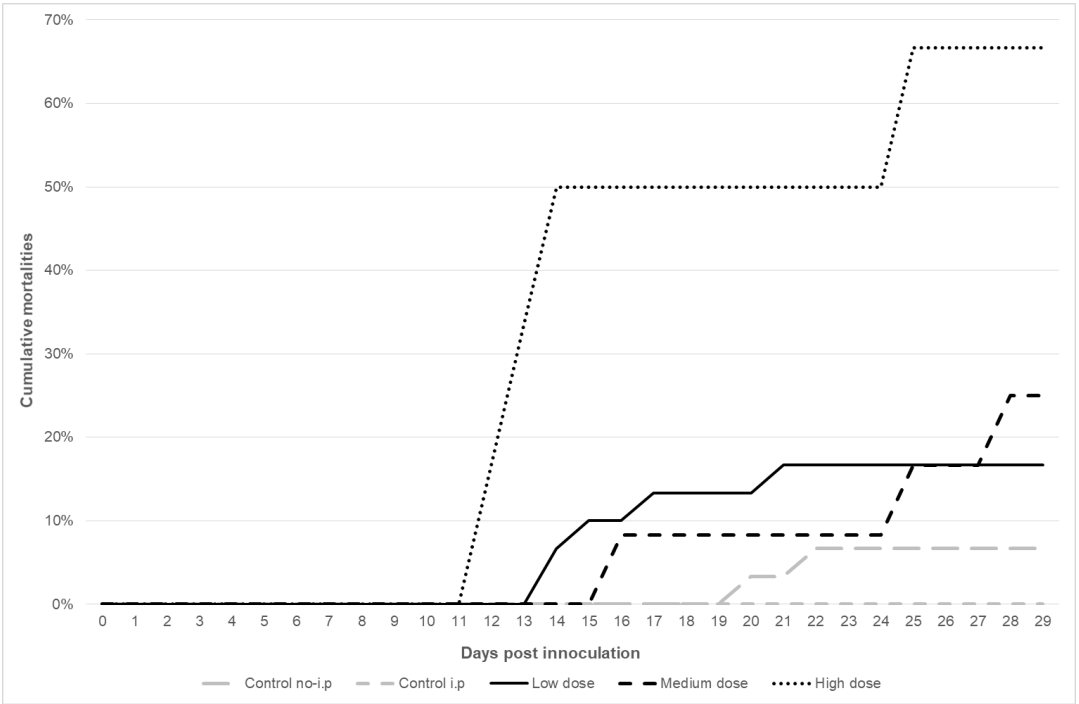


Figure 7.1. Cumulative mortalities of fish in the NZ-RLO1 study. High dose indicates that the fish were inoculated with a high dose of NZ-RLO1, medium dose indicated fish were inoculated with a medium dose of NZ-RLO1 while low dose indicates the fish were inoculated with a low dose of NZ-RLO1. Fish in the control no-i.p group did not receive any intraperitoneal injection while fish in the control i.p. group received an intraperitoneal injection that did not contain NZ-RLO1.

7.3.2 Gross pathology

At the completion of the study, fish that had been inoculated with NZ-RLO1 had an average weight (± SD) of 75 ± 16.5 which was significantly less than those in the control groups 87 ± 20 ($p < 0.001$). Fish inoculated with NZ-RLO1 had an average fork length (± SD) of 17.6 ± 1 which was significantly less than those in the control groups 18.3 ± 1 ($p < 0.001$). This significant difference was seen when one outlier in the control groups was removed (Figure 7.2) and was not included in the statistical analysis.

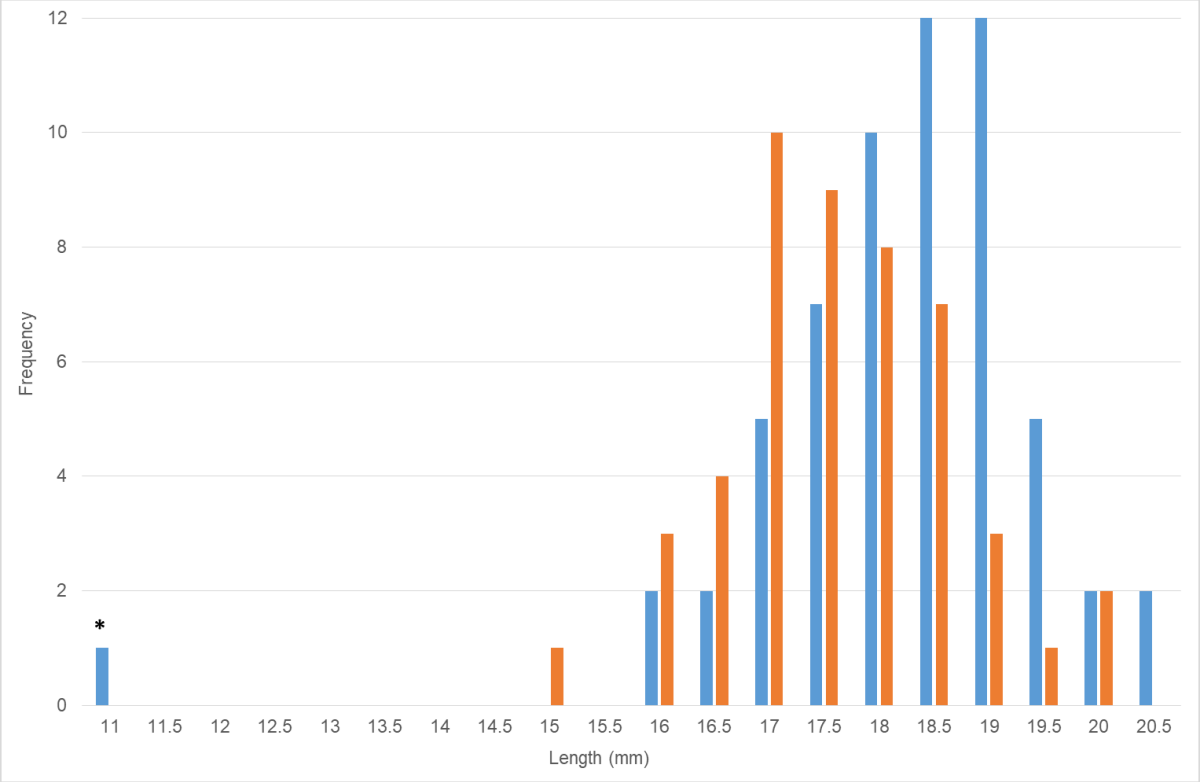


Figure 7.2. Lengths of fish in the group inoculated with NZ-RLO1 (Orange bars) and the control groups (Blue bars). Note the outlier (*) which was removed from the statistical analysis.

Although food intake was not objectively measured, fish in the high and medium dose groups were observed to feed less than fish in the low dose or control groups after inoculation with NZ-RLO1. These fish were also observed to be less active than those in the low dose or control groups.

Examination of blood smears revealed bacteria resembling NZ-RLO1 within circulating white blood cells (Figure 7.3). These NZ-RLO1 bacteria were observed in all six fish

from the high dose group, five of 12 fish (42%) from the medium dose group, and five of 30 fish (17%) from the low dose group.

Overall, 12 fish died after being inoculated with NZ-RLO1 before the completion of the study. As can be seen from Table 7.1, the most common findings on gross necropsy examination of these fish was petechial haemorrhage in the adipose tissue surrounding the pyloric caeca (Figure 7.4), enlarged spleen, petechiae in the ventral surface of the body including at the base of the fins and around the vent (Figure 7.5), red circular lesions with a cream margin in the liver (Figure 7.4), pale liver, pale gills, reddening on the brain, and blood spots in the eyes.

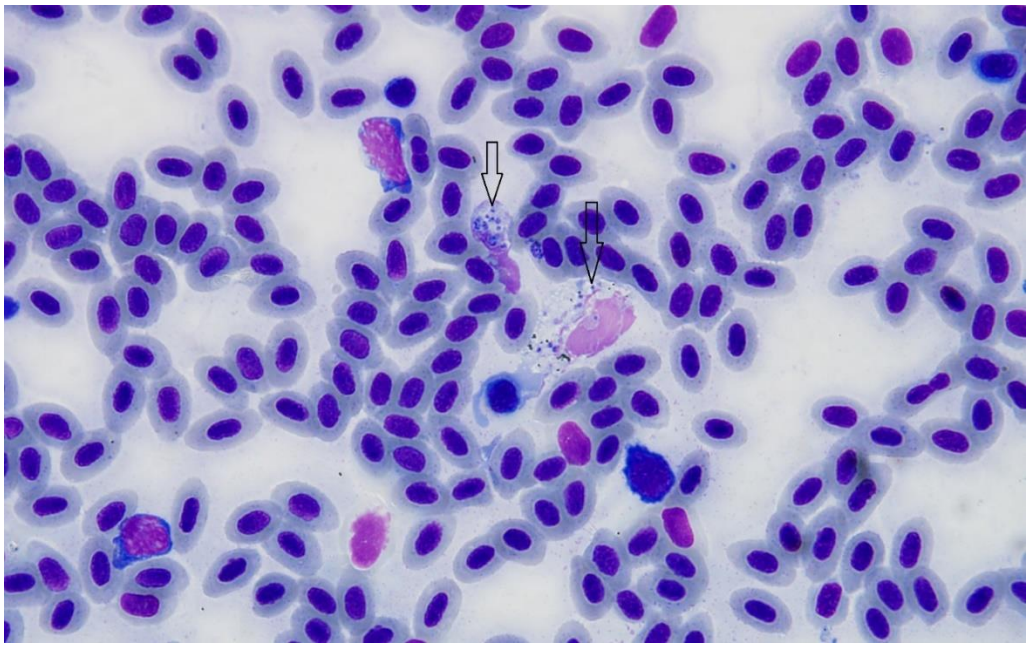


Figure 7.3. Blood smear of fish inoculated with NZ-RLO1. Blue stained coccid cells ~ 1 µm resembling NZ-RLO1 were observed within circulating white blood cells (arrows). Giemsa stain, 400 x magnification.

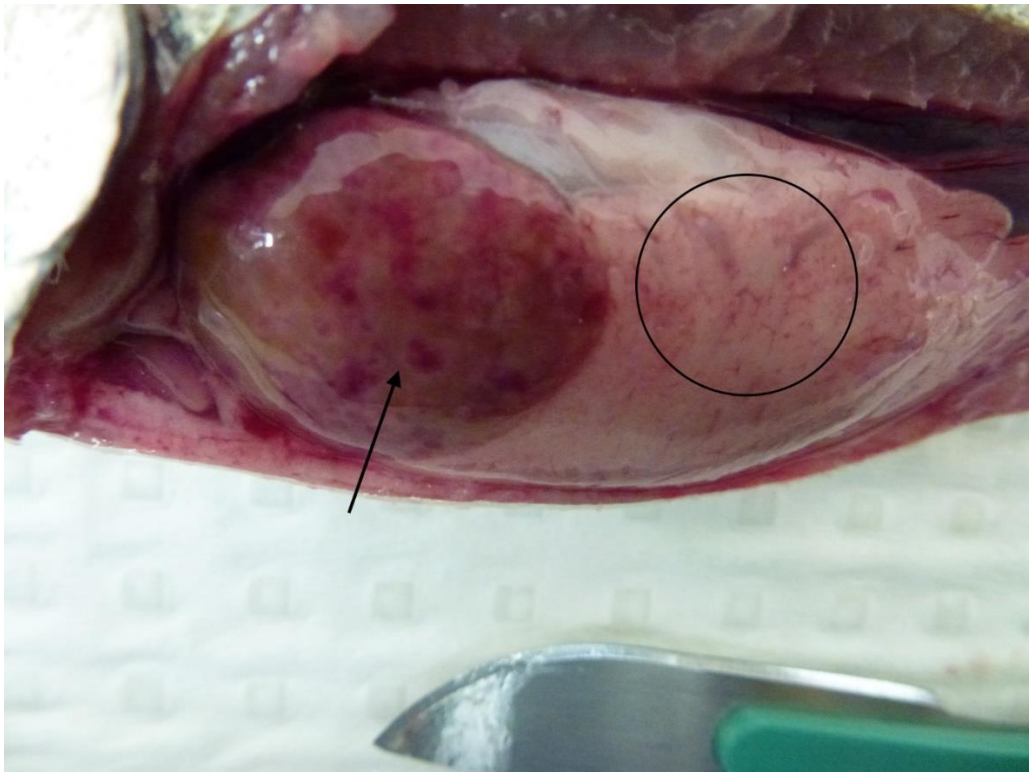


Figure 7.4. Petechial haemorrhage in the adipose tissue around the pyloric ceca (circle) and red circular lesions with cream margin within a pale liver (arrow) were commonly seen on necropsy examination of fish that died after being inoculated with NZ-RLO1.

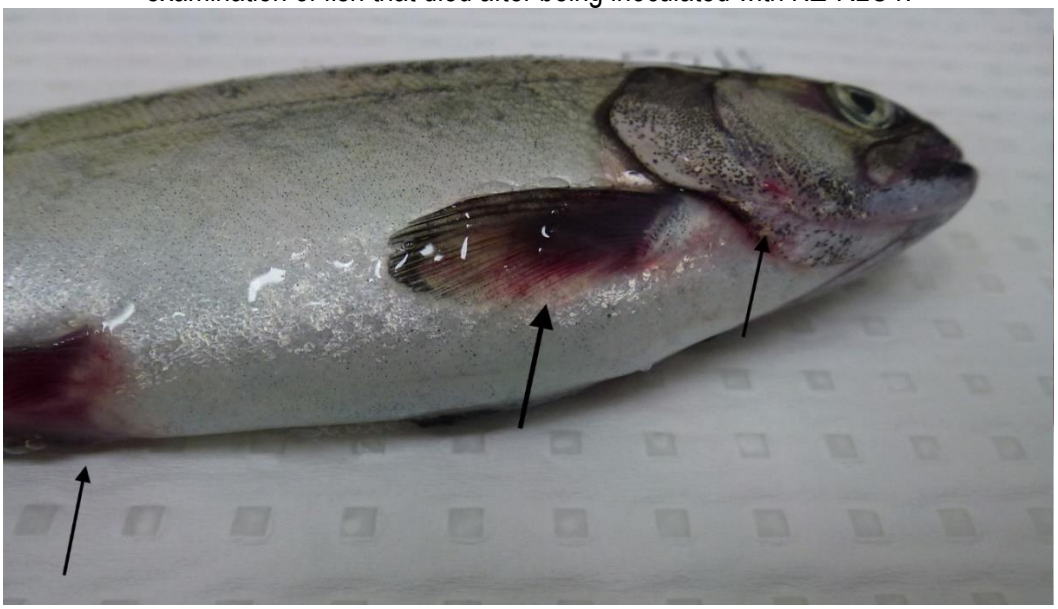


Figure 7.5. Petechiae at the base of the pectoral and pelvic fins as well as on the operculum (arrows) was commonly seen on necropsy examination of fish that died after being inoculated with NZ-RLO1.

Other findings that were less consistent were skin ulcers, reddening of the intestine, and pallor of the kidney. Skin ulcers were observed in fish inoculated with NZ-RLO1 from 20 dpi onwards. Skin ulcers were often visible as circular, approximately 2 mm in diameter, red foci often extending into the underlying musculature (Figure 7.6). Two

control fish in the no-i.p group that survived the study were observed to have scale loss with foci of reddened skin that did not extend into the underlying musculature. The foci of reddened skin from the control fish as well as the skin ulcers from the fish inoculated with NZ-RLO1 were taken for evaluation by qPCR for the presence of NZ-RLO1 DNA.

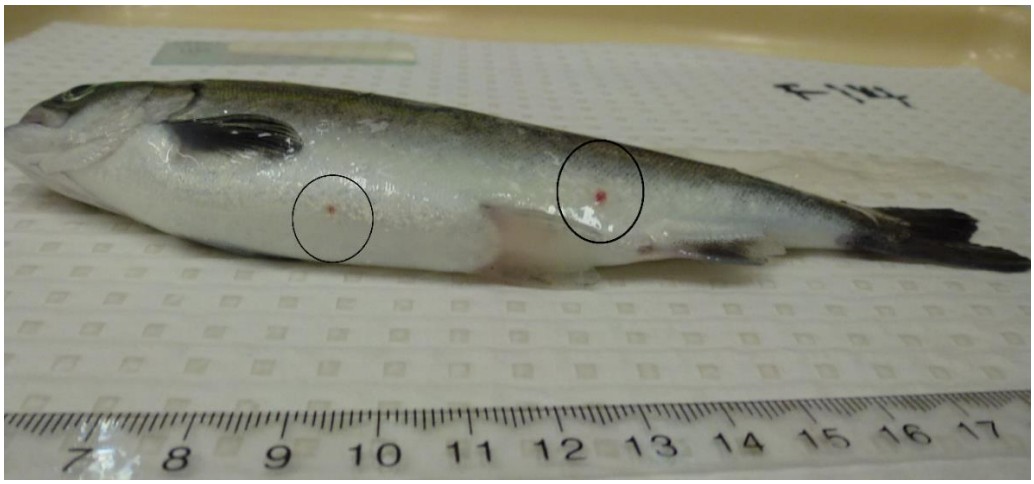


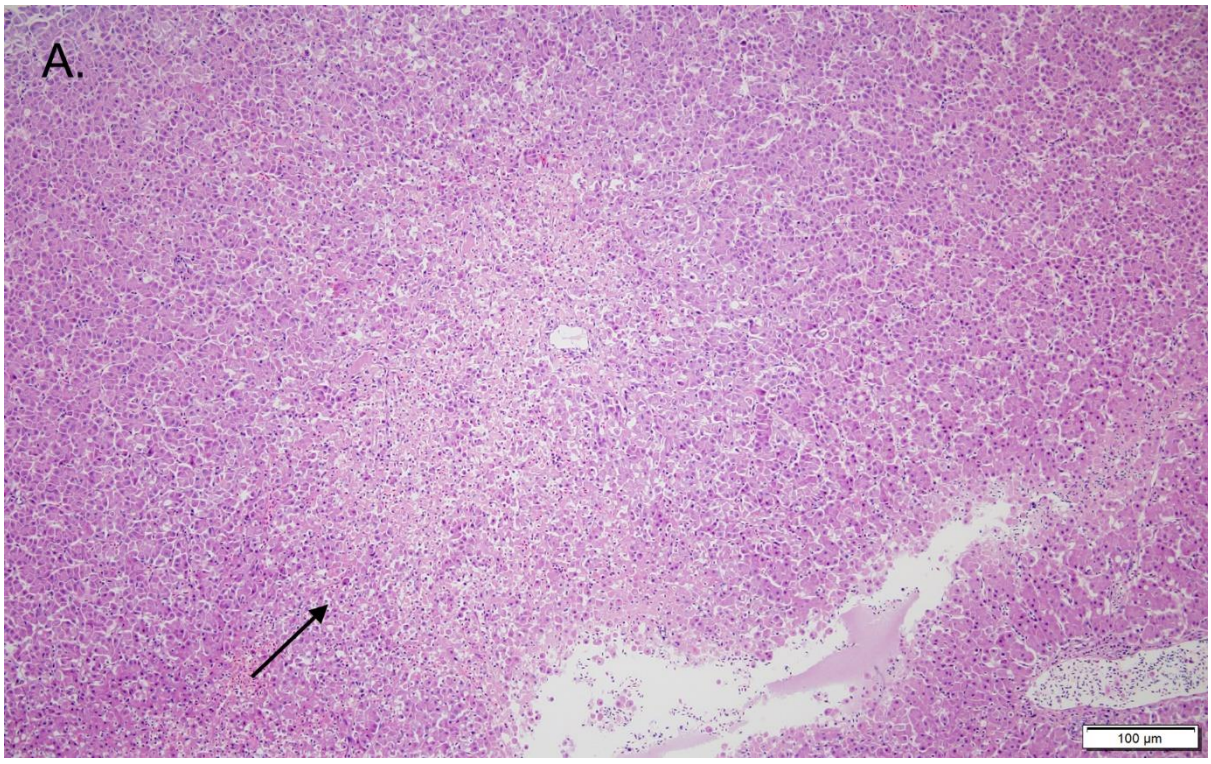
Figure 7.6. Skin ulcers (circles) observed in fish inoculated with NZ-RLO1 were grossly visible as approximately 1-2 mm diameter reddened foci that had white margins.

Table 7.1. Numbers of fish that developed each visible pathological change within the study. Fish that died during the study and fish that survived 30 days after inoculation are represented separately. High dose indicates that fish were inoculated with a high dose of NZ-RLO1, Medium dose indicates that fish were inoculated with a medium dose of NZ-RLO1, Low dose indicates that fish were inoculated with a low dose of NZ-RLO1, Control no-i.p indicates that fish were not inoculated and Control i.p indicates that fish were inoculated with sterile media that did not contain NZ-RLO1.

	Fish that died during the study				**Fish that survived the study**				
Gross pathology	**High dose (*n* = 4)**	**Medium dose (*n* = 3)**	**Low dose (*n* = 5)**	**Control no-i.p (*n* =2)**	**High dose (*n* = 2)**	**Medium dose (*n* = 9)**	**Low dose (*n* = 25)**	**Control no-i.p (*n* = 28)**	**Control i. p (*n* = 30)**
Enlarged spleen	3	1	4	0	0	1	2	0	0
Petechial haemorrhage in adipose tissue	3	1	5	0	0	1	8	1	0
Petechiae in the ventral surface of body	4	2	5	0	0	1	4	0	0
Petechiae at the base of one or all fins	4	2	5	1	1	1	6	1	0
Liver lesions	4	2	3	0	0	0	0	0	0
Pale liver	4	2	3	0	0	3	12	0	0
Pale gills	2	1	2	0	0	0	1	0	0
White patches on gills	0	1	0	0	0	0	4	0	0
Reddening on the brain	2	1	2	0	0	0	0	2	0
Blood spots in the eye	1	2	3	1	0	0	4	1	0
Skin ulcers	1	1	0	0	0	1	7	0	0
Redding of the intestine	2	0	0	1	0	0	2	0	0
Pale kidney	1	1	2	0	1	0	1	0	0
Pale heart	0	1	0	0	0	0	0	0	0

7.4.3 Histopathology

The most frequent histological lesion observed in fish that died after inoculation with NZ-RLO1 was multifocal hepatocellular necrosis. This lesion was observed in fish that died after being inoculated with all doses of NZ-RLO1 (Table 7.2). The necrotic foci were widespread within the liver parenchyma and appeared as loss of cell structure and increased eosinophilia within the affected cells. Some foci of necrosis were associated with mild lymphocytic and histiocytic inflammation suggesting that they were acute to subacute. Organisms that were suspected to be NZ-RLO1 were commonly seen as pairs or clusters of ~1 µm basophilic cocci within areas of hepatocellular necrosis. These presumptive NZ-RLO1 bacteria were present within the cytoplasm of inflammatory cells as well as in vacuoles within the cytoplasm of hepatocytes (Figures 7.7 A and B).



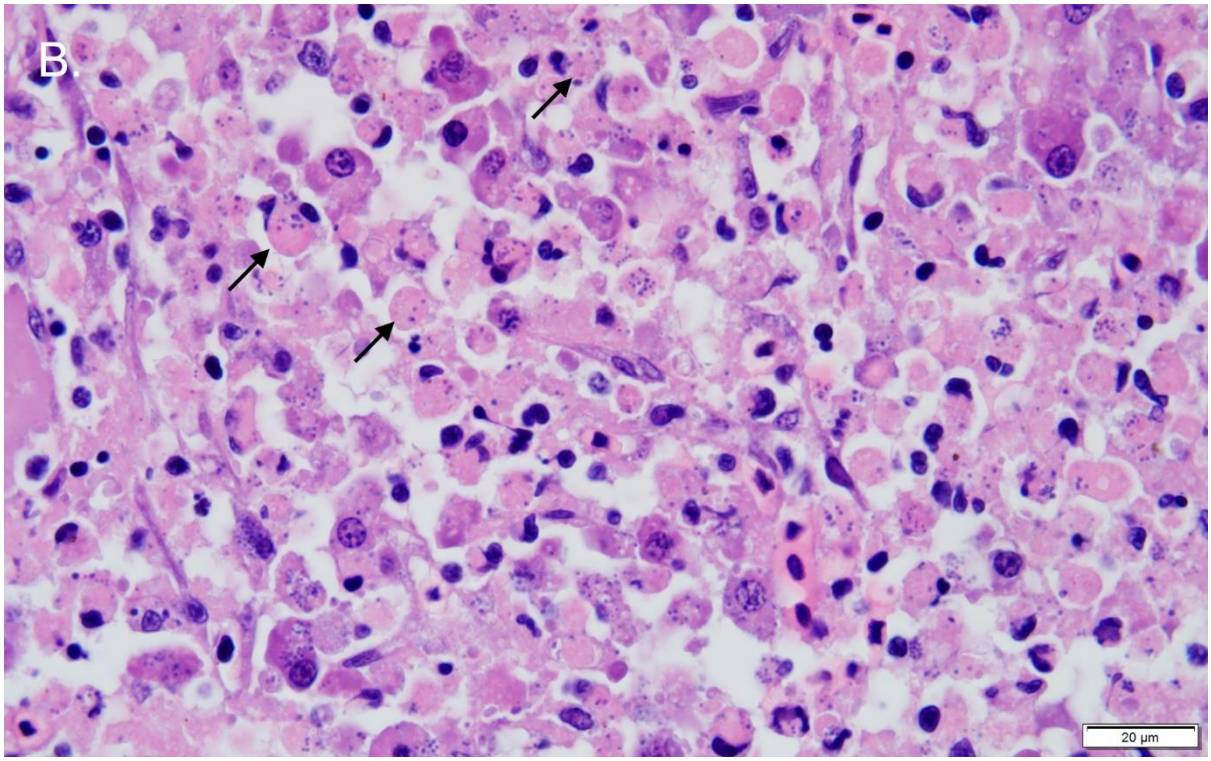


Figure 7.7. Liver from a fish that died after inoculation with a high dose of NZ-RLO1. **A.** Note the multi focal extensive areas of hepatocyte necrosis (arrow). H&E, 200 x magnification. **B.** Note the hepatocyte necrosis and the presence of NZ-RLO1 (basophilic cocci) within inflammatory cells and hepatocytes (arrows). H&E, 1000 X magnification.

