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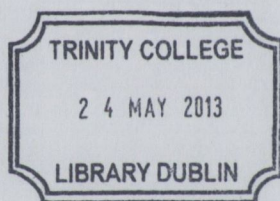
**The study of gill disease of cultured Atlantic salmon (*Salmo salar*, L.) in
Ireland with an emphasis on the role of epitheliocystis as a causative agent:
descriptive pathology, aetiology and epidemiology**

Susie Mitchell



**A thesis submitted in the fulfillment for the Degree of Doctor in
Philosophy to Trinity College, University of Dublin**

October 2011



Thesis 10035

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Summary

Gill disorders have emerged as one of the most important health issues in the farming of Atlantic salmon (*Salmo salar*) in Ireland in recent years. The aetiology of these conditions is frequently multifactorial. Environmental challenges such as harmful algal blooms and zooplankton swarms can be involved, in addition to a range of infectious agents. The importance of some of these agents in terms of fish health remains to be elucidated. One such agent is the causal organism of epitheliocystis. Epitheliocystis is an infectious condition that affects the gills and, less commonly, the skin of fish. It has been reported from more than 50 freshwater and marine species. It is characterised by intracellular cysts packed full of bacteria. Most of these bacteria to date have been identified as different members of the Order Chlamydiales. The different bacteria responsible for causing epitheliocystis in Atlantic salmon in Ireland were investigated using a combination of molecular techniques, histopathology and fluorescence *in situ* hybridization. The epidemiology of the condition and the pathology associated with different agents of epitheliocystis was described. A novel gill histopathological scoring system was developed during the study to enable pathology to be graded and compared over time and between cases.

Using 16s rRNA sequence data, the causative agent of epitheliocystis in freshwater Atlantic salmon was identified as '*Candidatus* Clavochlamydia salmonicola'. Prevalence of infection in four sites ranged from 75-100% and infection intensity was highly variable. No significant pathology was associated with infections. As a diagnostic tool molecular methods proved marginally more sensitive than histopathology. The fate of '*Candidatus* Clavochlamydia salmonicola' in Atlantic salmon post-seawater transfer was investigated by means of a 12-week marine longitudinal study. Both RT PCR and histopathology indicate that the organism disappears from the gills 4-6 weeks post-transfer. No evidence for a role of these *Chlamydiae* as agents of disease was observed.

Until very recently, no non-chlamydial species have been positively associated with epitheliocyst infections in fish. Investigations to identify the causal agent of epitheliocystis in the gills of diseased Atlantic salmon in a seawater farm on the west coast of Norway revealed a novel bacterium was responsible for at least some of the intracellular cysts. "*Candidatus* Branchiomonas cysticola" is the name currently proposed for this bacterium. A real-time PCR for this novel bacterium was developed during this study and samples from a number of farms in Norway and Ireland were investigated for the presence and levels of "*Ca. B. cysticola*". A significant association with it and the levels of cysts

observed on histopathology was uncovered. In many cases highly significant pathology was also associated with the cysts. Fluorescence *in situ* hybridization (FISH) using multiple probe hybridizations at different taxonomic levels provided further convincing evidence that "*Ca. B. cysticola*" was the main agent of epitheliocystis in many of the cases examined from both Norway and Ireland.

Both the transmission and mode of infection of the agents of epitheliocystis remain undetermined. Culture of intracellular bacteria such as chlamydiae is usually performed using appropriate cell lines or axenic amoebal culture. Despite numerous attempts no successful culture has been documented with any of the agents of epitheliocystis. Despite this, a challenge model was developed during this study. Transmission of infection was attempted by direct inoculation of the gills of challenge fish with gill material from infected fish. Even though the challenge was unsuccessful, the results may indicate that "*Ca. C. salmonicola*" requires an intermediate host to facilitate transmission. Further research will be required to elucidate this.

The work presented in this thesis has provided some novel findings on the epidemiology and pathology of epitheliocystis infections. Collectively these results significantly advance our knowledge of both the role of epitheliocystis in gill disease, and what areas of research should be prioritized to further our understanding of gill disorders in Atlantic salmon in the future.

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1. **Atlantic salmon, *Salmo salar* L., farmed in fresh water in Ireland is associated with "*Candidatus* *Clavochlamydia salmonicola*" infection.** *Journal of Fish Diseases*, 2010.
2. **A review of infectious gill disease in marine salmonid fish.** *Journal of Fish Diseases*, 2011.
3. **Gill pathology in farmed salmon associated with the jellyfish *Aurelia aurata*.** *Veterinary Record*, 2011.
4. **A novel histopathological gill scoring protocol for assessment of gill health during a longitudinal study in marine-farmed Atlantic salmon (*Salmo salar*).** *Aquaculture International*, 2011.
5. **Non-infectious gill disorders of marine salmonid fish.** *Reviews in Fish Biology and Fisheries*, 2011.
6. **Gill disease in marine farmed Atlantic salmon at four farms in Ireland.** *Veterinary Record*, 2011.
7. **A novel betaproteobacterial agent of gill epitheliocystis in seawater farmed Atlantic Salmon (*Salmo salar*).** *PLoS ONE* 7(3):e32696.doi:10.1371/journal.pone.0032696, 2012.