Examination of the pancreas and surrounding adipose tissue of fish that died after being inoculated with NZ-RLO1 revealed focal necrosis associated with mild to moderate neutrophilic inflammation. Intra-cytoplasmic bodies resembling NZ-RLO1 were frequently visible within infiltrating inflammatory cells in these areas of necrosis and inflammation (Figures 7.8 A and B).

Examination of the kidneys revealed mild to moderate individual cell necrosis and inflammation with apparent depletion of haematopoietic cells with NZ-RLO1 bacteria often visible within the inflammatory cells. Necrosis and depletion of the haemopoetic tissue within the kidney was seen in fish inoculated with NZ-RLO1 and was not seen in the control fish.

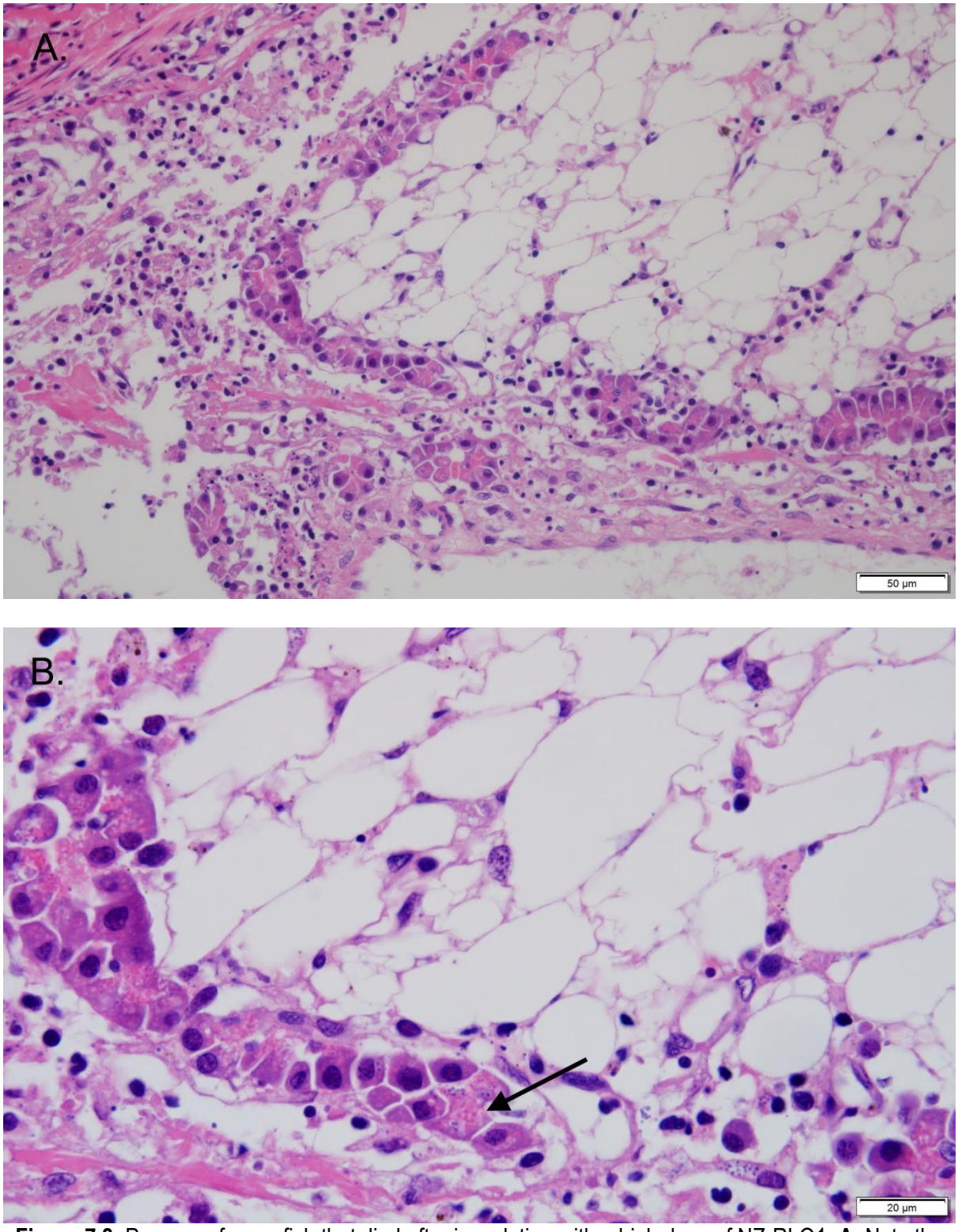
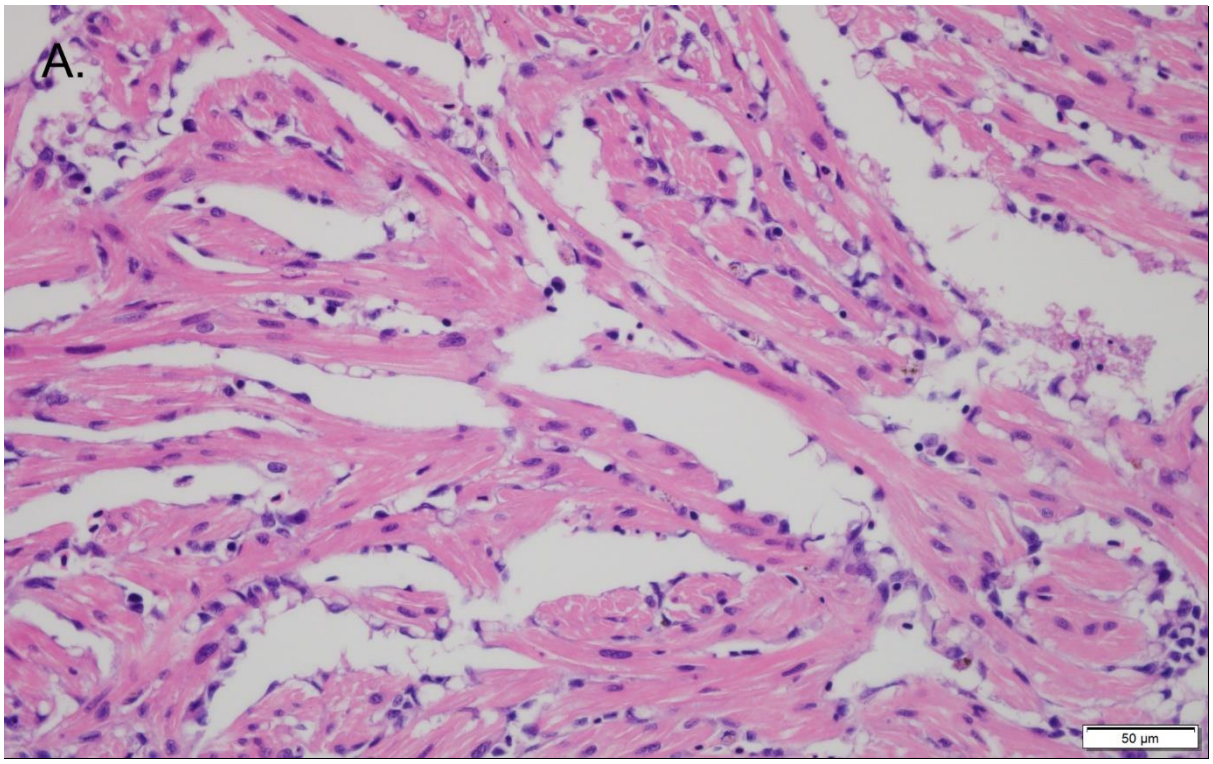


Figure 7.8. Pancreas from a fish that died after inoculation with a high dose of NZ-RLO1. **A.** Note the moderate infiltration of inflammatory cells within the surrounding adipose tissue. H&E, 400 x magnification. **B.** Note the presence of cocci within the cytoplasm of the exocrine pancreatic cells (arrow). H&E, 1000 x magnification.

Examination of the spleen of fish inoculated with NZ-RLO1 that died during the study showed multiple foci of eosinophilic fibrillary material. In addition, depletion of the

haemopoetic tissue and occasional lymphoid cellular depletion with associated necrosis and inflammation and the presence of bacteria resembling NZ-RLO1 within inflammatory cells. Loss of haematopoietic tissue and the presence of eosinophilic fibrillar material was observed to a lesser extent in fish that survived the study and this change was not observed in any of the control fish that died during the study. No significant lesions were visible within the spleen in any of the fish that survived the study.

Examination of the spongy and compact myocardium of fish that died during the study often revealed multifocal inflammation with the presence of increased neutrophils and macrophages, often associated with foci of eosinophilia and loss of cell detail interpreted as necrosis. Examination of the pericardium revealed focal areas of inflammation. Within areas of myocarditis, bacteria resembling NZ-RLO1 were commonly observed in circulating inflammatory cells as well as endothelial cells (Figures 7.9 A and B). Neither myocarditis nor pericarditis were observed in any control fish.



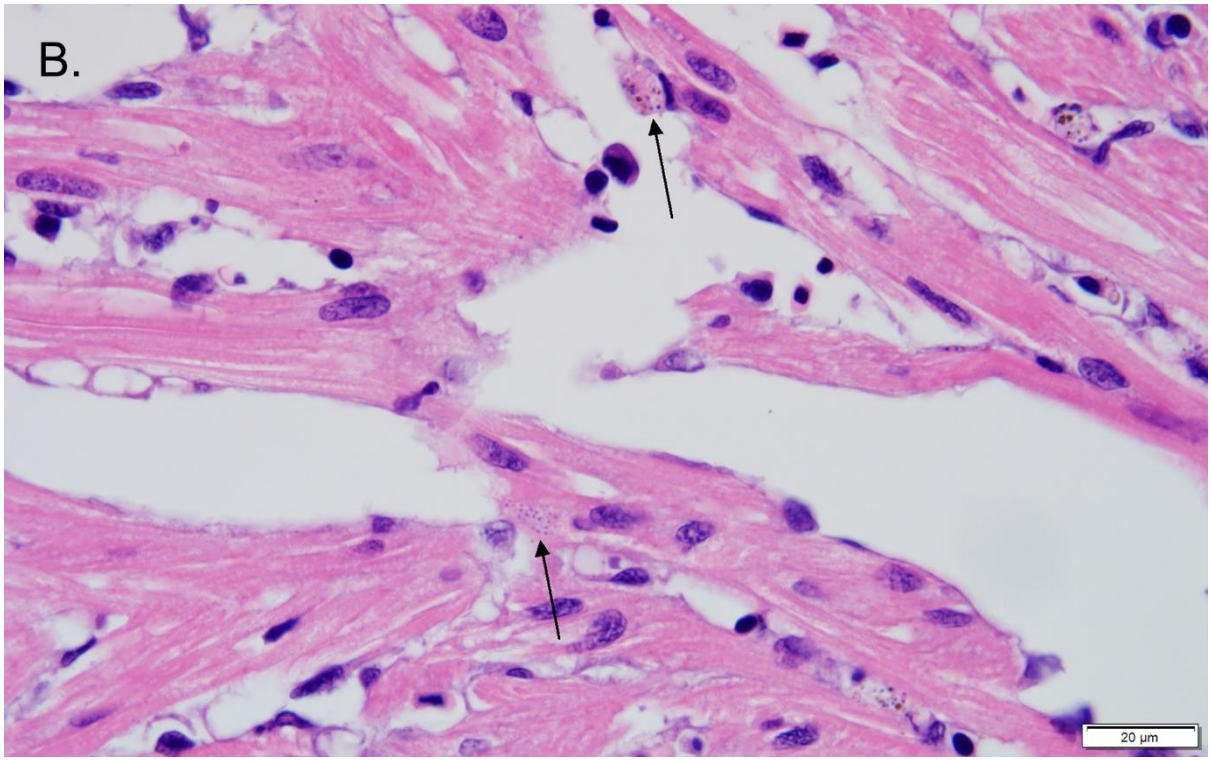


Figure 7.9. Heart from a fish that died during the study after being inoculated with a medium dose of NZ-RLO1. **A.** Note the inflammation in the inner spongy layer of the ventricle. H&E, 400X magnification. **B.** Note the presence of basophilic cocci within the cytoplasm of the endothelial cells (arrows). H&E, 1000 X magnification.

Examination of the brain revealed mild congestion of the blood vessels in the meninges and, occasionally, of blood vessels within the grey matter. Congestion was not associated with inflammation and was seen in both fish inoculated with NZ-RLO1 and in control fish.

Histological examination of the skin and muscle samples taken from the lateral line of all fish did not reveal significant changes. Examination of skin ulcers from one fish inoculated with a high dose and four fish inoculated with a low dose of NZ-RLO1 revealed loss of epithelium and basement membrane with inflammation extending into the stratum spongiosum and stratum compactum. This inflammation commonly extended through the hypodermis into the musculature (Figures 7.10 A and B). Bacteria interpreted as NZ-RLO1 were observed within the inflammatory and epithelial cells in these areas of inflammation (Figures 7.11 A and B). No other organisms were observed.

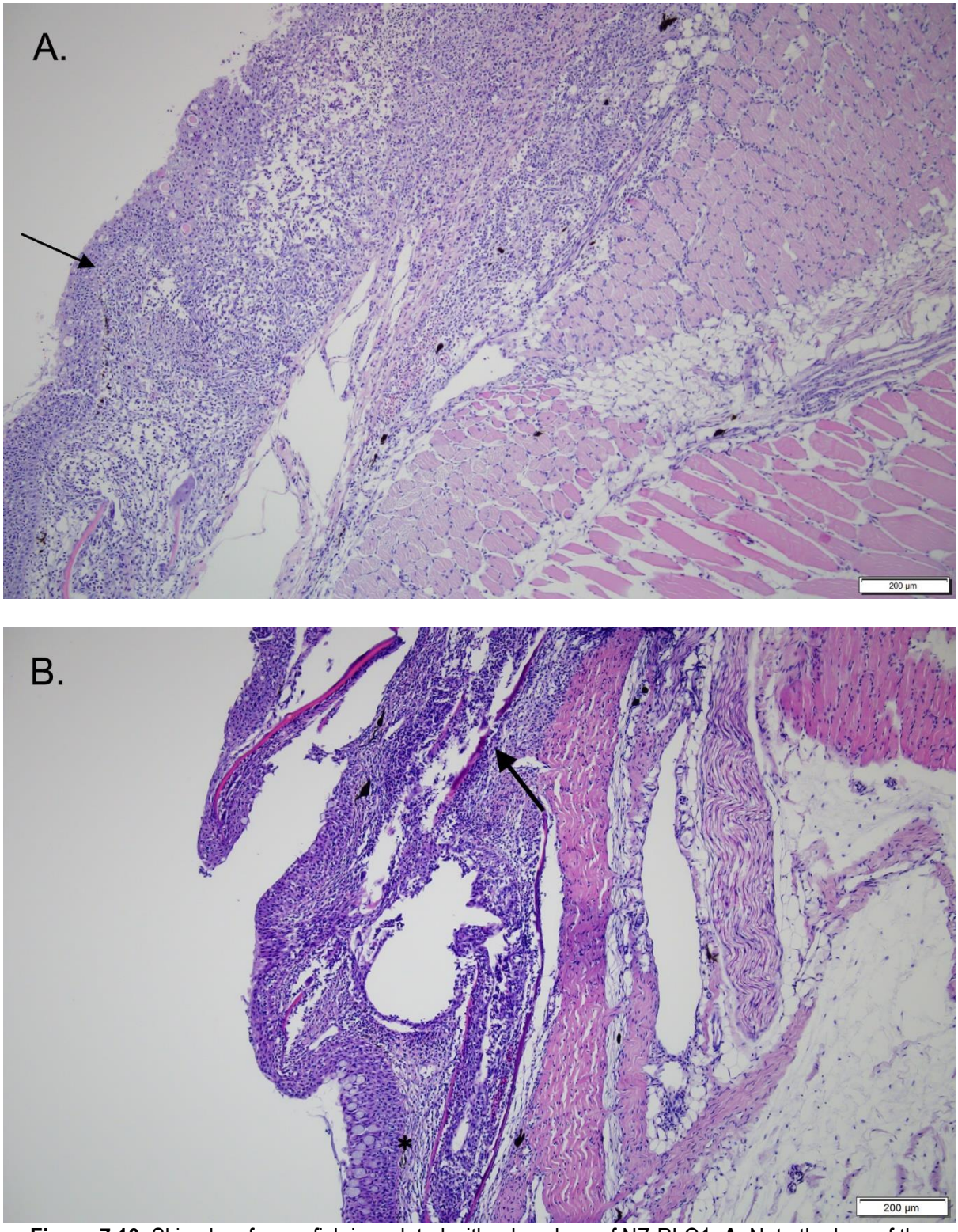


Figure 7.10. Skin ulcer from a fish inoculated with a low dose of NZ-RLO1. **A.** Note the loss of the basal membrane (arrow) and inflammation extending into the underlying dermis. H&E, 100 x magnification. **B**. Note the inflammation around the scale pockets (asterisk) as well as a dissolution of the scales (arrow). H&E, 100 x magnification.

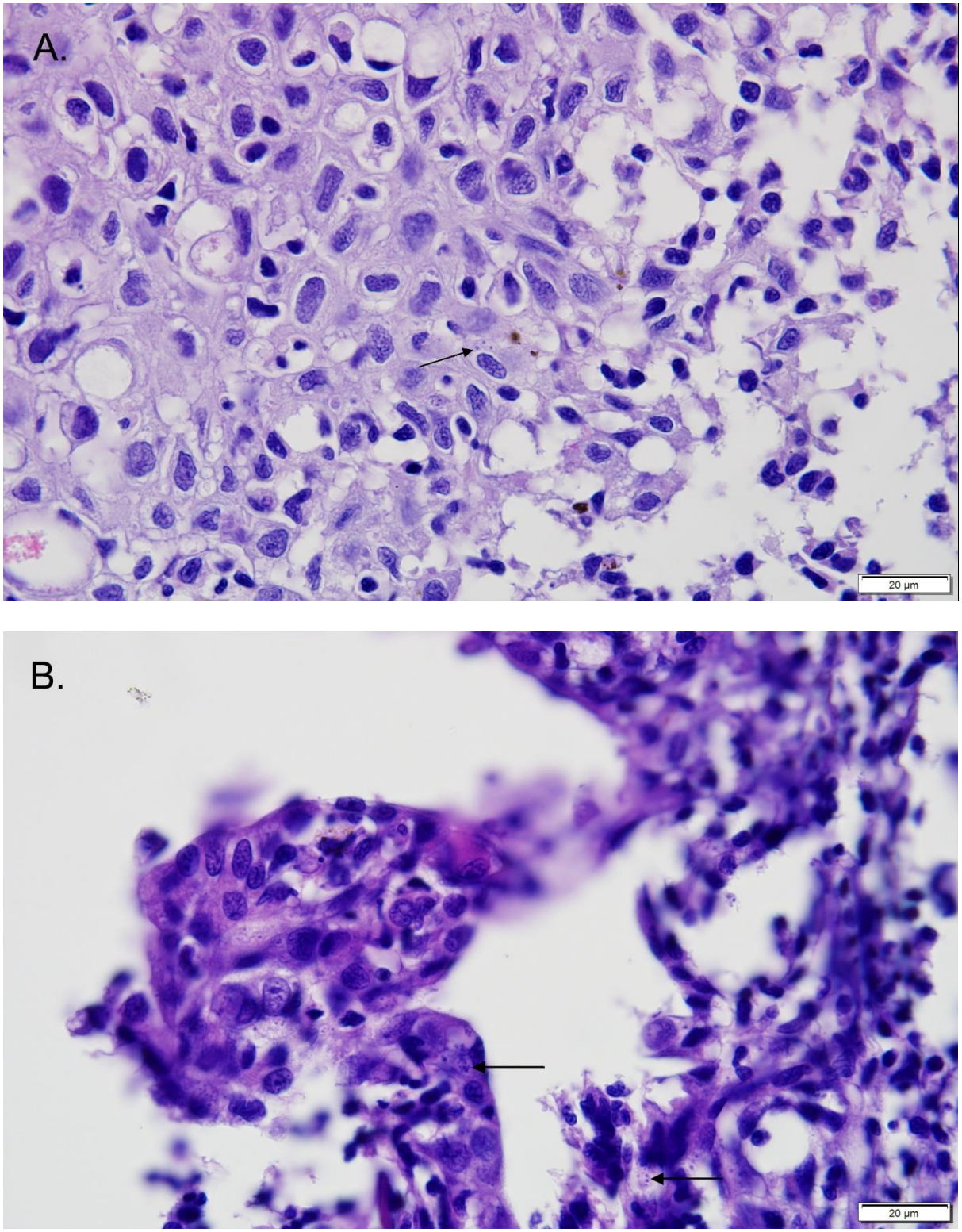
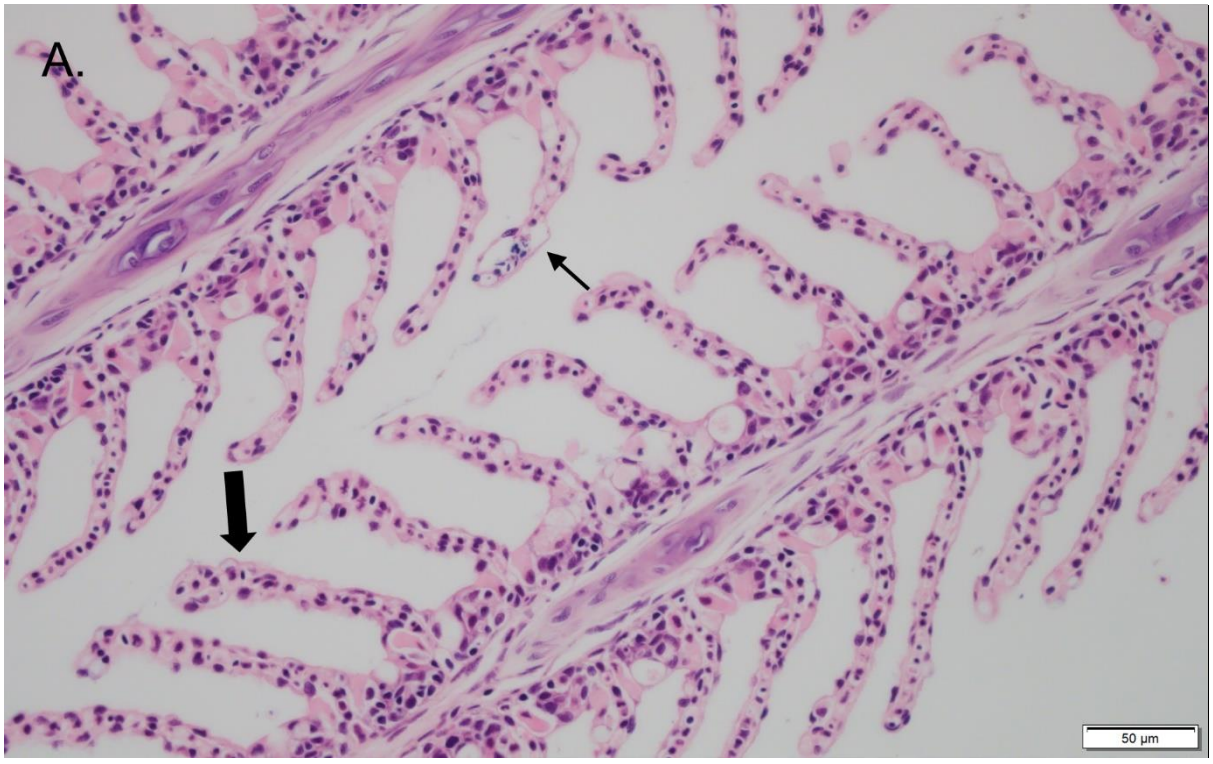


Figure 7.11. Skin ulcer from a fish inoculated with a low dose of NZ-RLO1. **A.** Note the presence of basophilic cocci within the cytoplasm of an epithelial cell (arrow). H&E, 1000 x magnification. **B.** Note the presence of cocci within the cytoplasm of inflammatory cells (arrows). H&E, 1000 x magnification.

Examination of the gills revealed epithelial lifting and hyperplasia resulting in focal lamellae fusion or clubbing of the filaments in fish inoculated with NZ-RLO1 and control

fish. Bacteria resembling NZ-RLO1 were occasionally observed within inflammatory cells in the primary and secondary lamellae from fish inoculated with all doses of NZ-RLO1 (Figures 7.12 A and B). The gills of two fish in the high dose group, two fish in the medium dose group, and three fish in the low dose group could not be critically evaluated due to post mortem autolysis.

Examination of sections of intestine of fish inoculated with NZ-RLO1 that died during the study revealed increased inflammatory cells in the adipose surrounding the intestine. In addition, there were mildly increased numbers of inflammatory cells visible within the mucosa of the intestine within some of these fish. Bacteria resembling NZ-RLO1 were occasionally observed within inflammatory cells in both of these areas (Figure 7.13).



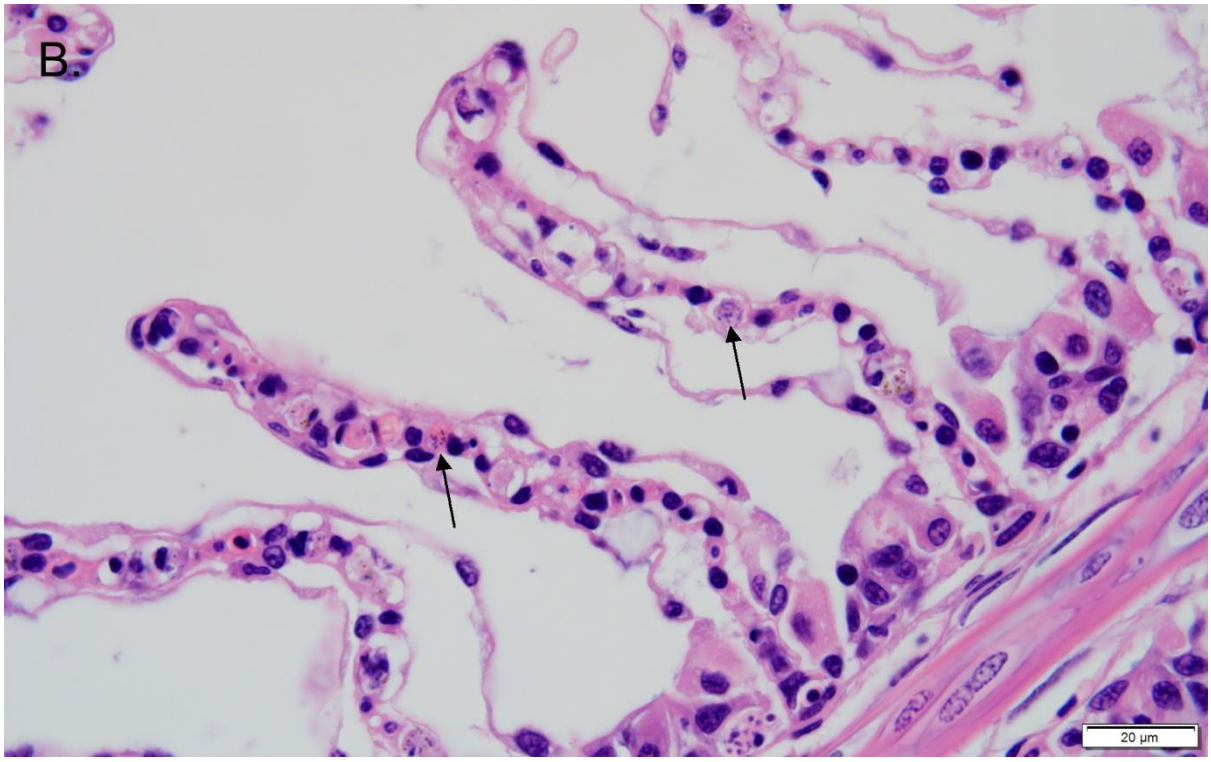


Figure 7.12. Gill taken from fish that died during the study after being inoculated with a high dose of NZ-RLO1. **A.** Note the epithelial lifting (thin arrow) and epithelial hyperplasia (thick arrow). H&E, 400 x magnification. **B**. Note the presence of basophilic cocci within the cytoplasm of inflammatory cells in the secondary lamellae (arrows) and the lifting of the epithelium from the pillar cells. H&E, 1000 x magnification.

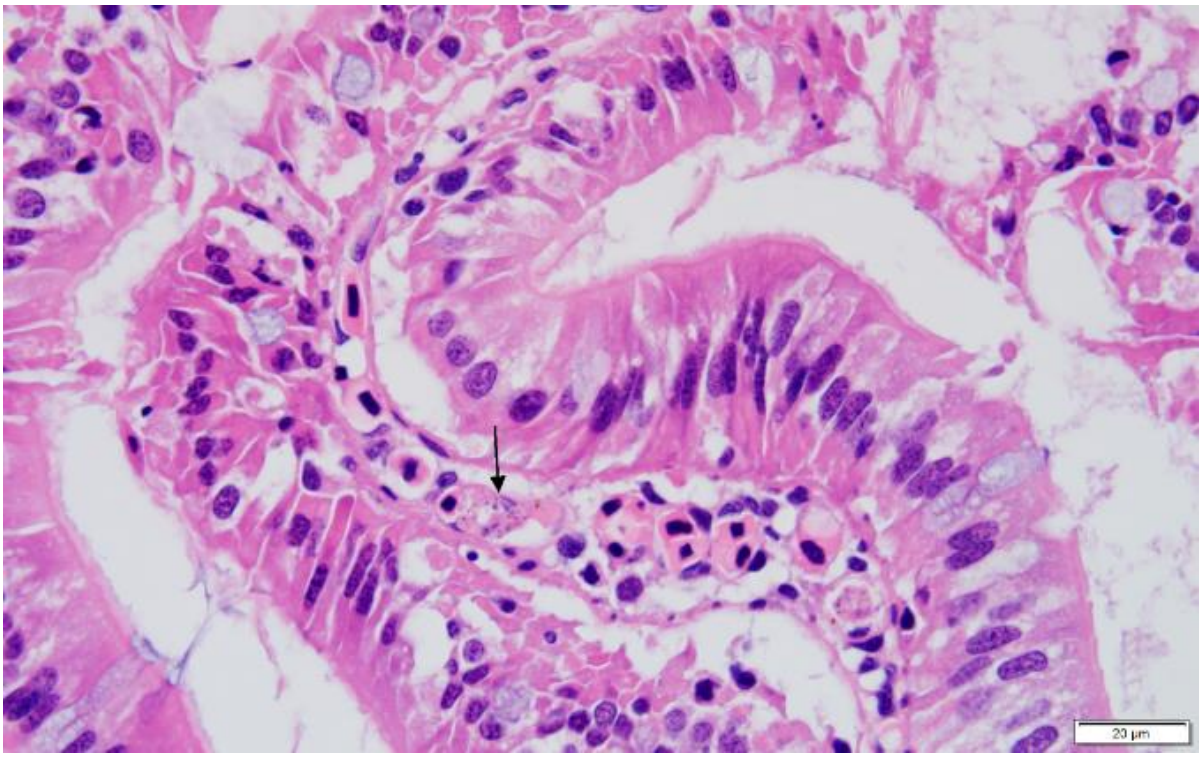


Figure 7.13. Section from around the mid-point of the intestine from a fish that died during the study after being inoculated with a medium dose of NZ-RLO1. Note the presence of basophilic cocci within the cytoplasm of inflammatory cells. H&E, 1000 x magnification.

Table 7.2. Numbers of fish that developed each microscopic lesion within the study. Fish that died during the study and fish that survived 30 days after inoculation are represented separately. High dose indicates that fish were inoculated with a high dose of NZ-RLO1, Medium dose indicates that fish were inoculated with a medium dose of NZ-RLO1, Low dose indicates that fish were inoculated with a low dose of NZ-RLO1, Control no-i.p indicates that fish were not inoculated and Control i.p indicates that fish were inoculated with sterile media that did not contain NZ-RLO1. Numbers in the table represent the number of fish where the microscopic lesions were observed (black number) out of the number of fish analysed (red numbers).

	Fish that died during the study				Fish that survived the study				
Histopathology	**High dose**	**Medium dose**	**Low dose**	**Control no-i.p**	**High dose**	**Medium dose**	**Low dose**	**Control no-i.p**	**Control i.p**
Hepatocellular necrosis	3/4	2/3	4/5	0/2	0/2	5/9	8/25	0/28	1/30
Pancreatitis	3/4	1/3	3/5	0/2	0/2	0/9	1/25	0/28	0/30
Steatitis	3/4	2/3	4/5	0/2	0/2	1/9	4/25	0/28	0/30
Renal necrosis	3/4	1/3	4/5	0/2	0/2	0/9	3/25	0/28	0/30
Splenic red blood cell depletion	3/4	1/3	2/5	0/2	0/2	1/9	4/25	0/28	0/30
Eosinophilic fibrillary material in spleen	4/4	2/3	3/5	0/2	0/2	0/9	1/25	0/28	0/30
Splenic necrosis and inflammation	3/4	1/3	5/5	0/2	0/2	0/9	0/25	0/28	0/30
Myocarditis	3/4	1/3	3/5	0/2	0/2	0/9	0/25	0/28	0/30
Pericarditis	1/4	1/3	0/5	0/2	0/2	0/9	3/25	0/28	0/30
Gill hyperplasia	2/2	1/2	2/3	0/2	1/2	5/8	11/24	13/28	8/29
Epithelial lifting	2/2	1/2	1/3	0/2	0/2	1/6	14/24	1/28	2/29
Congestion of the brain	2/4	3/3	2/5	1/2	0/2	0/9	5/25	0/28	2/30
Intestinal inflammation	2/4	1/3	2/5	0/2	0/2	0/9	0/25	0/28	0/30

Fish were evaluated using histology for the presence of bacteria resembling NZ-RLO1. Of fish that were inoculated with NZ-RLO1 that died during the study (*n* = 12), NZ-RLO1 was most commonly observed in the pancreas (eight of 12 fish, 67%), gills (five of eight fish, 63%) and the liver and spleen (seven of 12 fish, 58%). Of fish that survived the study, NZ-RLO1 was only observed in the gill (three of 34, 9%). A summary of all organs where NZ-RLO1-like bodies were observed histologically as a total from all inoculation groups in fish that survived and fish that died during the study is seen in figure 7.14.

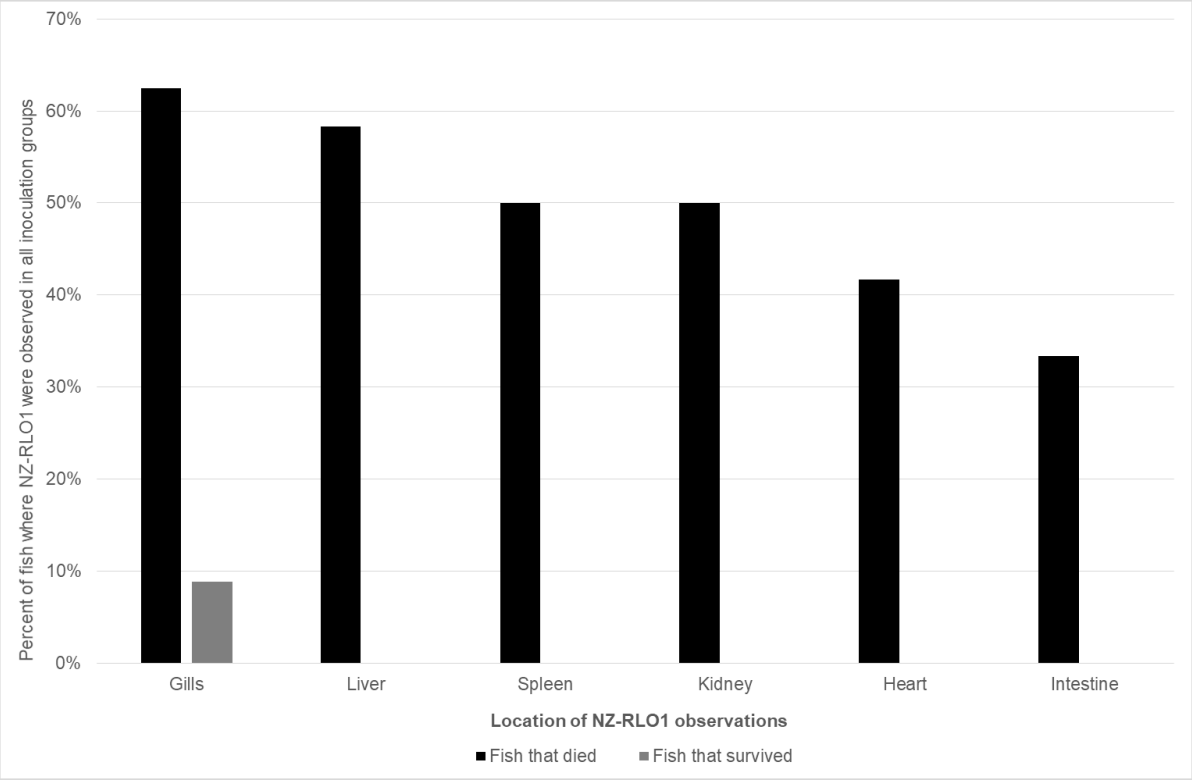


Figure 7.14. Location where NZ-RLO1 like bodies were most commonly observed using histology in all fish inoculated with NZ-RLO1. Fish that died during the study and fish that survived the study are represented separately.

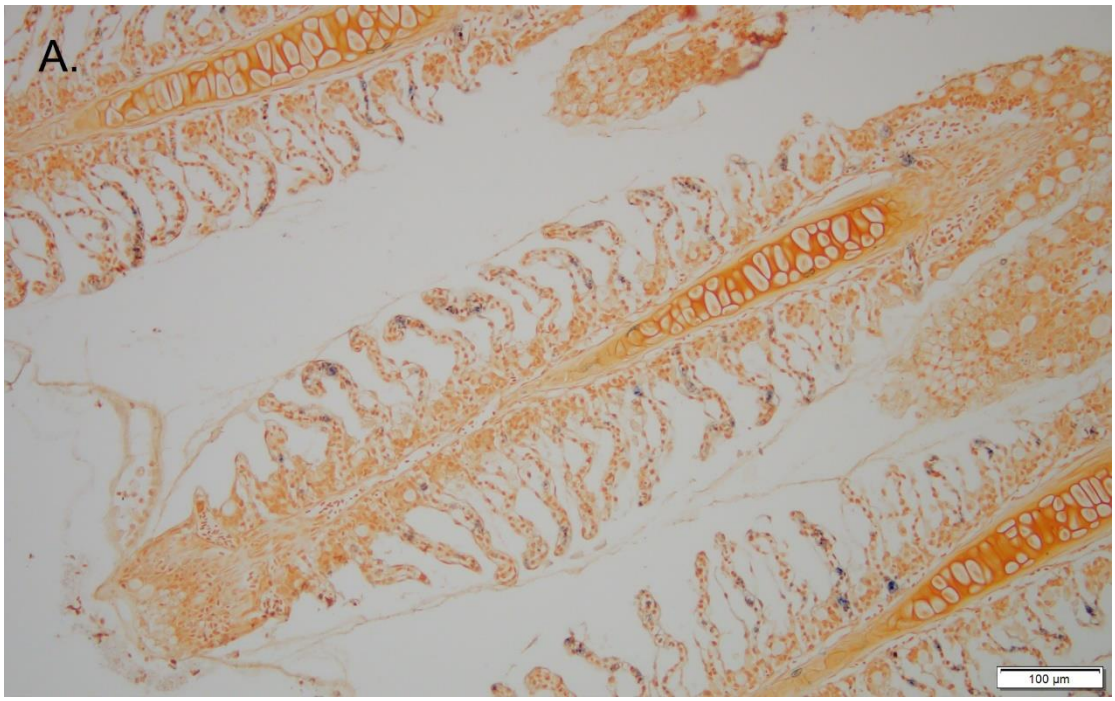
7.3.4 *In-situ* hybridization

In-situ hybridization was performed on sections from two fish inoculated with a high dose of NZ-RLO1 that died during the study and two fish from the low dose group that survived the study but showed mild signs of NZ-RLO1 infection using histology. Within the fish inoculated with a high dose of NZ-RLO1, hybridization to the ISH probe was visible in the spleen, liver, kidney, adipose surrounding the intestine and pyloric caeca,

pancreatic cells, gills (Figure 7.15 A), heart (Figure 7.15 B), and intestine. Probe hybridization was not observed in the brain of any fish analysed that were inoculated with a high dose of NZ-RLO1 and died during the study. When probe hybridization occurred, it was identified within areas of necrosis and inflammation. Probe hybridization was not observed in any sample from either fish that had been inoculated with a low dose of NZ-RLO1 that survived the study. Positive signals were observed in the positive control tissue and were not observed from the negative control tissue.

7.3.5 Recovery of NZ-RLO1 from tissue in cell culture and on agar

New Zealand rickettsia-like organism 1 was recovered from tissue using cell culture. Growth of NZ-RLO1 was identified by the observation of cytopathic effect (CPE). Growth indicated by CPE was observed from three of the six fish inoculated with a high dose, two of the 10 fish inoculated with a medium dose and four of the 12 fish inoculated with a low dose of NZ-RLO1. The CPE presented as a rounding of the cells followed by plaque formation, consistent with NZ-RLO CPE (Chapter 6). Amplification of NZ-RLO1 in cell culture was confirmed by qPCR (Table 7.3). Fish from the control groups displayed no CPE in cell culture after 28 days.



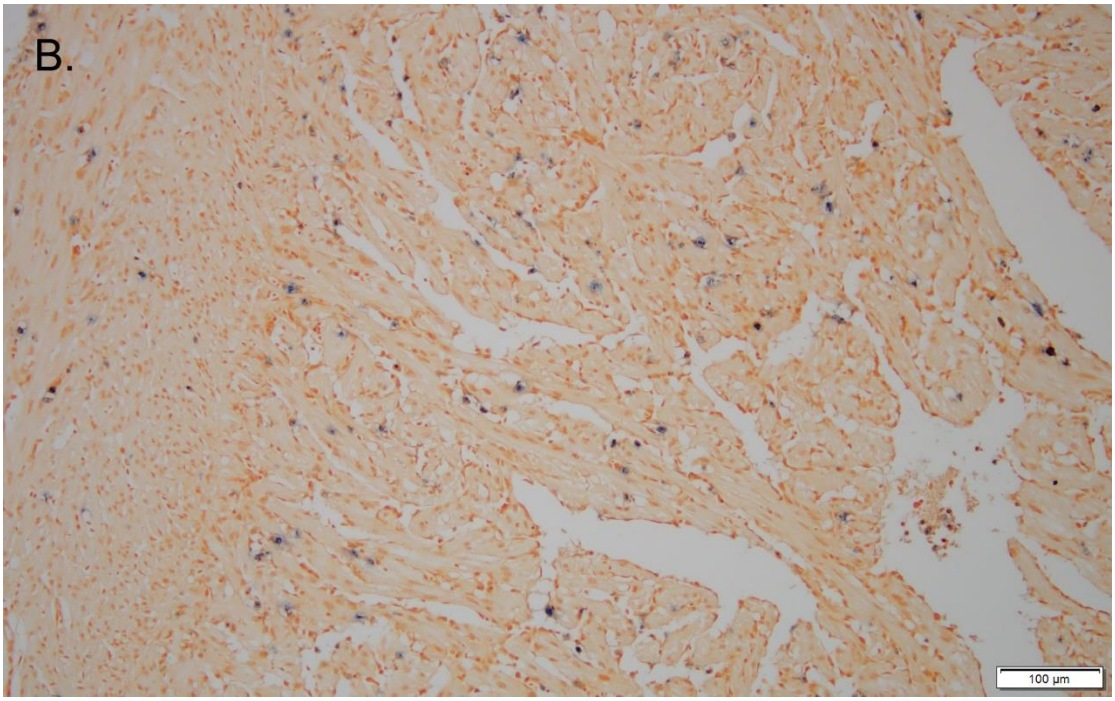


Figure 7.15. Gill (**A**) and heart (**B**) from fish that died during the study after being inoculated with a high dose of NZ-RLO1 and evaluated using *in-situ* hybridization. Note the probe hybridization, displayed as a dark blue signal on a background of brown staining. 200 x magnification.

New Zealand rickettsia-like organism 1 was recovered on CHAB + 5%. This growth was recovered from all three samples tested from fish inoculated with a high dose of NZ-RLO1, the single sample taken from a fish inoculated with a medium dose of NZ-RLO1, and two of the three samples taken from fish inoculated with a low dose of NZ-RLO1 that died during the study. Bacterial colonies of NZ-RLO1 were small, matt white with rough margins of variable sizes (Figure 7.16). Multiple colonies that were morphologically similar to NZ-RLO1 of various sizes from each agar plate were confirmed as NZ-RLO1 by qPCR.

Table 7.3. NZ-RLO1 qPCR cycle threshold (Ct) values and equivalent $TCID_{50}$ of cell-cultures with CPE to confirm growth of NZ-RLO1. P0= original tissue homogenate, P1= growth in cell culture material after 14 days incubation.

Inoculation dose	P0 Ct	$TCID_{50}$ equivalent	P1 Ct	$TCID_{50}$ equivalent
High	15.67	10^5	18.02	10^5
High	37.54	10	24.73	10^3
Medium	33.10	10^4	17.02	10^5
Low	24.63	10^3	21.74	10^4
Low	20.97	10^4	20.41	10^4

Table 7.4. NZ-RLO1 qPCR cycle threshold (Ct) values of all tissues at all treatments; high dose (H; *n* = 6) indicates that fish were inoculated with a high dose of NZ-RLO1, medium dose (M; *n* = 12) indicates that fish were inoculated with a medium dose of NZ-RLO1, and low dose (L; *n* = 30) indicates that fish were inoculated with a low dose of NZ-RLO1.

Infectious dose	Ct value				
	Kidney	Liver	Spleen	Digestive tract	Skin ulcer
H*[l]	*16.51*	*13.26^*	*15.52*	20.10	NA
H*[l]	*17.70*	*13.75^*	*19.02*	19.72	NA
H*	19.10	20.70	19.80	27.23	NA
H*	*30.04^*	*N*	*35.44*	35.31	26
H	N	N	N	N	NA
H	36.56	N	N	N	NA
M*	*19.67*	*15.15*	*19.34*	23.58	27.56
M*	*N^*	*N*	*N*	N	NA
M*	19.58	24.94	25.56	32.36	19.82
M	N	N	N	N	NA
M	31.44	N	38.51	N	NA
M	N	N	N	N	NA
M	35.69	N	N	36.06	NA
M	27.59	N	37.71	36.39	NA
M	35.49	N	N	N	NA
M	N	N	N	N	NA
M	N	N	38.76	N	27.42
M	N	N	N	N	NA
L*	22.06	23.80	23.28	25.31	NA
L*	35.54	28.4	37.14	19.41	NA
L*	17.37	14.64	14.68	18.33	NA
L*	*18.78*	*17.89^*	*19.44*	23.92	NA
L*	21.64	20.39	23.36	25.07	NA
L	*N*	*N*	*N*	N	NA
L	36.19	N	N	N	37.16
L	N	N	N	N	NA
L	N	N	N	N	NA
L	34.81	36.56	35.25	N	NA
L	*N*	*N*	*N*	N	NA
L	35.41	N	N	N	NA
L	*31.42^*	*36.13*	*N*	N	NA
L	33.69	36.19	N	37.63	26.59
L	33.51	37.57	33.96	32.94	22.65
L	33.17	39.37	N	N	NA
L	35.58	N	N	N	NA

L	36.23	N	N	35.15	NA
L	N	N	N	N	NA
L	N	N	N	N	31.61
L	N	N	N	N	NA
L[I]	N	N	N	36.37	NA
L	34.03	N	N	N	NA
L	N	37.11	N	N	NA
L	36.15	N	N	N	NA
L[I]	33.26	34.95	N	35.45	27.23
L	N	N	35.87	N	NA
L	34.86	N	N	N	27.53
L	39.59	N	N	38.28	NA
L	37.61	37.62	N	36.41	NA

Red cells = positive growth in cell culture; Blue cells = negative growth in cell culture; no colour = sample not tested for cell culture; N = negative qPCR result; NA = no lesion present. * = fish that died during the study, ^ = organs that were positive for NZ-RLO1 on agar, *italicised* = sample subjected to agar culture.[I] = fish tissues that were subjected to *in-situ* hybridization.

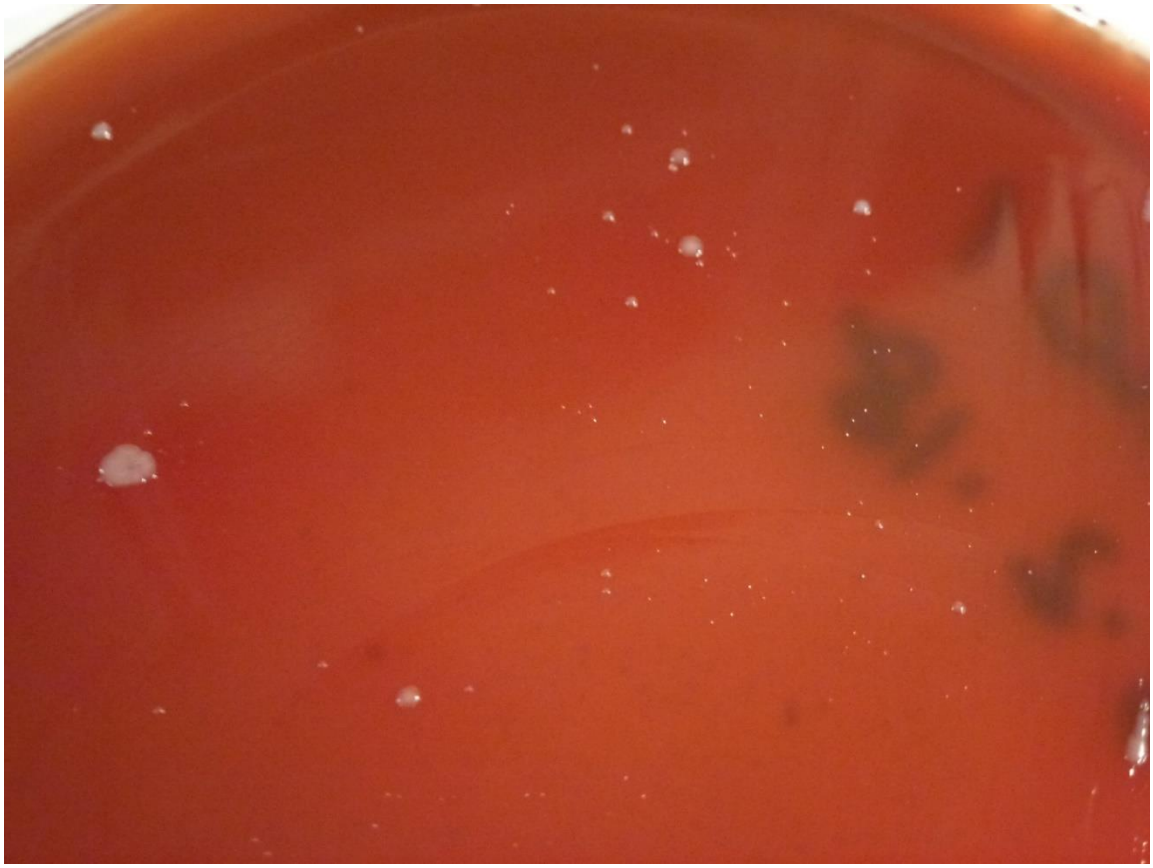


Figure 7.16. Bacterial colonies of New Zealand rickettsia-like organism 1 observed on cysteine heart agar supplemented with 5% sheep's blood agar following 28 days growth. Note the variable sized colonies.