1 Introduction and Literature Review

1.1 Introduction

Although the rate of growth of the aquaculture industry has slowed in the last few years it remains the fastest growing food-producing animal sector and is now estimated to supply approximately half of the total world fish food supply. Globally aquaculture is a massive industry and provides income and subsistence for many, in addition to a good quality supply of protein for billions of consumers worldwide (FAO, 2010). The total marine aquaculture production in 2009 has been estimated at approximately 20 million tonnes (if China is excluded this figure is reduced to 8 million tonnes).

Atlantic salmon (*Salmo salar* L.) is an important aquaculture species, particularly in the developed world. Sea cage culture of Atlantic salmon first began in the 1960s in Norway where early success prompted its spread to Scotland, Ireland, the Faroe Islands, Canada, the USA, Chile and Australia (Tasmania). Minor production also occurs in New Zealand, France and Spain. Farming of marine Atlantic salmon is a growing industry, and annual production is now approaching 2 million tonnes. Farmed Atlantic salmon currently constitute greater than 50 percent of the total global salmon market (FAO, 2010).

As the salmon farming industry has expanded, it has encountered several challenges. Disease currently provides a substantial cost to the industry in terms of treatments, mortality and loss of production. In addition to this, as individual farms have increased in size, disease problems have been compounded. There are a number of economically important diseases of Atlantic salmon of current concern, including sea-lice infestation, and infections with viruses such as salmonid alpha virus and infectious pancreatic necrosis virus (FAO, 2010). Depending on the year and environmental conditions, gill disease can also be a significant source of losses (Rodger, 2007).

The impact of gill disease in marine-farmed Atlantic salmon, *Salmo salar* L., has increased in Europe in the last decade, with Ireland, Norway and Scotland all reporting substantial losses (National Veterinary Institute, 2009; Rodger, 2007; David Cockerill, personal communication). In Ireland alone, it was the leading cause of mortality in farmed Atlantic salmon from 2003 – 2006

(Rodger, 2007), in addition to substantial production losses associated with sub-clinical disease.

There are numerous aetiological agents considered to be involved in or responsible for gill disease, and there are a range of clinical and pathological presentations (National Veterinary Institute, 2009; Rodger, 2007; Nowak and LaPatra, 2006; Roberts and Rodger, 2001). Gill disease has been responsible for large scale mass mortalities as well as poor growth and performance in farmed salmon and has been a serious financial burden for some sectors of the industry. The aetiology of these conditions is often multifactorial. Environmental challenges such as harmful algal blooms and zooplankton swarms can be involved, in addition to a range of infectious agents (Rodger, 2007). The importance of many of these agents in terms of fish health remains to be elucidated.

The agents that cause epitheliocystis are an example of this. Epitheliocystis is an infectious condition that affects the gills and, less commonly, the skin of fish. It has been reported from more than 50 freshwater and marine species (Nowak and LaPatra, 2006). It is characterised by intracellular cysts packed full of bacteria in the gills and less frequently the skin epithelia. Until recently, the bacteria responsible have been identified exclusively as different members of the Order Chlamydiales.

Most of the work on epitheliocystis has so far been limited to case studies and ultrastructural descriptions of the aetiological agent. Information regarding the epidemiology and pathogenicity of these organisms has not really progressed since the early investigations. Significant advances have, however, been achieved in the development of molecular tools to identify the species of bacteria involved (Draghi *et al.*, 2010, Polkinghorne *et al.*, 2010; Karlsen *et al.*, 2008; Meijer *et al.*, 2006; Draghi *et al.*, 2004). There is a substantial gap in knowledge regarding epitheliocystis and its impact on fish health, particularly in salmonids. In particular, its interactions, synergistic or otherwise, with other known gill pathogens of salmonids and also adverse environmental factors, has not been explored.

In the last number of years epitheliocystis has become a focus for attention in farmed Atlantic salmon in Ireland, Scotland and Norway, where it has been associated with numerous cases of gill-related mortality and disease. Substantial mortalities and loss of production in Norway have been attributed to proliferative gill inflammation (PGI), a gill disease of complex aetiology in which epitheliocystis is thought to play a part (Kvellestad *et al.*, 2005). Although PGI has not officially been

diagnosed outside Norway, disease syndromes with a similar clinical picture have been observed in clinical cases in both Ireland and Scotland (Hamish Rodger, pers. comm., Dave Cockeril, pers. comm.).