7.3.6 Evaluation of tissues using qPCR

Tissue samples from fish analysed at the beginning of the study showed no amplification when using the generic NZ-RLO qPCR. All DNA recovered from tissue samples were shown to be appropriate for PCR by amplification in the 18S rRNA gene.

By using the NZ-RLO1 specific qPCR, DNA was amplified from at least one tissue in five of six fish (83%) in the high dose group, seven of 12 fish (58%) in the medium dose group and 24 of 30 (80%) fish in the low dose group. Skin ulcers from fish inoculated with NZ-RLO1 (n = 12) contained NZ-RLO1 DNA (Table 7.4). The NZ-RLO1 qPCR did not amplify DNA from any tissues of the 60 control fish.

The specific NZ-RLO1 qPCR revealed the concentration of NZ-RLO1 DNA were highest in the liver for the high and medium dose groups. The difference in the Ct values between the different tissue types in the inoculation groups; high, medium, or low, were not significant (χ^2 = 1.83, p = 0.61 and χ^2 = 3.45, p = 0.33, respectively). When comparing the Ct values between the tissue types in the low dose group, a significant difference was seen between the kidney and the spleen ($p < 0.01$) with spleen showing a significantly higher abundance of NZ-RLO1 DNA (Figure 7.17).

When comparing the average Ct values derived from the tissues tested between each of the inoculation groups, no significant difference was seen (χ^2 = 5.51, p = 0.06).

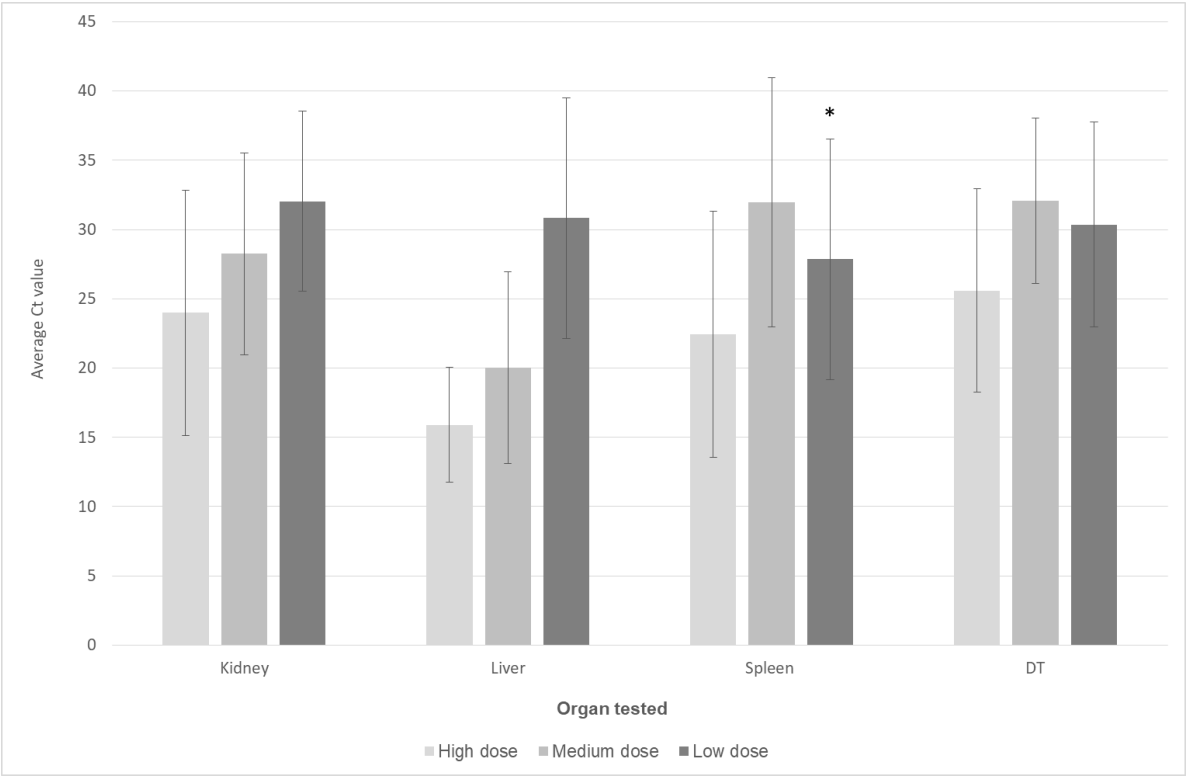


Figure 7.17. Average Ct values derived from the specific NZ-RLO1 qPCR detected in each organ within each of the experimental groups inoculated with NZ-RLO1. High dose indicates that fish were inoculated with a high dose of NZ-RLO1, Medium dose indicates that fish were inoculated with a medium dose of NZ-RLO1, and Low dose indicates that fish were inoculated with a low dose of NZ-RLO1. * = significantly different ($p < 0.01$) to low dose, kidney.

During the study, twelve fish inoculated with NZ-RLO1 died from all inoculation doses. Of these twelve fish, nine had an average Ct value of ≤ 30 from all internal organs, had bacteria that resembled NZ-RLO1 present in the blood smear and lesions histologically suggestive of NZ-RLO1 infection. Two of twelve fish had detectible NZ-RLO1 DNA but did not have bacteria resembling NZ-RLO1 present in the blood smear or histological lesions suggestive of NZ-RLO1 infection. One of these twelve fish did not have detectable NZ-RLO1 DNA, bacteria resembling NZ-RLO1 in the blood smear, or histological lesions suggestive of NZ-RLO1 infection. Of the 36 fish that survived the study and were inoculated with NZ-RLO1, none had a Ct < 30 from the internal organs using qPCR. Ten of these fish had histological lesions suggestive of NZ-RLO1 infection and two of these fish had organisms resembling NZ-RLO1 on a blood smear. Comparing the average NZ-RLO1 DNA Ct values from fish in each inoculation dose with the cumulative mortalities showed that when a higher level of NZ-RLO1 DNA was present higher cumulative mortality rates were observed. (Figure 7.18).

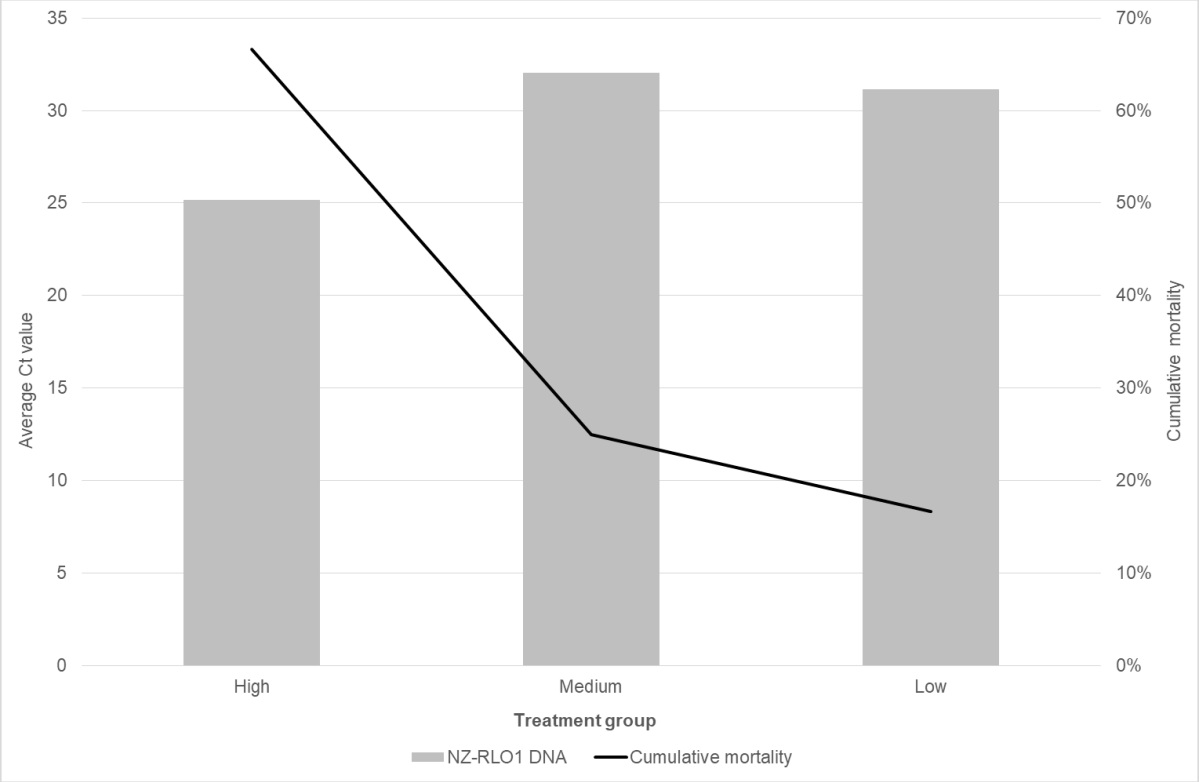


Figure 7.18. Summary of the average Ct values derived from the specific NZ-RLO1 qPCR from all internal organs of fish inoculated with either a high, medium, or low dose of NZ-RLO1 with the cumulative mortalities for each inoculation dose. High dose indicates that fish were inoculated with a high dose of NZ-RLO1, Medium dose indicates that fish were inoculated with a medium dose of NZ-RLO1, and Low dose indicates that fish were inoculated with a low dose of NZ-RLO1.

7.4 Discussion

Elevated mortalities of Chinook salmon at farmed sites in the Marlborough Sounds have occurred since 2012 (Norman et al., 2013). Two strains of NZ-RLO bacteria have been identified in fish from the summer mortalities (Chapter 2; Chapter 4) with the exact relationship between NZ-RLO strains and mortalities remaining unknown. To further investigate this relationship, the pathogenicity of NZ-RLO2 was evaluated and inoculated fish were shown to have high mortalities and disease (Chapter 6). To understand if NZ-RLO1 could also cause mortalities and disease in fish, this bacteria was injected intraperitoneally at three different doses high, medium, or low. The results of this study show NZ-RLO1 can cause death and disease in salmon and therefore could contribute to summer mortalities.

However, in the present study inoculation with NZ-RLO1 did not result in a significantly increased rate of mortality when compared to the controls. This may indicate that NZ-

RLO1 is not highly pathogenic to Chinook salmon. The estimated mortality rates that the sample sizes were based on for the present study (70%, 50%, or 30% for high, medium, or low dose) were higher than the actual mortality rates (63%, 25% or 17% for high, medium or low dose) leading to an assumed reduced power to reliably detect statistically significant differences between inoculated and control fish. To obtain a statistically conclusive result on this apparent low pathogenicity, an increased sample size would be required. An alternative hypothesis as to why NZ-RLO1 may not have not caused the expected mortality rates in the present study is due to passaging of NZ-RLO1. New Zealand rickettsia-like organism 1 had been passaged nine times in cell culture prior to amplification for the study. Passaging may have reduced the virulence of NZ-RLO1 which is common with pathogens serially re-amplified in the laboratory (Smith, 1988).

Although significantly higher mortalities were not observed in fish inoculated with NZ-RLO1 compared to the controls, a description of the clinical signs of disease occurring in fish inoculated with NZ-RLO1 were possible and similarities with fish analysed from summer mortalities were seen. A proportion of fish from summer mortalities have been observed to have petechiae on the ventral surface, petechiae in the internal fat, reddening of the intestine, pale livers, and skin ulcers. Fish inoculated with NZ-RLO1 that died during the study showed consistent pathology that included the presence of intra-cellular organisms found abundantly in inflammatory cells on blood smear, petechiae on the external ventral body surface and at the base of the fins, liver lesions and pale livers, petechiae on the internal adipose, splenic lesions, reddening of intestines, and myocarditis. These lesions were suggestive of a bacterial septicaemia, although some of these findings were irregularly observed in the fish. No control fish showed signs of disease by gross pathology or histopathology. As control fish did not show signs of disease but the fish inoculated with NZ-RLO1 did, this suggests that NZ-RLO1 was likely to be the cause of the observed lesions. Therefore, although NZ-RLO1 did not cause significant mortalities, it was associated with the development of disease within the inoculated fish, strongly suggesting that NZ-RLO1 can cause disease and mortalities in Chinook salmon.

Bacteria were visible within areas of inflammation and necrosis. As histological examination is unable to identify bacteria to species level, *in-situ* hybridization (ISH),

was used to confirm that the bacteria detected by histology were NZ-RLOs. A generic ISH probe was used in the present study, which can detect all strains of NZ-RLO as well as *P. salmonis*. As qPCR confirmed the presence of NZ-RLO1 within the tissues evaluated by ISH, no NZ-RLO DNA was present when screening fish at the beginning of the study, and *P. salmonis* has not been detected in New Zealand, it was considered unlikely that the ISH probe was identifying another strain of NZ-RLO. The demonstration of NZ-RLO1 in areas of necrosis and inflammation using ISH provides additional evidence that the bacteria was the cause of the observed tissue damage. Alternatively, it remains possible that NZ-RLO1 was present in areas of necrosis and inflammation without causing disease. For example, if NZ-RLO1 is able to invade inflammatory cells, areas with higher numbers of inflammatory cells could contain more bacteria without the bacteria being the cause of the disease. However, as higher inflammatory cells were not observed within the control fish that were under the same conditions this was considered less likely. Although only two fish were analysed that were inoculated with a low dose of NZ-RLO1, this did suggest the likely low sensitivity of ISH. This means that tissues where low levels of NZ-RLO1 are present are unlikely to show hybridization, a finding that was hypothesised as a reason for seeing no hybridization in the tissues evaluated from the summer mortality (Chapter 3). While the ISH probe was useful in the present study, it was not able to differentiate between NZ-RLO1 and NZ-RLO2. Therefore, the development of probes specific for NZ-RLO1 and NZ-RLO2 may be valuable to investigate naturally infected fish where a co-infection of the two NZ-RLO strains is possible.

Fish in groups inoculated with a high or medium dose of NZ-RLO1 were observed to be feeding less than those in the lower dose and control groups. Supporting this subjective observation was that there was a statistically significant difference between the length and weight of fish inoculated with NZ-RLO1 compared to the control groups. Reduced feeding means fish may become further immunocompromised. An immunocompromised fish will be less able to defend against a bacterial infection leading to increased replication of NZ-RLO1 and a heavy infection that may cause death. Additionally, fish of ill health are more likely to become co-infected with other pathogens within the environment which may further supress the immune system leading to the inability of the immune system to protect against infection from NZ-RLO1 or a secondary infection resulting in death. Moreover, fish that are of ill health are less

able to cope with other stressors such as increased seawater temperature. Increased seawater temperature may impact the mucosal barrier of the skin (Jensen et al. 2015), therefore compromising the first line of defence against pathogens. Additionally, this increase in seawater temperature may provide a more preferable temperature for bacterial replication, leading to an increased likelihood of the fish succumbing to a bacterial infection.

The presence of NZ-RLO1 in blood smears from fish inoculated with a high, medium, or low dose of NZ-RLO1 (100%, 42%, or 17% respectively) suggested haematogenous dissemination as a mechanism for distribution within the body. The high prevalence of NZ-RLO1 in the blood smears is in contrast to what was observed in fish inoculated with NZ-RLO2 in which the bacteria were observed at a lower frequency of fish inoculated with a high, medium, or low dose (33%, 17%, or 20% respectively; Chapter 6). This indicates NZ-RLO1 readily circulates via blood which may result in a systemic bacterial infection. If NZ-RLO1 is more capable at traveling through blood than NZ-RLO2, this may suggest it is able to cause widespread infection of multiple tissues in the body, regardless of location at which the bacteria initially infects the fish.

One of the most common lesions observed in fish that died after inoculation with NZ-RLO1 was petechiae in the external ventral body surface including at the base of the fins. Petechial haemorrhage is a common sign of disease in fish and can be caused by many different bacterial or viral pathogens. Petechial haemorrhage was only seen in fish that died during the study after inoculation with NZ-RLO1. Petechiae of the ventral surface was not seen in fish inoculated with NZ-RLO2. The reason for the difference in clinical signs between NZ-RLO1 and NZ-RLO2 is uncertain but could be due to a difference in virulence mechanisms. Virulence mechanisms vary with different strains of *P. salmonis* (Lagos et al., 2017) and this is likely to be true for NZ-RLO. Further genetic analysis assessing the virulence genes present between these two strains will provide a better understanding. Alternatively, the difference between the two strains of bacteria may be related to the abundance of NZ-RLO within the fish. In fish that died during the present study, NZ-RLO1 concentrations were up to 3-fold higher than in fish that died after being inoculated with NZ-RLO2 (Chapter 6). An

increased level of bacteria, such as seen in the fish inoculated with NZ-RLO1, may be required to cause this clinical sign of disease.

Petechial haemorrhage within the abdominal adipose was commonly observed in fish inoculated with NZ-RLO1, NZ-RLO2 (Chapter 6), and those evaluated during the summer mortalities (Chapter 2). Petechial haemorrhage within the abdominal adipose can also be found as a clinical sign of other diseases for example, viral haemorrhagic septicaemia, infectious haematopoietic necrosis virus, or *Moritella viscosa* and therefore cannot be considered a specific clinical sign for infection with NZ-RLO. Histological examination of the infected areas in fish inoculated with NZ-RLO1 revealed inflammation and necrosis within the adipose surrounding the pancreas and the exocrine pancreatic tissue. Within these areas of inflammation, bacteria confirmed as NZ-RLO1 by ISH were often visible. In fish evaluated during the summer mortalities, pancreatitis was not observed in any fish and mild steatitis was observed in two fish. Mild steatitis was consistent with a low level infection of NZ-RLO as demonstrated in the present study and Chapter 6. The difference between the fish from the summer mortalities and the laboratory inoculated fish may be due to a difference in infection level or inoculation route. Fish inoculated with NZ-RLO1 or NZ-RLO2 that died, frequently had a higher infection level compared to fish analysed in the summer mortalities. It is also possible that steatitis and pancreatitis may have been caused by the intraperitoneal injection in the present study, however as fish inoculated via the same method with sterile media did not have these lesions this is considered less likely. No lesions were observed by histological examination from the control fish that displayed minor petechial haemorrhage within the abdominal adipose and the aetiology remains unknown.

Gross pathology and histological examination revealed splenic lesions in fish inoculated with NZ-RLO1. Splenomegaly was seen in fish that died after being inoculated with NZ-RLO1 as well as a small number of fish that were inoculated and survived. Depletion of the haemopoetic tissue, eosinophilic fibrillar material as well as necrosis and inflammation was observed by histological examination in salmon that displayed splenomegaly. Depletion of haemopoetic tissue is consistent with a bacterial septicaemia causing anaemia and the increased inflammatory cells and eosinophilic fibrillar material is likely to have contributed to the gross observation of enlarged spleens. Pale kidneys and renal necrosis were only seen in fish that were inoculated

with NZ-RLO1 that died. Changes in the kidney were not seen in fish that were inoculated with NZ-RLO1 and survived, suggesting kidney pathology may only occur as a terminal event in disease due to NZ-RLO1.

Red circular lesions with a cream margin were commonly observed in the liver of fish inoculated with NZ-RLO1 that died. Histological examination of the lesions showed widespread hepatocellular necrosis with high numbers of intra-cytoplasmic NZ-RLO1 and using qPCR this tissue had a high abundance of NZ-RLO1 DNA. The histology and qPCR results suggest gross liver lesions may be due to a heavy infection with NZ-RLO1. Similar lesions in fish infected with *P. salmonis* are associated with a heavy infection (Cvitanich et al., 1991). Gross liver lesions were not observed in fish inoculated with NZ-RLO2 (Chapter 6), suggesting the cause may be specific to certain strains of NZ-RLO. Gross liver lesions of various descriptions can be caused by other pathogens, for example *Renibacterium salmoninarum* and *Vibrio* species and therefore should not be considered specific for NZ-RLO1 based on the gross pathology alone and further methods, such as histology or PCR, are required for confirmation. Gross liver lesions were not observed in fish evaluated during the summer mortalities as described in Chapter 2. However, gross liver lesions have been described in fish assessed on salmon farms in New Zealand during subsequent summer mortalities (New Zealand King Salmon, pers. comm.). If fish from the summer mortalities with gross liver lesions had been analysed by histology and qPCR, the detection of high numbers of NZ-RLO1 may have occurred which would have been consistent with this bacteria being the primary pathogen involved.

Results from the blood smear, histopathology, and qPCR varied between fish inoculated with NZ-RLO1 that died and those that survived. Of the fish that died, nine of 12 had NZ-RLO1 visible in the blood smear, showed histological lesions suggestive of NZ-RLO1 infection and had NZ-RLO1 Ct values lower than 30. In contrast, three of the fish that died after inoculation with NZ-RLO1 only variably had NZ-RLO1 visible in blood smears, histological lesions suggestive of NZ-RLO1 and all had NZ-RLO1 Ct values higher than 30. The results of these three fish were similar to those observed in inoculated fish that survived to the end of the study. Due to the results of the three tests not confirming NZ-RLO1 infection, this may indicate that the three fish that died during the study and were inoculated with NZ-RLO1, may not have died primarily due

to NZ-RLO1 infection. Death may have instead been due to the ill health of the fish as a consequence of an NZ-RLO1 infection making them more susceptible to death from other factors, for example a stressful laboratory environment. Samples evaluated from the summer mortalities using qPCR showed the presence of NZ-RLO1 DNA at Ct values greater than 30. Histological examination of fish from the summer mortalities showed seven of 10 fish had histopathology suggestive of an NZ-RLO infection. Comparison of results from the histology and qPCR from the summer mortalities and the present study suggest that most of the fish that died during the summer mortalities had lower concentrations of NZ-RLO1 DNA and therefore, these fish may not have died primarily due to NZ-RLO1 infection. Low level infection with NZ-RLO1 potentially made fish from the summer mortalities more susceptible to death from other factors such as infection from other pathogens or environmental changes. Alternatively, as fish evaluated from the summer mortalities were tested after peak mortality (Chapter 2), the fish analysed may be fish that survived the outbreak and were recovering, or those that were not susceptible to NZ-RLO infection.

Skin ulcers were observed in fish inoculated with NZ-RLO1 from all treatment groups from 20 days post inoculation. All skin ulcers evaluated contained NZ-RLO1 DNA suggesting NZ-RLO1 may have contributed to ulcer development. Alternatively, NZ-RLO1 may have been present in the skin ulcers as a secondary pathogen. Nevertheless, histological examination of skin ulcers revealed dermatitis and bacteria resembling NZ-RLO1 within inflammatory and epithelial cells suggesting NZ-RLO1 could be directly associated with skin ulcers and that the skin could be an important route of transmission for NZ-RLO1 in Chinook salmon. Additionally, skin ulcers observed in the present study resemble those described from fish infected with *P. salmonis* (Smith et al., 1999). In the present study, two control fish presented with scale loss and reddening of the underlying tissue. These areas did not contain NZ-RLO1 DNA. The scale loss and reddening were not grossly similar to the skin ulcers presented in fish inoculated with NZ-RLO1 and may be the result of the water being non-sterile with infection by other pathogens possible. Scale loss is a common sequallae of laboratory trials and while measures were taken to avoid this during handling, such as the use of rubber nets, it is often unavoidable. Bacterial culture from these areas may have helped determine the cause of this reddening however, this was outside the scope of this study.

Myocarditis was observed in the present study at a prevalence of 58% in fish that had died after inoculation with NZ-RLO1. This is higher than the 20% of fish that were found to have myocarditis during the initial investigation of summer mortalities (Chapter 2). Myocarditis is often seen due to a systemic bacterial infection (Rozas-Serri et al., 2017; Morrison et al., 2016). Fish evaluated in the present study had high infection levels of NZ-RLO1 which may account for the higher rate of myocarditis. This is in contrast to fish investigated during the summer mortalities that did not show consistent signs of bacterial septicaemia and therefore appeared to have a lower abundance of bacteria. Myocarditis was not seen in fish inoculated with NZ-RLO2 (Chapter 6) which may be due to the differences in virulence mechanisms between the two strains of NZ-RLO or due to the infection levels in the fish.

Reddening was observed in the intestine of fish inoculated with NZ-RLO1 in the present study and intestinal inflammation was observed within the mucosa or peri-intestinal adipose in 50% of all fish that were inoculated with NZ-RLO1 and died. Intestinal reddening and inflammation is a common observation in fish with a systemic bacterial infection (Ferguson, 2006). Intestinal reddening and inflammation was not observed in fish inoculated with NZ-RLO2 (Chapter 6). Intestinal reddening was observed in fish evaluated during the summer mortalities, although the diet of the fish was suggested to be the cause rather than the presence of a pathogen (Chapter 2). Intestinal inflammation was not observed histologically in the fish that died during summer mortalities (Chapter 2) although as post mortem autolysis of the intestine can affect the detection of inflammation (Ferguson, 2006) and fish from summer mortalities were samples 24 hours post mortem, this may explain the absence of intestinal inflammation in the summer mortalities. As the significance and the cause of the intestinal reddening observed in fish that died during the summer mortalities is uncertain, the presence of this change does not provide additional evidence that NZ-RLO1 was involved in the summer mortalities.

Gross pathology revealed white patches on the gills in fish inoculated with NZ-RLO1 that were not seen in any control fish. Histological examination of these areas of pallor on the gills revealed lamellae hyperplasia and an increase in inflammatory cells with NZ-RLO1 visible in one of the fish analysed. No other organisms were observed within

these areas of inflammation. The precise relationship between gill lesions and infection with NZ-RLO1 in Chinook salmon is currently unknown and further studies would be required to clarify this. Histological changes were visible in the gills of fish inoculated with NZ-RLO1 and control fish that revealed epithelial lifting and hyperplasia. This may be due to post mortem artefact (Strzyzewska, Szarek, & Babinska, 2016) or exposure to AQUI-S during the anaesthetising or euthanising process (Chance et al., 2018) and suggests gill pathology may not have been due to NZ-RLO1. Fish inoculated with NZ-RLO1 often exhibited intra-cytoplasmic bacteria within vacuolated inflammatory cells in the primary and secondary lamellae of the gills. These bacteria were confirmed as NZ-RLO1 using ISH and gills may be an important route of transmission in this disease. Gills were the only organ where NZ-RLO1 were observed in fish that survived which may suggest sampling of gills for qPCR testing in low level infections could be a useful diagnostic technique.

Growth of NZ-RLO1 on agar directly from infected tissue was demonstrated in the present study. Evaluation was only carried out on a small number of fish however, this demonstrated direct culture to be an alternative and more readily available technique than cell culture to recover NZ-RLO1 from infected tissues.

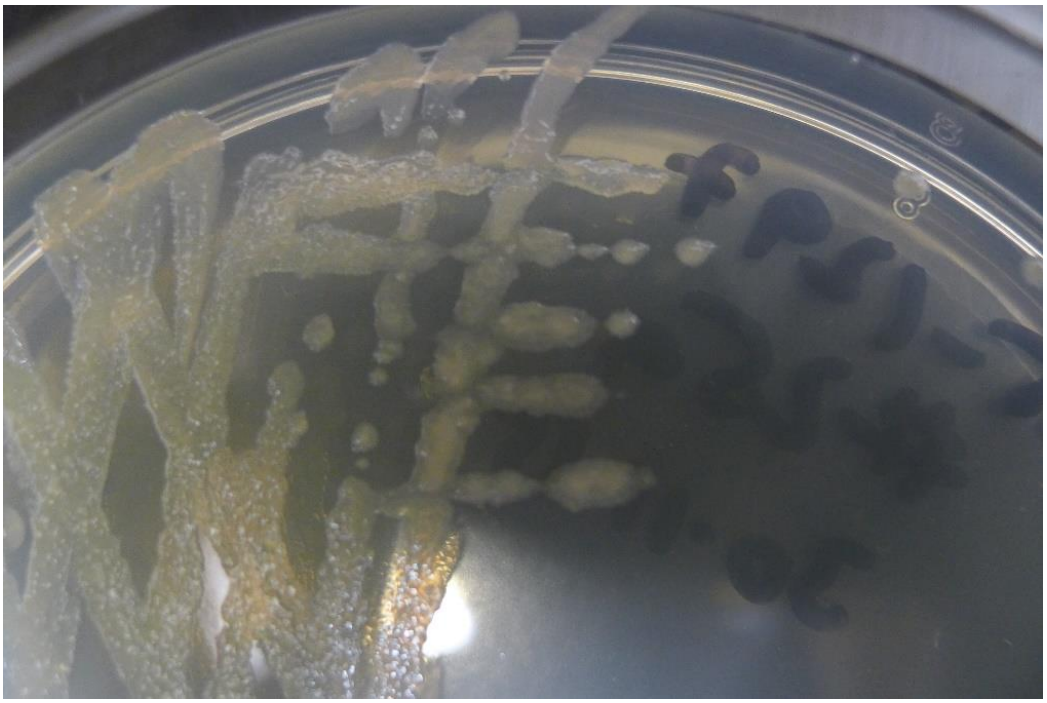
The Ct values from the specific NZ-RLO1 qPCR allowed the concentration of NZ-RLO1 DNA in the tissues to be analysed. Fish inoculated with a high or medium dose of NZ-RLO1 showed no significant difference in the concentration of NZ-RLO1 DNA between any of the organs tested. Fish inoculated with a low dose of NZ-RLO1 showed a significantly higher concentration of NZ-RLO1 DNA in the spleen compared to the kidney. Differences in the concentration of NZ-RLO1 DNA in the spleen and kidney of low dose fish may provide information on the progression of the disease. Possibly, a low level infection with NZ-RLO1 enables the fish immune system to manage disease progression by efficiently phagocytising the bacteria in the spleen leading to high concentrations of NZ-RLO1 DNA being detected in this organ. Phagocytosis of NZ-RLO1 in the spleen may limit the spread of NZ-RLO1 throughout the fish as well as preventing a bacterial septicaemia. Further studies, such as ISH on samples from a longitudinal study, would be required to explore this hypothesis.

Although studies between NZ-RLO1 and NZ-RLO2 cannot be directly compared, as they were not carried out concurrently, the present study suggests NZ-RLO1 may be less pathogenic than NZ-RLO2. During the present study, the initial inoculum of NZ-RLO1 was 1 log lower in concentration of viable cells than NZ-RLO2 as shown by the titration in cell lines. This was despite having similar levels of CPE in cell culture and Ct values as assessed by qPCR. This is another important consideration as to why these two studies cannot be compared. A reason for the discrepancy between the viability of the two NZ-RLO strains may be that NZ-RLO2 can survive for longer periods outside the host cells than NZ-RLO1 and that a reduced viability occurred during culture with a proportion of NZ-RLO1 bacteria dying once released from the host cell. Nevertheless, fish inoculated with NZ-RLO1 that died during the study showed high levels of NZ-RLO1 DNA and clear histological infection with NZ-RLO1. However, overall NZ-RLO1 caused fewer mortalities with a longer time to death with host tissues containing a higher concentration of bacterial DNA at death compared to NZ-RLO2. This suggests Chinook salmon may be able to survive a higher abundance of NZ-RLO1 than NZ-RLO2. For more conclusive results, a study carried out at the same time with the same concentration of viable cells on the same group of fish with both NZ-RLO1 and NZ-RLO2 would need to be conducted.

7.5 Conclusion

The present study demonstrated that NZ-RLO1 can cause mortalities and disease in Chinook salmon smolt when administered by i.p injection in a laboratory environment. No significant differences were seen in the mortality levels of fish inoculated with NZ-RLO1 compared to the controls, suggesting NZ-RLO1 was not highly pathogenic to Chinook salmon in this study. Fish inoculated with NZ-RLO1 that died demonstrated gross and histological changes suggestive of a bacterial septicaemia and changes in the liver were the most significant. The signs of disease in fish that were inoculated with NZ-RLO1 but survived the study showed similarities to those fish analysed during summer mortalities. Histology revealed NZ-RLO1 were often present in areas of necrosis and inflammation, and were recoverable in pure culture on cell culture and agar. These results suggest NZ-RLO1 can be pathogenic to Chinook salmon and therefore may have been a contributing factor in the summer mortalities in New Zealand.

Chapter 8 : Optimisation and validation of a PCR to detect viable *Tenacibaculum maritimum* in salmon skin tissue samples.



***Tenacibaculum maritimum* cultured on solid agar.**

This chapter has been published: Brosnahan CL, McDonald C, Georgiades E, Keeling SE, Jones BJ. (2019). Optimisation and validation of a PCR to detect viable *Tenacibaculum maritimum* in salmon skin tissue samples. *Journal of Microbiological Methods*, 159, 186-193.

8.1 Introduction

Tenacibaculum maritimum is a Gram-negative bacteria that is the causal agent of marine tenacibaculosis (also known as marine flexibacteriosis). Tenacibaculosis in finfish typically appears as the development of skin ulcers or erosions of the skin, fins, or within the mouth (Avendaño-Herrera et al., 2006). This disease is responsible for severe economic losses in marine aquaculture (Toranzo, Magarinos, & Romalde, 2005) with impacted species including Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), and sea bass (*Dicentrarchus labrax*) (Frelier, Elston, Loy, & Mincher, 1994; Holt, Sanders, Zinn, Fryer, & Pilcher, 1975; Bernardet, Kerouault, & Michel, 1994). In 2015, *T. maritimum* was isolated from farmed Chinook salmon (*Oncorhynchus tshawytscha*) experiencing summer mortalities in New Zealand

(Chapter 2). Since this detection, further testing has shown a widespread distribution of *T. maritimum* in Chinook salmon farms in New Zealand (Chapter 4).

During the initial testing of samples collected from the 2015 summer mortalities, only a small number of fish were found to be positive by bacterial culture leading to a perceived low prevalence. Culture of *T. maritimum* from field skin ulcer tissue can be challenging as its slow growth can allow the overgrowth by other environmental bacteria within the sample. Subsequent qPCR testing of these samples and of samples from other salmon farms revealed a higher prevalence of *T. maritimum* (Chapter 4). However, as the qPCR results of these samples often revealed a low concentration of target bacteria, it was difficult to determine the significance of these findings. Additionally, it could not be determined whether the qPCR results indicated viable bacteria that were likely to be causing disease or dead bacteria that may have been present incidentally within the lesions.

The viability of bacteria within a sample is traditionally determined by culture methods. However, nucleic acid intercalating dyes applied with PCR have been proposed as viability indicators. These assays, termed viability PCR (vPCR), use dyes that specifically penetrate the compromised membranes of dead cells and bind with their nucleic acid (Nogva et al., 2003; Nocker and Camper, 2009). The sample is then subjected to light of a specific wavelength to crosslink the dye with nucleic acid to form covalent bonds. Viability is determined as the presence of the crosslinked dyes within the DNA of a dead cell prevents amplification of the target DNA when PCR is performed.

Since vPCR introduction, a variety of dyes have been proposed including: ethidium monoazide (EMA), a derivative of ethidium bromide, (Nogva et al., 2003); propidium monoazide (PMA), a derivative of propidium iodide; and more recently, a mixture of the two (i.e. PEMAX). These dyes will bind with any nucleic acid present in the sample, therefore the effectiveness of vPCR will be impacted by a range of factors including the abundance of non-target organisms in the sample.

This study was conducted to validate and optimise a method for the use of vPCR to detect viable *T. maritimum* within skin ulcer samples collected from naturally-infected

fish. Determining the viability of the bacteria is important to understanding the pathogenesis of *T. maritimum* infections in fish. This is the first time this technology has reported to be optimised and validated on an aquatic animal pathogen and the results demonstrate the value of developing these assays for the investigation of infectious disease in aquaculture and wild fisheries.

8.2 Materials and methods

8.2.1 Bacterial strain and inoculum preparation

Tenacibaculum maritimum previously isolated from a Chinook salmon skin ulcer was used in this study (Chapter 2). The bacteria was revived from long-term storage on cryobeads (Cryobank, CA, USA) stored at -80°C. Inoculum was prepared from pure culture after aerobic incubation at 22°C for 48 hours in Tryptone, Yeast, Glucose broth with sea salt (TYG-M) (Cipriano, Schill, Teska, & Ford, 1996).

Bacterial concentration was calculated by serial dilution and plating onto TYG-M agar in triplicate. Agar plates were incubated for three days at 22°C prior to colony counts being conducted to determine the colony forming units per mL (CFU mL^{-1}).

8.2.2 *Tenacibaculum maritimum* non-viable (dead) cell stock production

To prepare dead *T. maritimum* cells for use in experiments, 1 mL of a 48 hour culture was heated at 99°C for 10 min in triplicate. The loss of viability was verified by inoculating the heat treated suspension on to TYG-M agar and into TYG-M broth incubated at 22°C for seven days. All heat-treated suspensions were cooled to room temperature prior to PEMAX treatment.

8.2.3 DNA extraction

DNA extractions were performed using the Qiagen QIAcube automated extraction robot using the QIAamp HT kit (Qiagen, Hilden, Germany).

Pure bacterial cultures and tissue homogenates greater than 100 µL were processed by centrifugation at 14,000 g for 5 min. The supernatant was then carefully removed

and the pellet was used for either immediate DNA extraction or stored at -20°C. DNA was extracted without pelleting for tissue homogenates of 100 µL or less. For both pellets and tissue homogenates, 180 µL of lysis buffer and 20 µL proteinase K were added and the sample lysed overnight at 56°C. Once lysed, samples were then processed following the manufacturer's tissue protocol.

8.2.4 Quantitative (qPCR) and nested conventional PCR

Both qPCR and nested conventional PCR were performed for all samples (Fringuelli et al., 2012; Cepeda and Santos, 2002) targeting an amplicon size of 155 and 400 base pairs (bp) respectively. To visualise amplicons from the conventional PCR, samples were loaded into a 1.5% agarose gel stained with GelRed (Biotium, Fremont, USA) for gel electrophoresis. Assays used a 2 µL DNA template for samples originating from pure culture, run in duplicate and a 2 and 5 µL DNA template for samples originating from tissue. DNA from a pure culture of *T. maritimum* was used as a positive control and no template controls with molecular grade water were run in each PCR.

The limit of detection (LOD) of the viability qPCR (v-qPCR) and viability conventional PCR (vPCR) assay were determined following optimisation of vPCR parameters. Live bacteria (10^1 to 10^6 CFU mL^{-1}) were diluted in a background of dead bacterial cells (10^6 CFU mL^{-1}) and treated with dye. Viable bacteria diluted in molecular grade water with no dye treatment were used as controls. All dilution series were carried out in triplicate. For v-qPCR, the LOD was determined to be the lowest bacterial concentration where the target molecules amplified during each replication cycle as shown by the cycle threshold (Ct) value. For vPCR, the LOD was assessed as the lowest dilution that all replicates produced amplicons of the expected size.

For the v-qPCR and qPCR, bacterial concentration was plotted against the corresponding Ct value and standard curves were generated by linear regression of plotted points. The amplification efficiency was assessed using the calculation $E = -1+10^{(-1/slope)}$.

8.2.5 Optimisation of vPCR protocol

PEMAX (GenIUL, Barcelona, Spain) was dissolved by adding sterile water to create a stock solution of 2 mM. Re-suspended dye was stored at -20°C. A maximum of five freeze thaw cycles was allowed. The following parameters were optimised: PEMAX concentration (including the use of a double dye exposure); PEMAX treatment time; PEMAX treatment temperature; light exposure time and the use of reaction buffers. All optimisation assays were carried out in triplicate. All samples were agitated during PEMAX treatment on a shaking platform (300 rpm) in the dark.

8.2.5.1 PEMAX concentration and treatment time

Aliquots (500 µL) of *T. maritimum* bacteria from a 48 hour culture (10^8 CFU mL^{-1}) were heat treated and dead bacteria harvested by centrifuging at 14,000 g for 5 min in vPCR tubes (GenIUL). The supernatant was removed and the pellet re-suspended in 245 µL phosphate buffered saline (PBS, pH 7.0-7.3; Gibco, Life Technologies, NY, USA). For optimisation of PEMAX concentration (10, 25, 50, or 100 µM) and treatment time (15, 20, or 30 min), prepared dead or live bacteria were incubated in the dark at room temperature (~22°C).

Subsequently, a 15 min photoperiod (PhAST blue LED light, GenIUL) was applied to all treated samples. The following controls were included in the assay: dead bacteria with PEMAX treatment at each concentration with no exposure to light (dead + PEMAX, - light); dead bacteria with no treatment (dead – PEMAX); dead bacteria with no PEMAX treatment with exposure to light (dead – PEMAX, + light) and live bacteria with no treatment (live – PEMAX).

8.2.5.2 PEMAX treatment temperature and photoperiod

Separate suspensions of live and dead bacteria were prepared as above. For temperature optimisation, samples were treated with PEMAX and incubated in the dark at 0, 4, 22, or 30°C followed by a 15 min photoperiod. Photoperiod was optimised (5, 10, 15, or 30 min), after the optimal incubation temperature was determined. For

both experiments, live and dead bacterial suspensions without PEMAX treatment were used as controls.

8.2.5.3 Reaction buffers

Separate pellets of live and dead bacteria, prepared as above, were re-suspended in the following reaction buffers: broth (TYG-M); sodium deoxycholate (0.01, 0.03, 0.1, or 0.3%) (Sigma-Aldrich, Missouri, USA); Triton-X 100 (0.1, 0.5, or 1%) (Sigma Aldrich); 1 X Reaction buffer + (GenIUL); pH (7, 7.5, 8, or 8.5); 0.85% saline (Fort Richard, Auckland, New Zealand) and artificial seawater (33 ppt (salt), pH 8.2, Forty Fathoms, Maryland, USA). Following re-suspension, the samples were treated using the optimal protocol. Live and dead bacterial suspensions without PEMAX treatment re-suspended in PBS were set up as controls.

8.2.5.4 Double dye exposure

Separate suspensions of live or dead bacteria were prepared in the appropriate reaction buffer. The re-suspended bacteria were then treated with 50 µM PEMAX and incubated in the dark for 15 min. Samples were then centrifuged at 14,000 g for 5 min, supernatant removed, and pellet re-suspended in 245 µL PBS. A further 50 µM PEMAX was added to some of these samples. All samples were then re-incubated in the dark for 15 min prior to a 15 min photoperiod. Live or dead bacterial suspensions without PEMAX treatment were used as controls.

8.2.6 Addition of bacteria (live or dead) to skin

Skin tissue from Chinook salmon originating from freshwater hatcheries were used to create tissue homogenates to spike with live or dead bacteria. These fish were selected as they were from populations known to be negative for *T. maritimum*. Homogenates of Chinook salmon skin were prepared in 0.85% saline (Fort Richard) at a dilution of 100 mg mL^{-1}. Aliquots of 245 µL of homogenate were used in the experiment. Mixtures of live or dead *T. maritimum* were used to assess the suitability of PEMAX treatment with mixed cells in tissue samples. All samples contained the same number of cells (i.e. 10^3 or 10^6 CFU mL^{-1}) with the ratio of live to dead bacteria

being adjusted from 100, 80, 60, 40, 20, or 0% live bacteria. Spiked skin tissue homogenates without PEMAX treatment were used as controls in both experiments.

8.2.7 High-throughput format

Homogenates of Chinook salmon skin were prepared as above. Mixtures of live or dead *T. maritimum* were prepared for comparison between two formats; individual vPCR tubes and a 96 well plate. All samples contained the same number of bacterial cells (i.e. 10^3 or 10^5 CFU mL^{-1}) with the ratio of live to dead being adjusted from 100, 50 or 0% live bacterial cells for 10^3 CFU mL^{-1} and 0 or 50% live bacterial cells for 10^5 CFU mL^{-1}. Aliquots of 100 µL of spiked tissue homogenates were run in parallel with the two formats. For vPCR tubes, the PhAST blue LED light system was used and for the 96 well plate, the photo activation universal light (PAUL; GenIUL) was used with 100% light exposure intensity.

8.2.8 Repeatability

Repeatability was carried out by two users within the same laboratory. Two identical panels of 20 artificially spiked skin samples were prepared by placing 100 mg of tissue in a 2 mL safe-lock tube with known amounts of live or dead bacteria. Samples were then processed using the optimal protocol by the two users in parallel. A suspension of live or dead *T. maritimum* culture (10^8 CFU mL^{-1}) with no treatment were used as controls.

8.2.9 Determining a cut off value for v-qPCR

A cut-off value was determined for interpretation of the v-qPCR results. All data from experiments performed in this study carried out using the optimised protocol were compiled and the % difference between the change in Ct value of the same sample with and without PEMAX dye treatment (% Δ Ct) was assessed. This was carried out by taking the following steps:

- Δ Ct = Ct treated – Ct not treated
- % Δ Ct treated = (Δ Ct / Ct treated)* 100
- % Δ Ct not treated = (Δ Ct / Ct not treated)* 100
- % Δ Ct = % Δ Ct not treated - % Δ Ct treated

Significant differences of % Δ Ct were analysed between samples containing live or dead bacteria. A cut-off value was determined for results indicative of samples likely to contain live bacteria. An accuracy of above 95% was considered acceptable.

8.2.10 Naturally infected samples

Thirty skin ulcers were sourced from farmed Chinook salmon thought to be infected with *T. maritimum* based on clinical signs of disease. Samples were processed 24 hours after collection. Ulcers was sub-sampled (~100 mg) and placed into a 2 mL safe-lock Eppendorf tube with a 5 mm sterile stainless steel ball. Saline (1 mL; 0.85%) was then added to the tube and the contents homogenised. One hundred µL of the homogenised sample was used for each of the following treatments: 1) heat treated + PEMAX; 2) + PEMAX; 3) – PEMAX and 4) grown in culture (plated onto agar at neat, 10^{-1}, 10^{-2} dilutions and incubated at 22°C for two weeks).

8.2.11 Enrichment protocol

Enrichment of artificially spiked samples was carried out to increase the sensitivity of the v-qPCR assay. Homogenates of Chinook salmon skin were prepared in 0.85% saline at a dilution of 100 mg mL^{-1}. The following final concentrations of *T. maritimum* were added (cells mL^{-1} (live : dead): $10:10^6$; $10^2:10^6$; $10^3:10^6$; $10^4:10^6$; $0:10^3$; $10^4:0$; $10^5:0$; $0:10^4$). Samples were processed immediately with two aliquots (100 µL each) processed as per the optimised protocol: + PEMAX; - PEMAX. At the same time, 300 µL of the homogenate was inoculated into 3 mL TYG-M broth for 48 hours at 22°C. After 48 hours, 2 mL of the broth was removed and centrifuged at 14,000 g for 5 min. The supernatant was carefully removed and discarded and the pellet was re-suspended in 200 µL 0.85% saline. This 200 µL was then divided into two for processing as above: + PEMAX; - PEMAX.

8.2.12 Statistical analysis

A generalised linear model was used to determine any significant differences between dye concentrations, time of incubation, incubation temperature, photoperiod, resuspension buffers, application of high-throughput platform and double dye exposure. Analysis was carried out in R version 3.5.2 (R Core Team, 2015) which examined the differences between Ct values when a qPCR test was conducted on samples with and without PEMAX treatment (R package *nlme*, Pinheiro et al., 2014). The response variable in the model was the Ct value which was log transformed to meet the assumptions of normality. To test for specific pair-wise differences between the treatment types, a multiple comparison procedure using Tukey contrasts was performed (R package *multcomp*, Hothorn et al., 2008). We used p values < 0.05 to determine statistical significance. For reaction buffers, differences were also assessed between dead bacteria compared with the controls (no dye treatment) and live bacteria (dye treated) compared with the controls. Pair-wise differences were assessed as above.

8.3 Results

8.3.1 *Tenacibaculum maritimum* dead stock production

Loss of viability by heating at 99°C for 10 min was confirmed as no growth was observed on either the agar or in the broth after seven days incubation.

8.3.2 Amplification efficiency and sensitivity of conventional-vPCR and q-vPCR

The LOD for v-qPCR was determined to be 10^3 CFU mL^{-1} (Figure 8.1). Below this bacterial concentration the Ct value did not increase (data not shown). The v-qPCR and qPCR was linear over the range of 10^3 to 10^6 CFU mL^{-1}. The corresponding amplification efficiencies were 86% (v-qPCR) and 107% (qPCR). As expected, the samples from the v-qPCR dilution that were not treated (live + dead samples – PEMAX) maintained a similar Ct value for all concentrations of live bacteria. The LOD of the conventional PCR was 10^3 CFU mL^{-1}. Below this bacterial concentration, production of amplicons was not consistent.

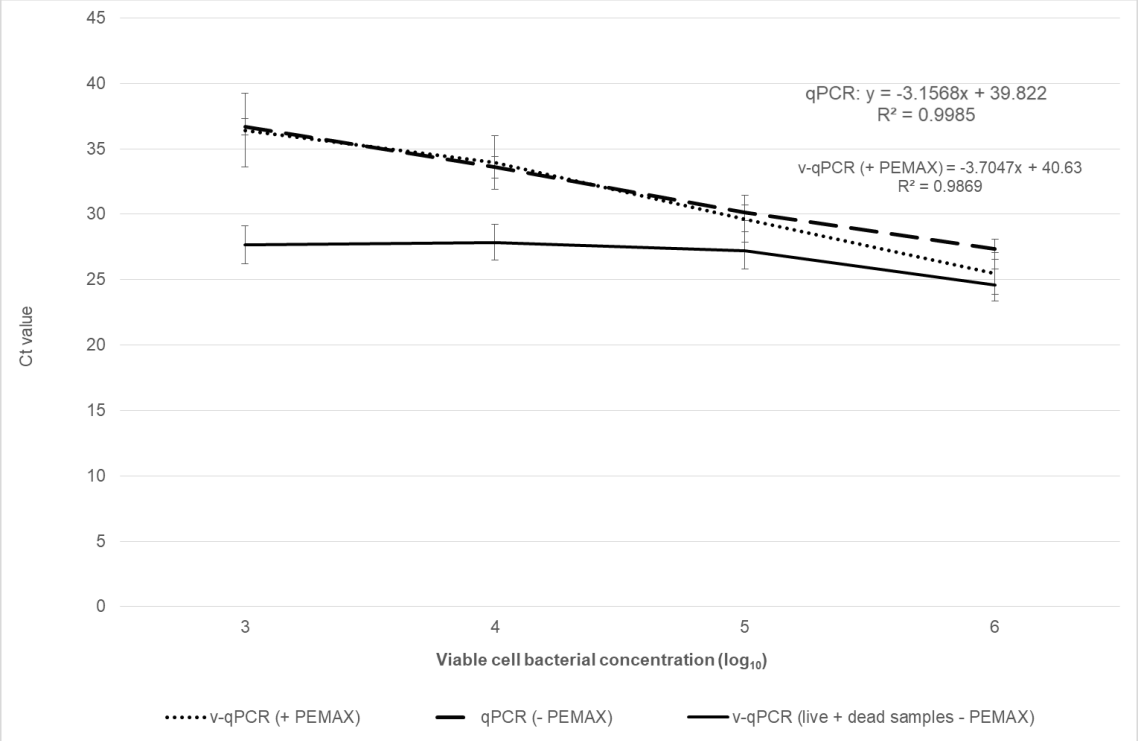


Figure 8.1. Serial dilutions of live *Tenacibaculum maritimum* cells (10^3 – 10^6 CFU mL^{-1}), *n* = 3. v-qPCR is diluted in the presence of 10^6 CFU mL^{-1} dead cells with '+PEMAX', treated with dye and 'live + dead samples – PEMAX' not treated with dye. qPCR (-PEMAX) is diluted in molecular grade water and not treated with PEMAX. Error bars represent the SD between the replicates.