The aims of the present study were to further our understanding of gill disease in farmed Atlantic salmon in general, with a focus on the role of epitheliocystis. We sought to explore the epidemiology and pathogenicity of epitheliocystis infections through longitudinal studies on selected sites and reactive sampling during outbreaks. The identity of the different causative agents of epitheliocystis in both freshwater and marine salmon in Ireland was established through molecular methods and fluorescence *in situ* hybridization. Gross and histopathology of epitheliocystis infections and interactions with other gill pathogens and environmental phenomena were investigated and described. A novel gill histopathological scoring system was devised enabling assessment of the severity of any gill pathology observed and to allow comparisons between cases. Finally attempts were made to create a viable challenge model to demonstrate infection dynamics of the freshwater agent of epitheliocystis, '*Candidatus* Clavochlamydia salmonicola'.

1.2 Literature Review

There are a range of different microbes associated with the gills in Atlantic salmon including a variety of parasites, viruses and bacteria. While the importance of many of these is clear in terms of disease, the significance of certain agents remains obscure. There are also several non-infectious causes of gill disease for farmed salmonids. These can be grouped into harmful algae, such as *Karenia mikimotoi*, harmful zooplankton, such as *Pelagia noctiluca*, other environmental challenges, such as pollutants, as well as nutritional and genetic or congenital causes. The interactions between infectious and non-infectious agents of gill disease have rarely been comprehensively explored, and the significance of many species of zooplankton in particular requires elucidation. This comprehensive review summarises much of the currently available published literature on both infectious and non-infectious causes of gill disease, in addition to the response of the gill to injury, assessment of gill histopathology and a focus on the disease condition epitheliocystis.

The salmonid gill – structure, function and response to injury

The salmonid gill is a complex vascular structure that functions primarily as a gas exchange organ, but also has important osmoregulatory and excretory functions. Salmonids have four pairs of gills or holobranchs, which are made up of filaments. The surface area of these lamellae is further increased by multiple secondary lamellae, situated at right angles to the primary lamellae. This creates a massive total surface area where the fish is in intimate contact with the environment. The squamous epithelium layer on the lamellae is one cell thick, providing a very thin cellular barrier between the blood and the external fluids. The cores of the lamellae are well vascularised and the position of the secondary lamellae allow for extremely efficient gas exchange via a counter-current flow system (Figure 1) (Perry and Laurent, 1993).

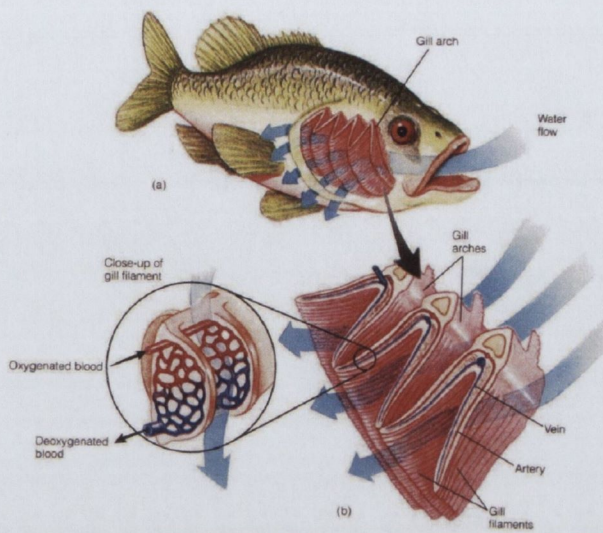


Figure 1. Illustration of the counter current flow system in a fish for oxygen uptake. a) Water flows into the buccal cavity and is pumped across the gills by the action of the operculae. b) Close up of the primary lamellae indicating the direction of water flow and the oxygenation of the lacunar capillaries (picture adapted from <http://www.betavak.nl/biologie/kieuw.htm>).

Other important functional cells that are present in the epithelium include structural pillar cells, mucous-producing goblet cells which produce the thin surface mucus layer of the gills and chloride cells which are involved in osmoregulation. The gills do not solely function as a gas exchange organ but are also the principle excretory organs in fish, where most of the nitrogenous waste is eliminated. They are also responsible for regulation of acid-base balance and body fluid pH. Finally, the gills serve to counter the effects of the osmotic and ionic gradients that provide an ongoing challenge to the fish in the aquatic environment (Perry and Laurent, 1993; Roberts and Rodger, 2001).