8.3.3 Optimisation

For v-qPCR, a higher dye concentration was more effective as shown by the increasing Ct values of samples containing dead bacteria. Significant differences were observed between the dye concentrations; 10 and 100 µM, 10 and 50 µM, 25 and 100 µM ($p < 0.01$, < 0.01 and 0.04 respectively). No significant differences were observed between any incubation times with each dye concentration (Figure 8.2). For vPCR, complete suppression of dead bacteria was not observed for any dye concentrations or incubation times tested. However at 100 µM dye for 30 min incubation time, 1/3 replicates showed complete suppression of dead bacteria.

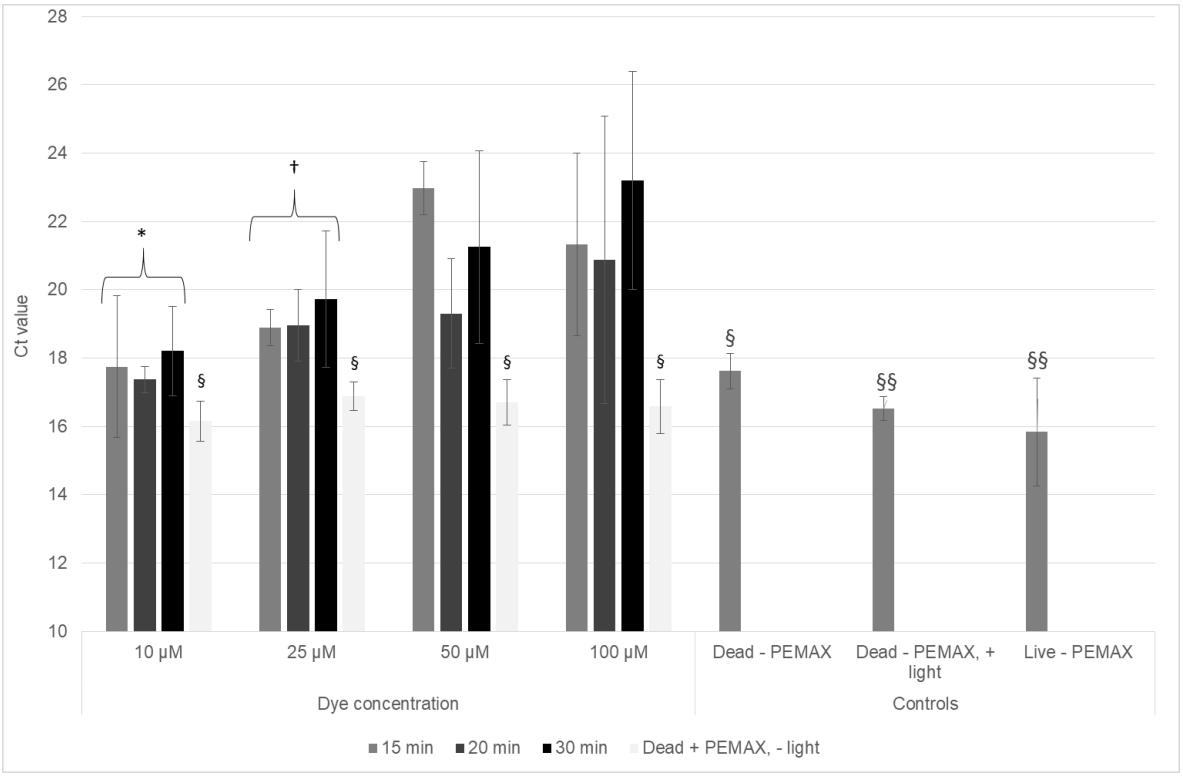


Figure 8.2. PEMAX concentrations and treatment times on dead cells, including controls. *n* = 3. * = significant difference to 50 and 100 µM dye concentration, dead treated cells ($p < 0.01$), † = significant difference to 100 µM dye concentration, dead treated cells ($p < 0.05$), § = significant difference from dead treated cells within the dye concentration ($p < 0.01$). § § = significant difference from dead treated cells at all dye concentrations ($p < 0.05$). Error bars = standard deviation. Error bars represent the SD between the replicates.

As shown in Figure 8.3, no significant difference was seen between any incubation temperatures trialled on dead treated bacteria. A significant difference was seen between ice (0°C) and 4°C in the live treated bacteria ($p = 0.03$). No significant difference was seen between the live bacteria at any other temperatures.

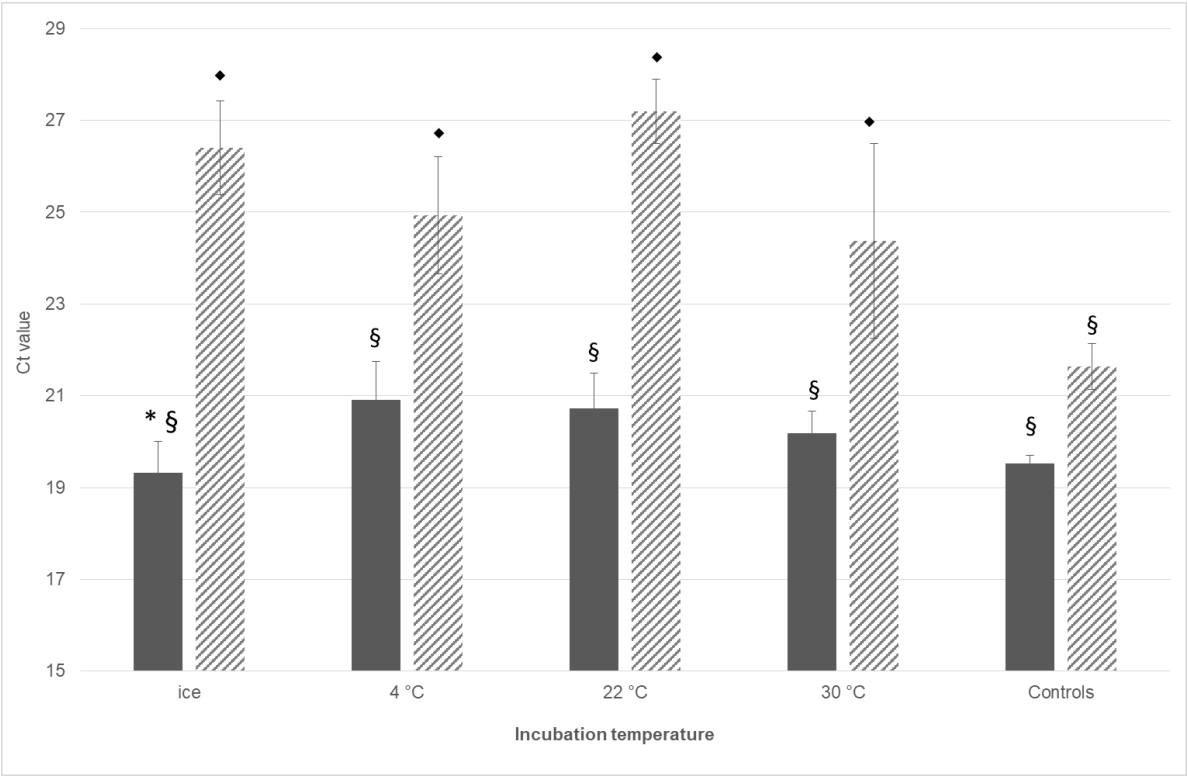


Figure 8.3. PEMAX treatment temperature on live (solid bar) and dead (hatched bar) *Tenacibaculum maritimum*, including controls. n = 3. § = significant difference from dead treated samples at all incubation temperatures ($p < 0.01$), * = significant difference from live treated cells at 4°C ($p < 0.05$), ◆ = no significant difference observed between all dead treated cells. Error bars represent the SD between the replicates.

Photoperiod on dead treated bacteria was found to be significantly better at 15 min compared to 5, 10 or 30 min (p = 0.05, 0.01 and 0.05, respectively) (Figure 8.4). No significant difference was seen between the live treated bacteria at any photoperiod trialled. In the vPCR, complete suppression of dead cells was not seen at any incubation temperature or light exposure time tested. Amplification was detected in all samples that contained live bacteria.

Compared to the other reaction buffers trialled in the v-qPCR, the penetration and binding of PEMAX on dead cells was significantly lower when re-suspended in broth or seawater (Figure 8.5). The addition of reaction buffers had no influence on live bacteria treated with PEMAX. For vPCR, amplification was observed in all samples containing live cells. Weak or no amplification was seen in at least one replicate of samples containing dead bacteria with the reaction buffers 0.01% SD, 0.1 and 0.5% Triton X-100, and saline. There was a significant difference between the dead not treated bacteria (control) compared to the dead treated bacteria, the live and dead

treated bacteria, and the live not treated bacteria (control) and the dead treated bacteria (all $p < 0.01$). No significant difference was seen between the live treated bacteria and the live or dead not treated bacteria (controls) or the live and dead not treated bacteria (controls).

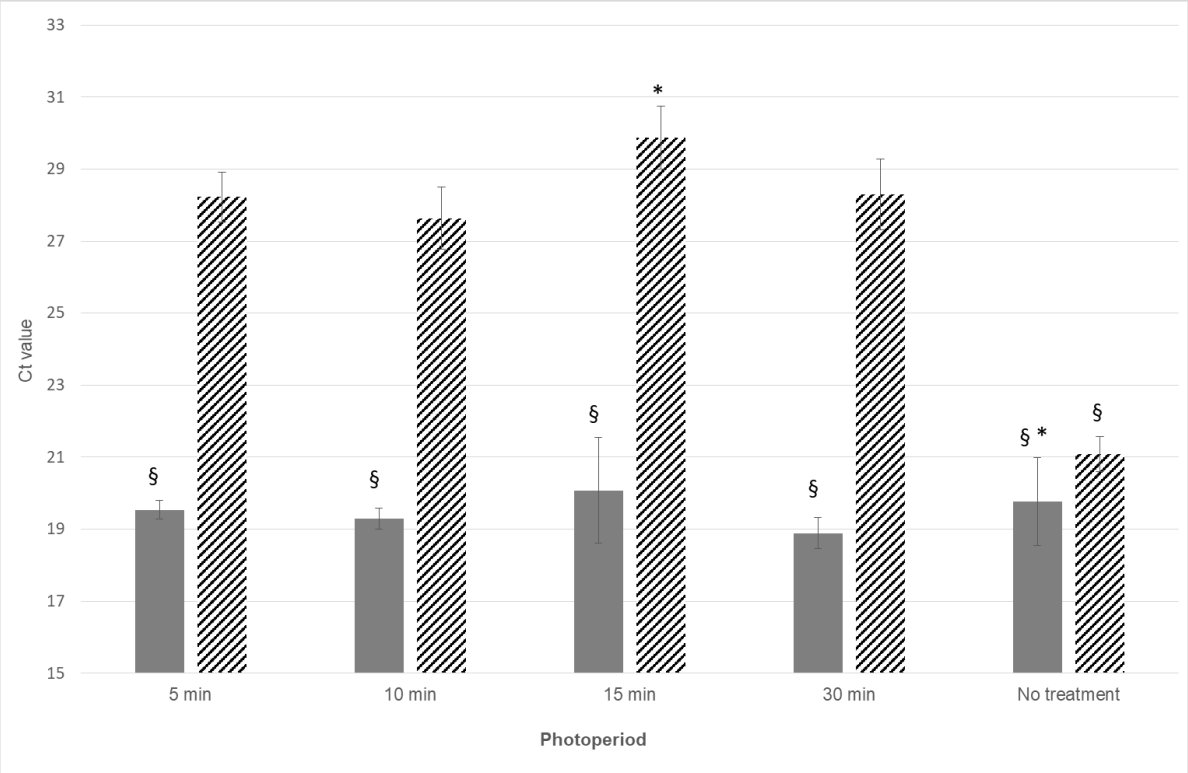


Figure 8.4. Light exposure times on live (solid bar) and dead (hatched bar) *Tenacibaculum maritimum*, including controls. $n = 3$. * = significant difference from dead treated bacteria exposed to 5, 10 or 30 min photoperiod ($p < 0.05$), § = significant difference from dead treated bacteria independent of light exposure time ($p < 0.01$). * = significant difference from dead bacteria no treatment ($p < 0.01$). Error bars represent the SD between the replicates.

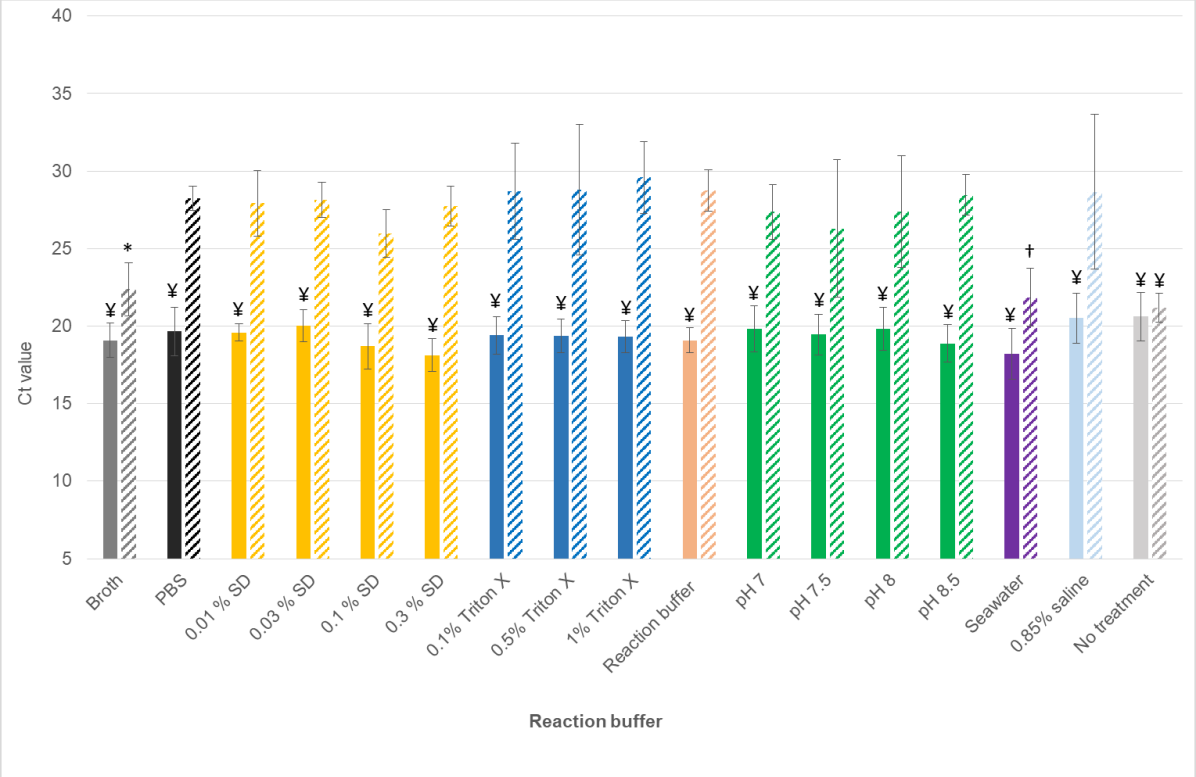


Figure 8.5. Resuspension buffers on PEMAX treated live (solid bar) and dead (hatched bar) *Tenacibaculum maritimum* of the same concentration exposed to PEMAX with controls. *n* = 3. * = significant difference from dead treated bacteria re-suspended in: 0.01, 0.03, 0.3% SD, 0.1, 0.5 and 1% Triton X-100, PBS, pH 8 and 8.5, reaction buffer and saline ($p < 0.05$). † = significant difference from dead treated bacteria re-suspended in: 0.1, 0.03, 0.03% SD, 0.1, 0.5 and 1% Triton X-100, PBS, pH 7, 8 and 8.5, reaction buffer and saline ($p < 0.05$). ¥ = significant difference from all dead treated bacteria ($p < 0.01$). Error bars represent the SD between the replicates.

No significant difference was detected using a double dose of PEMAX for live or dead bacteria when analysing the v-qPCR results (Figure 8.6). Amplification was seen in all samples containing live and dead bacteria for the vPCR. The optimal method used for the rest of this study are summarised in Table 8.1.

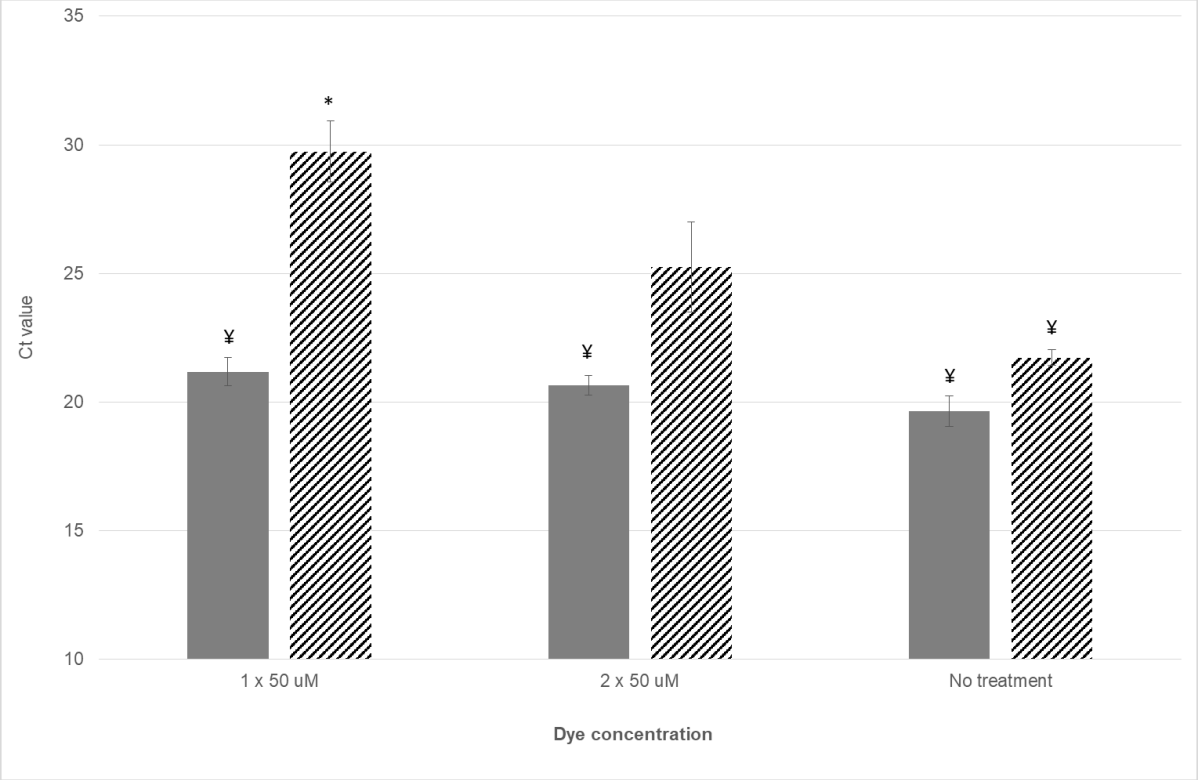


Figure 8.6. Cycle threshold values of live (solid bar) and dead (hatched bar) *Tenacibaculum maritimum* exposed to a double dose of PEMAX dye. n = 3. * = significant difference from 2 x 50 µM (p = 0.03). ¥ = significant difference from dead treated bacteria ($p < 0.01$). Error bars represent the SD between the replicates.

Table 8.1. Summary of optimised conditions for *Tenacibaculum maritimum* used in this study.

Variable	Optimal parameters
LOD	10^3 CFU mL^{-1}
Inactivation method	99 °C for 10 min
PEMAX concentration	50 µM
PEMAX treatment time	15 min
PEMAX treatment temperature	22 °C
Exposure to light	15 min, 100% light intensity
Reaction buffer	0.85% saline

8.3.4 Assessing live bacteria in artificially spiked tissue samples

Treated samples containing only dead bacteria (10^3 CFU mL^{-1} in skin tissue), were on average 7.83 Ct higher than samples containing live cells. The number of replicates producing an amplicon via vPCR was lower when the number of live bacteria in the sample was lower. No amplicons were produced in the conventional vPCR when only dead bacteria were present.

Treated samples containing only dead bacteria, (10^6 CFU mL^{-1} in skin tissue), were on average 10.57 Ct higher than samples containing viable cells. The vPCR assay produced amplicons when samples contained live bacteria and no amplicons were produced in the vPCR when only dead bacteria were present.

For both bacterial concentrations, the Ct values in the v-qPCR became higher as more dead cells were present. This trend was more evident at the higher bacterial concentration (Table 8.2; Figure 8.7).

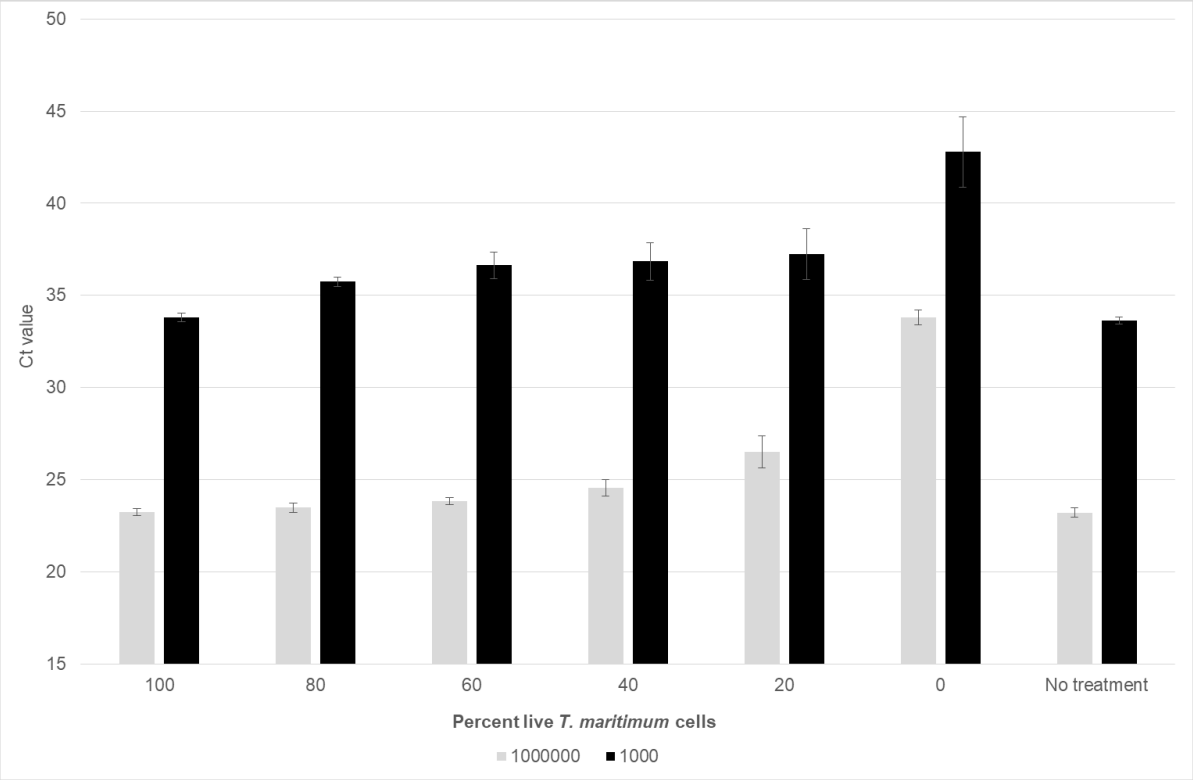


Figure 8.7. Percentage of live and dead *Tenacibaculum maritimum* spiked in skin tissue with a starting concentration of 10^3 and 10^6 CFU mL-1, including controls that contained 100% live bacteria (no treatment). *n* = 3. Error bars represent the SD between the replicates.

Table 8.2. Conventional vPCR for reaction buffers trialled with PEMAX. The number in last two columns equals the number of replicates that produced an amplicon in the vPCR.

Percent live cells in sample matrix	vPCR result; 10^7 CFU mL^{-1}	vPCR result; 10^4 CFU mL^{-1}
100	3	3
80	3	3
60	3	2
40	3	2
20	3	1
0	0	0

8.3.5 High-throughput testing

No significant differences were observed in the Ct values from the v-qPCR between the two platforms (p = 0.27). The results from the vPCR on the two platforms were the same: no amplicons were produced when only dead bacteria were present and amplicons were produced when live or a mix of live and dead bacteria were present at both bacterial concentrations.

8.3.6 Repeatability

No significant difference was seen between the Ct results of the two users (p = 0.71). Sample 20 of the panel contained skin tissue not spiked with *T. maritimum*. This sample gave no signal in the v-qPCR or vPCR for either user.

Two samples did not give consistent results for the vPCR between the two users. These were both at the lowest bacterial concentration (10^2 CFU mL^{-1}), i.e. lower than the assay LOD (Table 8.3).

Table 8.3. Ct values (threshold autocaculated) from repeatability testing of two users in the same lab. P = Positive, N = Negative. Red = treated with dye. * samples that were not consistent between users in the vPCR. Samples in boxes are below the LOD of the assay. c = control samples not treated. SD = standard deviation.

Sample	v-qPCR, user 1 (Ct)	v-qPCR, user 2 (Ct)	Average	SD	vPCR, user 1	vPCR, user 2
10^6 L	27.81	28.18	28.00	0.26	P	P
10^6 L	26.58	27.26	26.92	0.48	P	P
10^6 D	36.46	36.78	36.62	0.23	N	N
10^6 D	26.94	26.62	26.78	0.23	P	P
10^5 L 10^3 D	31.94	32.29	32.12	0.25	P	P
10^5 L 10^3 D	31.55	31.61	31.58	0.04	P	P
10^4 D	39.07	40.75	39.91	1.19	N	N
10^4 D	36.23	38.17	37.20	1.38	P	P
10^4 L	33.00	33.40	33.20	0.29	P	P
10^4 L	33.40	32.54	32.97	0.61	P	P
10^3 L	38.58	38.88	38.73	0.22	N	N
10^3 L	36.02	36.18	36.10	0.12	P	P
10^3 D	39.98	38.63	39.31	0.96	N	N
10^3 D	35.91	35.67	35.79	0.17	P	P
10^3 L 10^3 D	37.92	38.36	38.14	0.31	P	P
10^3 L 10^3 D	36.50	37.51	37.00	0.71	P	P
10^2 D	>45	>45	-	-	N	N
10^2 D	41.57	43.85	42.71	1.61	P	P
10^2 L*	42.59	41.65	42.12	0.66	P	N
10^2 L	38.86	38.80	38.83	0.04	P	P
10^6 L 10^6 D	27.39	28.32	27.86	0.66	P	P
10^6 L 10^6 D	26.21	26.71	26.46	0.36	P	P
10^4 D	36.15	37.55	36.85	0.99	N	N
10^4 D	34.37	35.37	34.87	0.71	P	P
10^4 L 10^2 D	34.94	35.35	35.14	0.29	P	P
10^4 L 10^2 D	33.29	33.53	33.41	0.17	P	P
10^5 D	39.95	42.78	41.36	2.00	N	N
10^5 D	30.13	30.92	30.52	0.56	P	P
10^5 L	30.68	31.03	30.86	0.25	P	P
10^5 L	29.92	29.79	29.86	0.09	P	P

Sample	v-qPCR, user 1 (Ct)	v-qPCR, user 2 (Ct)	Average	SD	vPCR, user 1	vPCR, user 2
10^6 D	28.24	29.32	28.78	0.76	N	N
10^6 D	26.60	27.70	27.15	0.78	P	P
10^2 D	N	N	N	-	N	N
10^2 D*	39.71	41.97	40.84	1.59	P	N
10^2 L	41.97	41.33	41.65	0.45	N	N
10^2 L	40.37	39.15	39.76	0.86	P	P
10^5 L 10^5 D	34.82	34.99	34.90	0.12	P	P
10^5 L 10^5 D	33.47	33.22	33.35	0.18	P	P
10^8 L[c]	24.56	23.89	24.23	0.47	P	P
10^8 D[c]	26.87	26.08	26.48	0.56	P	P

8.3.7 Interpretation of qPCR results

Results from all tests performed using the optimal protocol were compiled (*n* =103) and the % Δ Ct value determined (Table 8.4).

Table 8.4. % Δ Ct between PEMAX treated and PEMAX untreated samples containing live cells, dead cells, or a mix of cells. "Live" = samples that contained a mix of live and dead cells, "dead" = only dead cells were present.

Sample Type (*n*)	% Δ Ct†
Live (76)	0.37 (0-2.51)
Dead (27)*	5.09 (0.36 - 10.64)

*Six cases showed a Ct value in the PEMAX untreated samples but were not detected in the PEMAX treated samples.
† A significant difference was seen between the live and dead samples ($p < 0.0001$).

A % Δ Ct of 2.5 between PEMAX treated and untreated samples was determined to predict if live bacteria are present in the sample. For example, a change of ≤ 2.5% indicates live bacteria are present and a change of ≥ 2.5% indicates no live cells are present. Above the LOD of the assay, this value has a 98% accuracy. Under the LOD of the assay, ≤ 10^3 CFU mL^{-1} (Ct 36), the accuracy decreased to 88%.

8.4.8 Assessing viability in naturally affected tissue samples

Detection of *T. maritimum* in naturally infected tissue was assessed by the following methods: qPCR, PCR, v-qPCR, vPCR, and culture.

Of the samples that had no PEMAX treatment, 23 of 30 produced an amplification curve with an average Ct value of 37.09 in the qPCR and 16 of 30 samples produced an amplicon in the PCR. For the samples that had PEMAX treatment with no heat treatment, 11 of 30 produced an amplification curve with an average Ct value of 37.97 in the v-qPCR and three of 30 produced an amplicon in the vPCR. Of the 11 v-qPCR positive samples, eight were below the LOD of the assay (> Ct 36) thus the results could not be reliably interpreted. For the heat killed and PEMAX treated samples, 11 of 30 produced an amplification curve with an average Ct value of 37.94 in the v-qPCR and no samples produced an amplicon in the vPCR.

Using the indicative % Δ Ct value of the 11 samples that produced an amplification curve in the v-qPCR, seven samples had a value of ≤ 2.5% and four had a value of ≥ 2.5%. This indicates that seven samples are likely to have live cells present and four samples are likely to have no live cells present. However, as only three of these samples were above the LOD of the assay, these were the only samples that could be reliably assessed. Of these three samples (samples 16, 25 and 27), all values were ≤ 2.5% indicating live cells were present. Two of these (samples 16 and 27) were verified as containing live cells by a positive culture result and also produced a positive amplicon in the vPCR. Sample 25 was not positive by culture or vPCR. As expected, the heat killed PEMAX treated samples all had a value of ≥ 2.5% in the v-qPCR (Table 8.5).

8.3.9 Enrichment protocol

When using the % Δ Ct value on samples processed immediately, samples that contained a low concentration of live bacteria (< 10^4 CFU mL^{-1}) in a background containing a high concentration of dead cells were analysed as unlikely to contain live bacteria (≥ 2.5%, Figure 8.6). When these samples underwent enrichment, however analysis showed live bacteria were likely to be present (% Δ Ct < 2.5%). Samples that

contained only dead bacteria at a low concentration had a % Δ Ct value of ≥ 2.5% or no amplification when processed immediately and after enrichment. Compared to the samples processed immediately these samples also had a higher Ct value after enrichment.

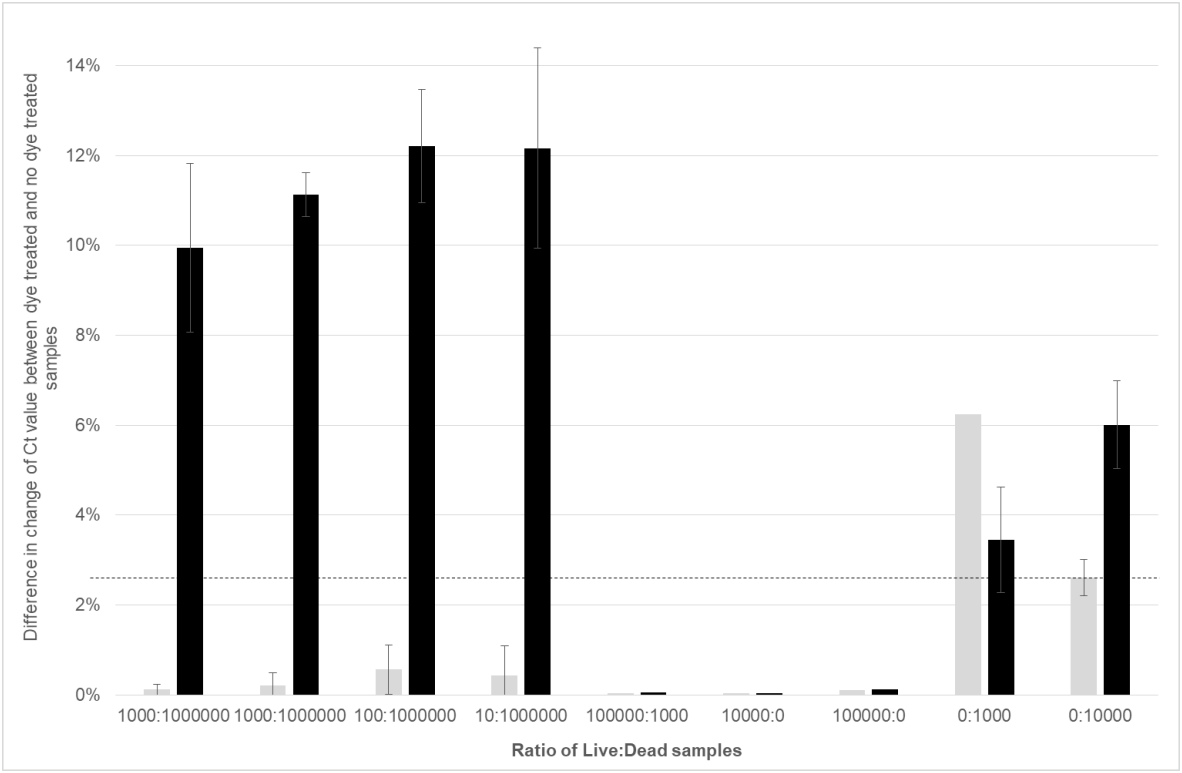


Figure 8.8. % difference for samples processed immediately (black bars) and processed after enrichment (grey bars). Dashed line = cut off value for cells that are live (2.5%). *n* = 3. Error bars represent the SD between the replicates. Samples with no error bars only had one replicate amplify in the v-qPCR.

Table 8.5. Naturally infected fish tissue samples processed with and without dye treatment in both conventional and qPCR as well as culture on agar. H = heat killed, N = no fluorescent signal in qPCR. *=below the detection limit of the assay.

	qPCR (Ct value)				Conventional PCR amplicon			
Sample	qPCR	v-qPCR	H + v-qPCR	% Δ Ct	PCR	v-PCR	H+ vPCR	Culture confirmation
1	37.75	39.67*	39.28	0.25	+	-	-	-
2	40.95	N	N		+	-	-	-
3	42.25	N	N		-	-	-	-
4	35.32	37.81*	39.51	0.46	+	-	-	-
5	N	N	N		-	-	-	-
6	N	N	N		-	-	-	-
7	30.43	36.58*	37.16	3.40	+	-	-	-
8	39.12	N	N		+	-	-	-
9	32.02	38.28*	37.2	3.20	+	-	-	-
10	N	N	N		-	-	-	-
11	33.03	36.2*	37.45	0.84	+	+	-	-
12	N	N	N		-	-	-	-
13	40.81	N	N		-	-	-	-
14	35.81	40.54*	42.14	1.54	+	-	-	-
15	39.86	N	N		+	-	-	-
16	30.37	33.02	35.25	0.70	+	+	-	+
17	30.81	39.16*	37.34	5.78	+	-	-	-
18	42.61	N	N		-	-	-	-
19	42.62	N	N		-	-	-	-
20	N	N	N		-	-	-	-
21	39.68	N	N		-	-	-	-
22	41	N	N		-	-	-	-
23	N	N	N		-	-	-	-
24	N	N	N		-	-	-	-
25	29.64	33.05	33.31	1.19	+	-	-	-
26	43.68	N	N		-	-	-	-
27	32.07	35.85	37.87	1.24	+	+	-	+
28	33.13	38.82*	40.83	2.52	+	-	-	-
29	38.65	N	N		+	-	-	-
30	41.5	N	N		+	-	-	-
Average	37.09	37.97	37.94					
SD	4.72	1.63	2.46					

8.4 Discussion

The present study represents the first time vPCR has been optimised and validated for an aquatic pathogen. This study further shows the potential of vPCR to be a rapid, sensitive and reliable method to detect live cells in a complex sample matrix. Rapid methods to detect live bacteria is important in many fields of study, including to inform decisions about a pathogen incursion, differentiate vaccine strains (dead cells) from an infection, and to provide information of the level of live bacteria present to determine the risk of infection. In the authors' experience, the use of traditional culture methods to answer these questions can be problematic, particularly if the level of infection is low. Importantly, this study assessed the suitability of v-qPCR and vPCR to identify live cells in a skin tissue matrix where environmental bacteria are expected to be present in relatively high numbers.

The following factors were considered to optimise the viability assays: PEMAX concentration (including a double dye exposure), PEMAX incubation time, PEMAX incubation temperature, photoperiod, and the use of reaction buffers. Dye concentration was the only parameter to have any significant impact on v-qPCR with *T. maritimum.* However, increasing the dye concentration above a certain level did not improve assay effectiveness. Importantly, it was found that none of the concentrations tested were able to penetrate the cell wall of live bacteria and subsequently impair the amplification of their DNA. One of the three live bacterial replicates in the vPCR showed complete suppression at the highest dye concentration with the longest incubation time. However, this result was not consistent between all replicates nor was this trend of reduced amplification seen in the v-qPCR.

Incubation temperature was shown to have no effect on dead treated bacteria, however at 4°C there was a negative impact on live treated bacteria compared with live treated bacteria at a lower temperature. However, this trend was not consistent with higher temperatures not showing a significant difference to live bacteria treated at the lowest temperature.

In contrast to other studies (Codony et al., 2015; Takahashi, Gao, Miya, Kuda, & Kimura, 2017), the use of reaction buffers did not significantly improve the efficiency

of the assay for *T. maritimum*. In the present study, reaction buffers either did not improve the assay or resulted in lowered effectiveness, for example when re-suspended in seawater or broth. The effectiveness of ethidium bromide, the chemical EMA is derived from, is known to be influenced by the presence of salt by either competition between the sodium ions and the dye molecules (Graves, Watkins, & Yielding, 1981) or due to osmotic shock of the salinity (Shi et al., 2011). In the present study, the lowered effectiveness observed is likely due to competition between the dye and the sodium ions in matrices of high salt content, i.e. in seawater or broth. Interestingly, no reductions in efficiency were observed when processing artificially infected skin tissue samples of fish originating from seawater compared to pure culture. However, this would be an important consideration if the sample matrix changed, for example if this protocol was used to detect *T. maritimum* in seawater.

The vPCR showed complete suppression in samples that contained only dead bacteria when the bacterial concentrations were ≤ 10^6 CFU mL^{-1}. It is documented that longer PCR products result in better or complete suppression of amplicons (Seidel, Strathmann, & Nocker, 2017; Banihashemi, van Dyke, & Huck, 2012). This is due to the dye binding in a certain stoichiometry. That is, the probability that a binding event has occurred and inhibition of amplification during PCR is higher when longer amplicons are amplified. Although vPCR has shown to be repeatable and reproducible it was shown to be less reliable than v-qPCR when used on artificially spiked samples at low bacterial concentrations. Additionally, conventional PCR is time consuming, does not lend itself to high-throughput processes and with nested conventional PCR, has an inherent risk of contamination that may produce inaccurate results. For these reasons, a v-qPCR protocol is preferred and was optimised in the present study.

The results of the optimised v-qPCR protocol on artificially and naturally infected tissue samples indicated that the tissue matrix was not interfering with assay performance. The protocol was shown to be robust and transferable with different users producing equivalent results. Testing the v-qPCR on naturally infected tissue demonstrated a lower likelihood of overestimating the infectivity of *T. maritimum* in the sample compared to using qPCR and PCR. The high presence of dead bacteria within these samples could be an artefact of field sampling leading to cross contamination or it could be an accurate result of dead bacteria within the skin lesion. Selection of the

piece of tissue for testing can affect analysis of pathogen viability. Therefore, skin lesions should be sampled from the leading edge as the target pathogen is most likely to be viable and invading the tissue, whereas in the centre of the lesion secondary bacteria are more likely and the target pathogen may no longer be viable (Buller, 2014).

Three of the naturally infected tissue samples were within the LOD of the assay (i.e. < Ct 36). These three samples were found to contain viable cells based on the % Δ Ct and two of them were confirmed positive by culture. Detection by culture from these samples proved difficult with heavy mixed environmental growth and as a result it is possible that the culture result for the one negative sample was inaccurate. Repeat processing of naturally infected tissues with an enrichment protocol prior to v-qPCR and culture may help to clarify these results. An enrichment study to detect *Helicobacter pylori* in naturally infected water samples by Santiago, Moreno, & Ferrús (2015) produced similar results. Santiago et al., (2015) used two methods on samples that were qPCR positive prior to enrichment and negative after enrichment; v-qPCR and direct viable count combined with fluorescent *in-situ* hybridization (DVC-FISH). This showed that the qPCR positive results were from nonviable bacteria and that qPCR is not a suitable indicator for determining the infective potential of a sample.
The artificially and naturally infected tissue samples along with the repeatability experiments performed in this study highlight the difficulties of interpretation of lower bacterial concentrations. The enrichment protocol produced a reliable method to detect viable cells at low bacterial concentrations in artificially spiked skin tissue. Samples that contained only dead bacteria were either not amplified or had a much higher Ct value after enrichment. This effect is unlikely due to the dilution of the sample as the process ensured the same amount of homogenate was used after enrichment. This effect is more likely due to DNA degradation of the heat treated samples. This degradation of DNA during the heat process was demonstrated occasionally in the present study between the Ct results of the live and dead control samples and it is unknown if this same rate of degradation would occur in naturally infected tissue samples. Although the additional enrichment step adds extra time to the process, it remains more efficient than traditional culture methods.

The present study observed a reduced amplification efficiency of the v-qPCR with live cells diluted in a background of dead cells. This could be due to the carryover of dye or dead cell debris from the sample interfering with the PCR reaction. However this is unlikely to affect the results of v-qPCR for detection of viable *T. maritimum* if the concentration of bacteria is above the LOD. When the concentration of the sample is below the LOD, the enrichment protocol should be employed to improve the effectiveness of v-qPCR. As the LOD is unknown prior to testing the sample, it is recommended to use the enrichment protocol as a standard method. The linear range and LOD for v-qPCR and vPCR of *T. maritimum* in the present study is consistent with previous studies (Dinu and Bach, 2012; Maće et al., 2013; Thanh, Agustí, Mader, Appel, & Codony, 2017; Daranas et al., 2018). Similarly, Fittipaldi, Nocker, & Codony (2012) found that v-qPCR for live bacterial concentrations < 10^3 CFU mL^{-1} in the presence of a high number of dead bacteria was unreliable.

The present study has shown the importance of a thorough validation process for both the target pathogen and sample matrix with clearly defined limitations. Live *T. maritimum* in skin tissue, can be detected using vPCR or q-vPCR by calculating the % Δ Ct. The % Δ Ct method has been shown to be a reproducible and reliable method when the amount of cells present are above the LOD of the assay. This sensitivity is expected to be within the range of an infectious dose of *T. maritimum*. The detection limit reported in the present study is below the lowest published infectious doses for this pathogen of 6.36 x 10^5 CFU mL^{-1} (Frisch et al., 2018; Rahman, Suga, Kanai, & Sugihara, 2015; van Gelderen et al., 2010). Moreover, the natural load on the gills of fish infected with *T. maritimum* has been reported to be equivalent of 10^{10} CFU mL^{-1} by qPCR (Downes et al., 2018)

8.5 Conclusion

The developed protocol has shown reliable detection of live *T. maritimum* from skin tissue above 10^3 CFU mL^{-1} in artificially spiked samples. The detection of live *T. maritimum* in naturally infected samples shows the utility of this assay to investigate disease pathogenesis. However, further samples will need to be tested to improve v-qPCR assay confidence. Additionally, as the method can be transferred to a 96 well plate format, high-throughput processing to increase efficiency is possible. An

enrichment protocol is recommended to allow detection of low numbers of live bacteria with and without a background of high concentration of dead bacteria.

Chapter 9 : General Discussion

In the summer months of 2015, mortalities of up to 70% occurred in farmed Chinook salmon in the Marlborough Sounds, termed ‘summer mortalities’. Understanding disease and mortalities of farmed Chinook salmon is crucial for effective management and improving animal welfare, which ultimately leads to industry growth. A lack of understanding of the reasons for these summer mortalities guided the research in this thesis. In summary, this body of research detected bacterial species new to New Zealand (Chapter 2). Certain strains of these bacterial species were shown to have an association with Chinook salmon affected by summer mortalities (Chapter 4) and their pathogenicity was further investigated (Chapters 6 and 7). Additionally, new diagnostic tools were developed to identify these bacteria more effectively (Chapters 3, 5, and 8). Overall, these findings increased our understanding of the newly detected bacteria and advanced the field of fish health in New Zealand.

9.1 Pathogen detection.

Two potential pathogens were detected from Chinook salmon investigated during summer mortalities in 2015; New Zealand rickettsia-like organism (NZ-RLO) and *Tenacibaculum maritimum* (Chapter 2). This was the first detection of NZ-RLO in New Zealand salmonids. While NZ-RLO was detected by PCR, the bacteria visible on histology were not initially able to be confirmed as NZ-RLO by *in-situ* hybridization or transmission electron microscopy (Chapter 3). *Tenacibaculum maritimum* had not previously been associated with disease in fish in New Zealand (Diggles et al., 2002). Furthermore, it was unclear if previous confirmation of *T. maritimum* had been carried out using PCR and nucleotide sequencing. This confirmation is necessary as five other species of *Tenacibaculum* are recognised and biochemical analysis does not always allow for differentiation of the species (Fernández-Álvarez and Santos, 2018). *Tenacibaculum maritimum* in the present study was isolated using bacteriology amongst a mixed culture from a skin ulcer and confirmed by PCR and nucleotide sequencing.

Investigating the relationship of both NZ-RLO and *T. maritimum* with summer mortalities was important as the presence or detection of an infectious agent does not

imply the presence or causation of disease (LaPatra et al 1998). Pathogens can be ubiquitous in the environment or included as part of the microbiome of fish without causing disease. Alternatively, a pathogen can be a primary pathogen causing disease in otherwise healthy hosts due to its virulence or the host immune system not being able to provide adequate defence. It is important to note that it is also possible for a ubiquitous pathogen to normally be asymptomatic, but cause disease if host or environmental factors are altered in a way that favours the pathogen. Additionally, a one pathogen-one disease model does not adequately explain all diseases (Vayssier-Taussat et al., 2015) and it is possible that a pathobiome in combination with the host and environment led to the summer mortalities. Due to the low prevalence of NZ-RLO and *T. maritimum* detected in the analysed fish, there was uncertainty as to the relationship of these pathogens with the observed mortalities.

NZ-RLO is an intracellular bacteria closely related to *Piscirickettsia salmonis*. *Piscirickettsia salmonis* isolates are genetically heterogeneous with isolates grouped into either EM-90-like or LF-89-like (Bohle et al., 2014; Mandakovic et al., 2016). Genetic information of NZ-RLO are incomplete and therefore it cannot be determined which grouping, if any, NZ-RLO may align with. *Piscirickettsia salmonis* is known to cause variable levels of mortality with a range of clinical signs in salmonid and other teleost hosts with the highest mortalities reported in Atlantic salmon farmed in Chile (Sernapesca, 2016). The mortality rates caused by *P. salmonis* differ depending on the strain, the host it is found in, and the environment (House et al., 1999). New Zealand rickettsia-like organisms differ genetically from *P. salmonis.* Chinook salmon is the only salmon species farmed in New Zealand which is in contrast to other salmon producing countries who predominantly farm Atlantic salmon. Furthermore, there are numerous environmental differences between farmed salmon sites globally. Therefore, due to the differences in the organism, the host, and the environment it was unclear whether NZ-RLO infection of Chinook salmon could be expected to have significant similarities to *P. salmonis* infections of other salmonid hosts.

Tenacibaculum maritimum causes disease and mortality in a wide range of hosts globally with juvenile fish likely to suffer more severely (Toranzo et al., 2005). In previous reports of disease due to *T. maritimum*, bacterial colonies were easily identifiable in histological sections of infected tissue (Handlinger et al., 1997) and

bacteria was isolated in pure culture from infected tissue (Cepeda and Santos 2002). In contrast, bacteria were not visible histologically and culture did not generate pure growth of *T. maritimum* from fish investigated during these summer mortalities. Furthermore, qPCR testing indicated low levels of bacterial DNA without the comparative samples growing in culture. The low levels of bacteria suggested that *T. maritimum* may not be causing disease in these fish and instead may have been an incidental finding. Virulence has been shown to differ between isolates of *T. maritimum*, including those with the same sequence type based on analysis of the housekeeping genes (Frish et al., 2018). Therefore, full genome studies are the most efficient way to identify genes accounting for virulence between the different isolates (Pérez-Pascual et al., 2017).

9.2 Distribution and phylogenetic analysis of *Tenacibaculum maritimum* and NZ-RLO within farmed salmon populations.

9.2.1 *Tenacibaculum maritimum*.

Tenacibaculum maritimum was detected throughout farmed salmon populations in both clinically affected and unaffected fish leading to the conclusion that this bacteria was unlikely to be involved in the summer mortalities as a primary pathogen (Chapter 4). However, the involvement of *T. maritimum* as a co-factor or secondary pathogen cannot be ruled out as it has been reported as a significant pathogen of finfish globally causing signs of disease similar to those observed in summer mortalities (Wakabayashi et al., 1984, Carson et al., 1992). Furthermore, seawater temperatures above 15°C have been implicated as a risk factor for infection with *T. maritimum* (Avendaño-Herrera et al., 2006), a consistent finding in the New Zealand summer mortalities.

Genetic analysis using multi-locus sequence typing of *T. maritimum* revealed the New Zealand isolates were identical to isolates from rainbow trout and Atlantic salmon in Tasmania (Chapter 4). However, this does not necessarily mean all of these isolates will have the same pathogenicity (Frish et al., 2018). The relationship of New Zealand *T. maritimum* with Australian isolates coupled with its widespread distribution is indicative of the endemic nature of this bacteria. It is possible that *T. maritimum* arrived

with the importations of salmonids via ova into New Zealand from around the world, including Tasmania, between the late 1800s and early 1900s as *T. maritimum* can be vertically transmitted (Toranzo et al., 2005). Alternatively, it may have been an inhabitant in the New Zealand environment pre-salmonid introduction adapting as a pathogen to New Zealand salmonids over time. *Tenacibaculum maritimum* can also affect non-salmonid marine fish (Avendaño-Herrera et al., 2006), another potential pathway of arrival and dispersal into New Zealand. Providing information on the origin of *T. maritimum* would require further work such as single-nucleotide polymorphisms as demonstrated by a recent study with the fish pathogen *Yersinia ruckeri* (Barnes et al., 2016).

Tenacibaculum maritimum was not associated with clinical disease in fish from all locations where summer mortalities occurred and was detected at low levels from the internal organs in fish analysed from the summer mortality of two salmon indicating infrequent systemic infection. Due to this, *T. maritimum* was considered a secondary pathogen in the summer mortalities and may be contributing to the ill health of these fish. If *T. maritimum* was shown to be an important secondary pathogen in New Zealand Chinook salmon, management of this bacteria may be required. *Tenacibaculum maritimum* is relatively homogenous, however three serotypes have been identified (Avendaño-Herrera, Magriños, Morinigo, Romalde, & Toranzo et al., 2005). If vaccines were to be implemented into New Zealand to manage any diseases caused by *T. maritimum* further characterisation, including knowledge of the serotype, of the New Zealand isolates would need to be determined as there is evidence to suggest vaccines are serotype specific (Romalde et al., 2005).

9.2.2 New Zealand rickettsia-like organism.

New Zealand rickettsia-like organisms were found to be genetically diverse with three strains identified; NZ-RLO1, NZ-RLO2, and NZ-RLO3 (Chapter 4) with NZ-RLO1 and NZ-RLO2 detected from the Marlborough Sounds and NZ-RLO3 detected in Canterbury. The internal transcribed spacer (ITS) region and the 16S rRNA gene (Chapters 2 and 4) were used to determine the strain types. The presence of multiple strains of NZ-RLO may suggest the bacteria has been present in New Zealand for long

enough to have evolved geographical differences. Alternatively, three separate incursions of the bacteria may have occurred over time.

Multiple strains of *P. salmonis* or *piscirickettsia-like* organisms is typical in regions where they are identified, for example Australia (Morrison et al., 2016), Chile (Rozas-Serri et al., 2017), and Ireland (Reid et al., 2004). Analysis carried out in the present study was on two genes which was insufficient to provide information to confirm if NZ-RLO strains are endemic or if they are from more recent incursions. Recently, analysis of NZ-RLO1 and NZ-RLO2 of the predicted proteomes was performed (Gias et al., 2018). Results from this analysis suggests separate incursions were less likely as the New Zealand strains clustered together and were significantly similar to each other rather than to the *P. salmonis* strains they were compared with.

Both NZ-RLO1 and NZ-RLO2 were associated with clinical disease in fish from locations where summer mortalities occurred. In contrast, NZ-RLO3 was detected in fish that were not clinically diseased and from a region where summer mortalities had not been recorded (Chapter 4). Therefore, as NZ-RLO1 and NZ-RLO2 appeared most likely to be primary pathogens in the summer mortalities, their pathogenicity in Chinook salmon was assessed.

9.3 Pathogenicity of NZ-RLO1 and NZ-RLO2

New Zealand rickettsia-like organism 2 appeared to cause higher mortalities with a lower abundance of bacteria than NZ-RLO1 (Chapters 6 and 7). Differences in virulence is also known for different strains of *P. salmonis* (Morrison et al., 2016; Rozas-Serri et al., 2017; Smith et al., 1999; Yuksel, 2003). The NZ-RLO studies were not carried out simultaneously, which would be necessary to confirm any differences in pathogenicity between the two strains.

Fish inoculated with a high concentration of NZ-RLO1 first died at 12 days post inoculation (dpi) with 33% of fish remaining by the end of the study, fish inoculated with a medium concentration of NZ-RLO1 first died at 16 dpi with 75% of fish remaining by the end of the study, and fish inoculated with a low concentration of NZ-RLO1 first died at 14 dpi with 83% of fish remaining by the end of the study. In this study, it

appeared that NZ-RLO1 was not highly pathogenic as the levels of mortality from inoculated fish were not significantly different to the controls. This study also suggested that unless NZ-RLO1 were found in high abundance within fish tissues, it is unlikely that NZ-RLO1 could cause high levels of mortality, for example those seen in the initial summer mortality (Chapter 2). These low levels of mortality in fish infected with NZ-RLO1 are consistent with results from natural infections in a closely related strain; Tasmanian-RLO (Zanaithan, 2012). An increase in mortalities of fish naturally infected with Tasmanian-RLO is often observed when there is a co-infection with the Tasmanian reovirus or increased seawater temperature (Zanaithan, 2012). It is highly likely that temperature is also a risk factor in the New Zealand summer mortalities as mortalities only occur when seawater temperatures are elevated. Due to limited published information on Tasmanian-RLO it cannot be determined if NZ-RLO1 and Tasmanian-RLO are the same.

Pathology observed in fish investigated during the summer mortalities (Chapter 2), where NZ-RLO1 was detected, showed dissimilarities to fish that were inoculated with NZ-RLO1 and died (Chapter 7). Fish inoculated with NZ-RLO1 that died showed high levels of NZ-RLO1 DNA (< 30 Ct), histology consistent with an NZ-RLO1 infection, and visualisation of intracellular NZ-RLO1 within host cells. This high NZ-RLO1 DNA concentration, extensive histology, or visualisation of NZ-RLO1 was not consistently seen in fish investigated during the summer mortalities or fish experimentally inoculated with NZ-RLO1 that survived. These dissimilarities highlight that it cannot be concluded NZ-RLO1 was the primary cause of the summer mortalities. Fish experimentally inoculated with NZ-RLO1 that survived the study showed similar signs of disease to the fish investigated from the summer mortalities, suggesting NZ-RLO1 was likely to be involved in the mortalities but possibly not as a primary pathogen (Chapter 2). It is important to remember that the initial investigation into summer mortalities occurred more than two months post peak mortality (Chapter 2). Delays in testing samples from peak mortality created challenges as the level of the primary pathogen would have reduced due to the highly infected fish perishing. It is feasible that fish sampled at peak mortality would have had a high concentration of NZ-RLO1 DNA and would have showed clear histology consistent with an NZ-RLO1 infection, however this remains unknown. Ideally, sampling would occur throughout mortality

events to increase the understanding of the relationship between any pathogens detected and the mortalities.

If NZ-RLO1 is involved as a co-factor in summer mortalities, it is hypothesised that a low level infection with NZ-RLO1 causes ill health to the salmon and other risk factors then contribute to the elevated mortalities. Fish experimentally inoculated with NZ-RLO1 showed significantly decreased growth rates by the end of the challenge trial compared to the controls. This reduced growth rate would also contribute to the immunosuppression and ill health of the fish. A key risk factor that should be investigated is high seawater temperatures. Summer mortalities occur when the seawater temperatures are at their highest, often over 17°C, which is associated with a high risk of disease for Chinook salmon (Carter, 2005). Additionally, the summer period was a time when NZ-RLO DNA was detected at its highest prevalence (Chapter 4).

If NZ-RLO1 has been present in New Zealand for an extended period of time, Chinook salmon may have evolved adaptive immunity against NZ-RLO1, potentially explaining the lower levels of mortalities. Wild sea-going brood-stock are unable to be obtained by commercial salmon farmers in New Zealand for breeding (Fisheries Act 1996) so adaptive immunity would only be achieved if there was continued exposure of brood-stock or juveniles to NZ-RLO1. Determining if NZ-RLO1 is present in freshwater, where brood-stock and juveniles are maintained, would help to explain any relationship between adaptive immunity and NZ-RLO1 infection. If Chinook salmon does not have adaptive immunity against NZ-RLO1, it may be that NZ-RLO1 is of low pathogenicity. To understand this, virulence mechanisms of NZ-RLO1 and the factors that influence host susceptibility would need to be determined.

Fish experimentally inoculated with NZ-RLO2 resulted in high mortalities with a direct relationship between inoculation dose and onset of mortality. These high mortalities are consistent with levels of mortalities observed in fish infected with *P. salmonis* (House et al., 1999; Rozas-Serri et al., 2017). Fish inoculated with a high concentration of NZ-RLO2 died between 6 and 8 dpi, fish inoculated with a medium concentration of NZ-RLO2 died between 7 and 23 dpi and fish inoculated with a low concentration of NZ-RLO2 first died at 11 dpi with 37% of fish remaining by the end of the study. The

high level of mortality in fish inoculated with NZ-RLO2 may have been due to the unnatural route of infection by intraperitoneal injection. The route of infection directly transferred the pathogen internally, not allowing the fish immune system time to mount a defence. Furthermore, a study conducted comparing signs of disease and mortalities between the intraperitoneal injection and cohabitation route of infection with *P. salmonis* expressed the same levels of mortalities and signs of disease with the cohabitating fish having a delayed onset of mortality (Meza et al., 2019). This suggests the mortality levels and development of disease with NZ-RLO2 may be irrespective of route of infection.

A potentially significant difference between intraperitoneal inoculation and natural infection by NZ-RLO2 is the immune systems role of preventing widespread infection. While it is unknown exactly how NZ-RLO2 infects fish, evidence from *P. salmonis* suggests that bacteria are present externally and the innate and adaptive immune system can prevent entry into the body and dissemination. However, if bacteria gain entry into the body, the bacteria are able to infect white blood cells, replicate and infect more host cells becoming systemic. In fish evaluated from summer mortalities, NZ-RLO2 bacterial DNA was detected most commonly in skin ulcers (Chapter 4) and rarely in the internal organs indicating NZ-RLO2 does not frequently cause a systemic infection via a natural infection. This was in contrast to NZ-RLO1 which was detected most commonly within the internal organs in naturally infected fish. Possibly, the innate immune system can provide adequate protection and defence for NZ-RLO2 at the skin and mucus barrier. However, if NZ-RLO2 does enter the fish as seen in the inoculation studies, the innate and adaptive immune system may not be able to act rapidly enough to defend against NZ-RLO2 and prevent disease. Further studies on the immunity of these fish against NZ-RLO2 would need to be performed to understand any differences of the innate and adaptive immune system in systemic and mucosal immunity. Vaccines targeting the mucosal immune system may increase the antibody response to NZ-RLO within the mucosal layer resulting in increased protection.

Evidence from the inoculation studies suggests NZ-RLO2 is highly pathogenic and likely to be playing a role as a primary pathogen in the summer mortalities. However, this bacteria was not detected when fish were evaluated from the initial summer mortalities (Chapter 2). This absence of NZ-RLO2 may have been due to the low

sample size of 10 fish. If NZ-RLO2 was at a low prevalence within the population, this sample size would be inadequate for confident detection. As fish were analysed after peak mortality, fish infected with NZ-RLO2 may have already perished. Based on the high levels of mortalities observed in the inoculation studies (Chapter 6) it is possible that NZ-RLO2 was the primary cause of the high mortalities with a low prevalence remaining in the surviving population after the peak mortality. Alternatively, NZ-RLO2 may not have been present in the population at this site. This is considered less likely as NZ-RLO2 was identified in affected fish from the same site when testing was conducted five months later on a larger number of samples (Chapter 4). Another explanation for why NZ-RLO2 was not detected may be that it was not involved in summer mortalities and had been found as an incidental environmental organism causing disease when fish were immunocompromised.

These inoculation studies showed NZ-RLO strains have different tissue tropisms. New Zealand rickettsia-like organism 1 was found most abundantly in the liver and NZ-RLO2 was found most abundantly in the pancreatic cells and surrounding adipose using histology. Naturally infected fish were found to contain both strains of NZ-RLO by PCR (Chapter 4). Further inoculation studies with a co-infection of NZ-RLOs would provide an understanding of the combined impact on disease and mortalities. A co-infection may increase disease and mortalities by competition of the two strains within the host, or a cumulative effect of the strains targeting different organs. Co-infections with different strains of *P. salmonis* in the same animals have not been conducted but may account for some of the variation in efficacy of vaccines (Price et al., 2017). Additionally, the role of *T. maritimum* in summer mortalities should be considered. While evidence suggests this bacteria is not directly related to the mortalities, its presence as an important secondary pathogen should be investigated. For further understanding of the summer mortalities a co-infection study should be carried out including NZ-RLOs and *T. maritimum.*

The most consistent external clinical sign of disease in fish from summer mortalities was the presence of skin ulcers. Skin ulcers can be detrimental for fish by causing both a breach in the primary defence against pathogens as well as affecting the regulation of osmotic balance. If skin ulcers are present, fish are more likely to be susceptible to secondary infections through invading bacteria. Additionally, skin

damage is a predisposing factor to infection with *T. maritimum* (Handlinger et al., 1997). Skin ulcers in summer mortalities are likely to be multifactorial and have a range of causes not just pathogens for example, nettle animals, handing damage, fish to fish contact, or net damage. However, during the inoculation studies, skin ulcers developed in some fish at the later stages of the trial. While these studies could not confirm that the skin ulcers were caused by NZ-RLO, they strongly suggest an association of NZ-RLO and skin ulcers (Chapters 6 and 7). Fish inoculated with NZ-RLO1 or NZ-RLO2 developed skin ulcers (21% and 25% respectively) which suggests skin ulcers are not pathognomonic for NZ-RLO infection however it does suggest some skin ulcers of summer mortalities are likely to be due to NZ-RLO. Alternatively, NZ-RLO may alter the skin defences which predisposes fish to infection by other agents that cause skin ulcers. Immersion or patch contact studies would provide valuable information as to the development of skin ulcers caused by NZ-RLO.

The NZ-RLO2 inoculation study suggested the possible impact an increased in water temperature may have on fish infected with NZ-RLO (Chapter 6). This increase was accidental and not a controlled study therefore further work would need to be conducted to draw conclusions. However, it is appealing to hypothesise that an increased seawater temperature in conjunction with an NZ-RLO infection was a likely cause of the summer mortalities. The outbreak of any clinical disease depends on the interaction between the host, pathogen, and the environment, not just the presence of the pathogen (Snieszko, 1974). Increased temperature and disease is commonly reported in association with bacterial infections (Rees et al., 2014; Magnadóttir et al., 1999; Zanaithan, 2012). Summer mortalities occur in the warmest months when the prevalence of NZ-RLO has been observed to be greatest (Chapter 4). If a higher seawater temperature increases susceptibility to infection with NZ-RLO or has an adverse effect on fish infected with NZ-RLO, management strategies to reduce other stressors may reduce mortalities as well as the amplification and spread of this disease. Strategies to minimise risk factors for the emergence of disease may include reduced stocking densities and reduced handling (Murray and Peeler, 2005).

9.4. Future perspectives

Following on from this study, two areas of research to provide further understanding and management of NZ-RLO or *T. maritimum* would be: investigation of potential reservoirs of NZ-RLO and efficacy of vaccines.

9.4.1. Reservoirs

Natural reservoirs of both NZ-RLO and *T. maritimum* in New Zealand are currently unknown. Chinook salmon show signs of infection two to three months after transfer to seawater (New Zealand King Salmon, pers. comm.). This suggests a reservoir maintaining bacteria for re-infection is required in the marine environment if the disease is not vertically transmitted or present in freshwater. Transfer from freshwater to seawater is a stressful period in the production cycle of farmed Chinook salmon where as well as stress, scale loss, and immunosuppression often develop (Franklin, Davison, & Forster, 1992). These factors may increase the chance of infection by any pathogenic bacteria that the fish may be in contact with.

Reservoirs within the marine environment may exist in biofouling assemblages, organisms on the sea floor, transient wild fish populations, salmon already on the farm, or particles within the water column. Sea-pen nets are prone to biofouling which restrict water flow, reducing the amount of oxygen fish receive (Floerl, Sunde, & Bloecher, 2016). A practice of New Zealand salmon farms is to regularly clean biofouling organisms off nets that, once removed, remain in the environment and settles under the sea-pens where pathogens can replicate and therefore re-infect fish. Furthermore, net cleaning dislodges fine particulates in suspension as well as potential nematocysts from anemones and jellyfish, which can be a part of a biofouling assemblage, leading to stings and gill irritation of salmon in the pens (Floerl et al., 2016). In addition, nettle-like animals can cause a breach in the skin, leading to an entry point for pathogenic infection by NZ-RLO, *T. maritimum*, or other pathogens. There is also some evidence that nematocysts or jellyfish can be carriers or vectors of disease. Research from Ferguson et al., (2010), demonstrated jellyfish as a potential vector for *T. maritimum* and it remains a possibility they could also be a vector for NZ-RLO. Moreover, isopods and copepods were present on fish investigated during the summer mortalities

(Chapter 2) and have been suggested vectors of bacteria (Barker, Braden, Coombs & Boyce, 2009). There is evidence that NZ-RLO can grow on insect cell lines (Ministry for Primary Industries, unpublished data) suggesting crustaceans such as copepods or isopods could transmit NZ-RLO. The isopods or copepods found on fish during the summer mortalities were not tested for *T. maritimum* or NZ-RLO and their capability to harbour these pathogens remains unknown.

The host range of NZ-RLO is currently unknown and it is probable that this bacteria does not only infect salmonids. Closely related strains to NZ-RLO, *P. salmonis* and piscirickettsia-like organisms, are not host specific and are known to infect the following non-salmonid hosts: white seabass, *Atractoscion nobilis,* black seabass, *Dicentrarchus labrax* (M.F., Chen et al., 2000; Mauel et al., 2005; Comps et al., 1996; Athanassopoulou et al., 2004), tilapia, *Oreochromis* species. (Iregui et al., 2011; Mauel et al., 2003; Mauel et al., 2005) and grouper, *Epinephelus melanostigma* (S.C., Chen et al., 2000). *Tenacibaculum maritimum* is also not host specific and includes both salmonid and non-salmonid hosts, for example: Turbot, *Scophthalmus maximus* (Devesa, Barja, & Toranzo, 1989; Pazos, Santos, Núñez, & Toranzo, 1993), rainbow trout, striped trumpeter, *Latris lineata*, and greenback flounder *Rhombosolea tapiriña* (Handlinger et al., 1997). As *T. maritimum* is not host specific and it remains possible that NZ-RLO is not either, wild fish that come into contact with farmed salmon may be possible reservoirs for these bacteria.

Identifying if there is a reservoir for NZ-RLOs and *T. maritimum* may enable management practices for removal or mitigation of re-infection. If a reservoir cannot be determined, a practice that can reduce amplification of bacteria in the environment, and therefore disease, is fallowing and year class separation. This period of time for fallowing will differ depending on the target disease to be reduce. Fallowing reduces the probability of disease transfer to the next production generation by creating a break in the disease cycle (Midtyng, Grave, & Horsberg, 2011). Fallowing has been used in the management of *P. salmonis* with a time of 50 days to three months suggested (Olivares and Marshall, 2010; Price et al., 2017) and is part of best biosecurity practices for aquaculture (Georgiades, Fraser, & Jones, 2016). Separation of year classes works in a similar way by breaking the disease cycle through prevention of

disease transfer from mature fish to generally more susceptible and stressed juvenile fish during the transition to seawater.

9.4.2. Vaccines

This thesis has shown that NZ-RLO1 and NZ-RLO2 can be pathogenic to Chinook salmon and therefore likely to be contributing to the summer mortalities and the ill health of the fish. Due to this pathogenicity, vaccines to protect against NZ-RLO infection are currently being used in New Zealand. The vaccine in use has been designed to protect Atlantic salmon against *P. salmonis*. The efficacy of this vaccine in preventing NZ-RLO infection of Chinook salmon has not been determined and no clinical trials conducted. Efficacy of vaccines depends on the time and frequency of administration (Figueroa et al., 2017; Maisey, Montero, & Christodoulides, 2017; Tandberg et al., 2017). Studies have also suggested that when fish are co-infected, protection offered by the vaccine is reduced and it has been suggested that the co-infection is likely to dominate over the protective effects of the pathogen that is vaccinated for (Figueroa et al., 2017). This is an important reason why efficacy trials should be carried out in both a laboratory environment and in the field. Evaluating the efficacy of the vaccine in fish infected with both strains of NZ-RLO and fish infected with both NZ-RLO and another pathogen, for example *T. maritimum* should be a priority. If the available commercial vaccines are not effective against NZ-RLO, there is little value in subjecting the fish to the additional stress of vaccination. Indeed, the additional stress of vaccination may increase the risk of infection by NZ-RLO by causing a transient immunosuppression (Jensen, 2018). Whole genome sequencing to determine the complete genome of both NZ-RLO1 and NZ-RLO2 would provide information to improve vaccine development for specific strains of NZ-RLO. This could be achieved by identifying surface-exposed antigens that could be directly involved in pathogenesis of the organism to be used as vaccine candidates (Moriel et al., 2008). Alternatively, comparative genomics could be used to determine the difference in virulence mechanisms between strains of the same pathogen. This analysis could also help to appreciate if vaccines protective against *P. salmonis* would protect against NZ-RLO in Chinook salmon.

If the current vaccines are proven inefficacious, antibiotics targeting skin could be evaluated to reduce NZ-RLO infection. However, antibiotic success against bacterial pathogens is variable (Price et al., 2016) and may lead to pathogen resistance over time (Miller and Harbottle, 2018). Furthermore, antibiotic usage can have a negative impact on the marketing of New Zealand salmon that are often marketed on an antibiotic free industry (Wilson, 2013).

9.5 Summary statement

To be able to effectively manage diseases in aquaculture, an understanding of the pathogen(s) is crucial. This research has shown that NZ-RLO1 and NZ-RLO2 can cause disease and mortalities in Chinook salmon within a laboratory environment. The signs of disease produced in these experimental infections showed similarities to those fish naturally affected during summer mortalities, suggesting NZ-RLOs were likely to be involved. This research has also offered new diagnostic tools for the detection of NZ-RLOs and *T. maritimum*. These diagnostic tools will be important for the continued monitoring of these pathogens in farmed Chinook salmon populations. This research will provide valuable information for the aquaculture industry, fish health specialists, diagnostic laboratories, and fish disease scientists in New Zealand. While this research improves our knowledge and understanding, further work is needed to build upon these initial studies to improve the health and welfare of farmed Chinook salmon for the continued growth of this industry.

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Appendix

List of publications and statement of contribution

Brosnahan, C.L., Ha, H.J., Booth, K., McFadden, A.M.J., Jones, J.B. (2017) First report of a rickettsia-like organism from farmed Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), in New Zealand. *New Zealand Journal of Marine and Freshwater Research,* 51, 356-369.

Brosnahan, C.L., Munday, J., Ha, H.J., Preece, M., Jones, J.B. (2018). New Zealand rickettsia-like organism (NZ-RLO) and *Tenacibaculum maritimum*: Distribution and phylogeny in farmed Chinook salmon (*Oncorhynchus tshawytscha*). *Journal of Fish Diseases,* 42, 85-95.

Brosnahan, C.L., Davie, P.S., Munday, J.S., Kennedy, L., Preece, M., Barnes, S., Jones, J.B., McDonald, W.L. (2019) Pathogenicity of the bacterium New Zealand rickettsia-like organism (NZ-RLO2) in Chinook salmon (*Oncorhynchus tshawytscha*, Walbaum) smolt. *Diseases of Aquatic Organisms*, 134(3), 175-187.

Brosnahan, C.L., McDonald, C., Georgiades, E., Keeling, S.E., Jones, B.J. (2019). Optimisation and validation of a PCR to detect viable *Tenacibaculum maritimum* in salmon skin tissue samples. *Journal of Microbiological Methods,* 159, 186-193.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Cara Brosnahan
Name/title of Primary Supervisor:	John Munday
Name of Research Output and full reference:	
Brosnahan, C.L., Ha, H.J., Booth, K., McFadden, A.M.J., Jones, J.B. (2017) First report of a rickettsia-like organism from farmed Chinook salmon, Oncorhynchus tshawytscha (Walbaum), in New Zealand. New Zealand Journal of Marine and Freshwater	
In which Chapter is the Manuscript /Published work:	Chapter 2
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	80
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
Experimental design, conduction of tests, analysis and production of manuscript.	
For manuscripts intended for publication please indicate target journal:	
See above	
Candidate's Signature:	
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Primary Supervisor's Signature:	John Munday Digitally signed by John Munday Date: 2019.10.30 09:09:31 +13'00'
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Name/title of Primary Supervisor:	John Munday
Name of Research Output and full reference:	
Brosnahan CL, Munday J, Ha HJ, Preece M, Jones JB. (2018). New Zealand rickettsia-like organism (NZ-RLO) and Tenacibaculum maritimum: Distribution and phylogeny in farmed Chinook salmon (Oncorhynchus tshawytscha). Journal of Fish Disease	
In which Chapter is the Manuscript /Published work:	Chapter 4
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and	
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For manuscripts intended for publication please indicate target journal:	
See above	
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Brosnahan CL, Davie PS, Munday JS, Kennedy L, Preece M, Barnes S, Jones JB, McDonald WL. (2019) Pathogenicity of the bacterium New Zealand rickettsia-like organism (NZ-RLO2) in Chinook salmon (Oncorhynchus tshawytscha, Walbaum) smolt	
In which Chapter is the Manuscript /Published work:	Chapter 6
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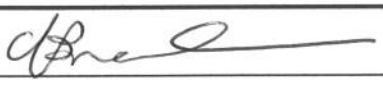
Candidate's Signature:	
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STATEMENT OF CONTRIBUTION
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We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Cara Brosnahan
Name/title of Primary Supervisor:	John Munday
Name of Research Output and full reference:	
Brosnahan CL, McDonald C, Georgiades E, Keeling SE, Jones BJ. (2019). Optimisation and validation of a PCR to detect viable Tenacibaculum maritimum in salmon skin tissue samples. Journal of Microbiological Methods, 159, 186-193.	
In which Chapter is the Manuscript /Published work:	Chapter 8
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