The mucus layer produced by the goblet cells on the surface of the lamellae is extremely important in terms of the first line of defence of the gills. It protects the epithelium from toxins, pollutants and other noxious substances and also provides a physical and chemical barrier to the entry of pathogens. The mucus serves to trap pathogens and with a high rate of turnover removes them from the gill surface. In addition to this, the mucin that makes up the layer contains many anti-microbial peptides, lysozymes, lectins and proteases (Ellis, 2001). Innate immune mechanisms are thought to be more important in the gills than any specific responses (Ellis, 2001).

The responses of the gill to injury are remarkably conserved, regardless of the initial insult. Epithelial hyperplasia, lamellar fusion and the presence of necrotic or degenerating cells are three commonly observed histopathological changes, regardless of the insult (Roberts and Rodger, 2001). Even though host cell proliferation in the form of epithelial hyperplasia may be induced by and even benefit intracellular pathogens, it has been suggested that this is actually a protective response, being a means of reducing the surface area of the gills available for pathogen colonization and/or toxic substances (Speare and Ferguson, 2006). Unfortunately the reaction is often excessive and results in severe compromise to the physiological functions of the fish. Hyperplastic and fused gill filaments have a greatly reduced surface area for gas exchange, excretion and osmoregulation. Another common response of the gills to injury is an increase in mucus production, and a change in the quality of the mucus in terms of an increase in viscosity. While having clear protective benefits, excessive mucus production can also result in impeding gill function, by effectively increasing the distance between the gill vasculature and the environment.

One of the most outstanding characteristics of gill epithelia is its ability to heal after a gill disease event. Complete reversal of the pathological changes can occur in time, as long as the host is not overly compromised systemically and the environmental conditions are optimal. This healing occurs remarkably without any scarring or fibrosis (Ferguson, 2006).

Assessment and description of gill health and disease

From a clinical perspective, gill disease can be described through a combination of behaviour, clinical signs and gross- and histopathology. Fish may display increased respiratory rate, flaring of operculae, swimming at an elevated position in the water column or gathering near a water inflow (where the water flow rate is highest), coughing, increased mucus production on the gills, bleeding in the anesthetic bin while being examined, a reduction in feeding or finally, an increase in mortality, all of which may indicate the presence of gill disease (Rodger, 2007). However, histopathology is generally the most definitive means of diagnosis, as in many cases clinical and gross pathological signs can be obscure and significant lesions may only be visible on microscopy. Scoring systems are frequently employed in both veterinary and human histopathology to grade the severity of disease (Brink *et al.*, 2010; Westin *et al.*, 1999). Few such systems have been employed in fish to date, the

exception being studies on gonadal alterations post exposure to endocrine disrupting chemicals in species such as the fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*) and the zebrafish (*Danio rerio*) (Johnson *et al.*, 2009). Bernet *et al.* (1999) described some useful histopathological alterations in the gills as part of a multi-organ scoring protocol for aquatic pollution, however some of the criteria are quite complex, and geared towards the effects of toxins on gill epithelia.

In general, the use of scoring and grading in histopathology aims to facilitate treatment decisions and non-biased comparisons of results from different studies. Optimization of reproducibility and reliability between observations is crucial for a system to be useful. The basic parameters should be easy to identify and selected as criteria that are usually affected in response to an insult. The response of gill epithelia to injury is relatively conserved which favors the use of such a system. As mentioned above, epithelial hyperplasia, lamellar fusion and the presence of necrotic or degenerating cells are the three most commonly observed histopathological changes in the gills (Roberts and Rodger, 2001). To be useful, a scoring system must find the appropriate balance between complexity and practicality. A suitable gill scoring system in piscine histopathology could provide a valuable tool for the clinician in aiding the farmer to make management decisions about his fish, such as those related to treatments, feeding or oxygenation strategies. It could also be applied in clinical research or field trials involving studies on gill health and pathology, to further our understanding of disease in these tissues.

Non-infectious causes of Gill Disease

Harmful algae

Reports of algal blooms affecting finfish or salmonid culture are numerous. Algal blooms can be termed 'harmful' when these occurrences impact on human public health, wildlife and fisheries or aquaculture. Approximately 4,000 marine microalgae have been described to date. Of these between 60 and 80 species are identified as being toxic and 200 species as potential causes of harmful algal blooms (HABs) (Sournia, 1995; Smayda, 1997). Some of the main species which have been associated with pathology and mortality in fish are listed in Table 1.

Table 1. Examples of marine phytoplankton species associated with fish mortalities (modified from Rodger *et al.*, 2011).

Phytoplankton species	Finfish affected	Country/Region	Details
<i>Karenia mikimotoi</i>	<i>Gadus morhua</i> , <i>S. salar</i>	Ireland	Cod hatchery juveniles killed, 500 mortalities of farmed salmon
	<i>S. salar</i>	Scotland	Gill damage in farmed salmon
	<i>S. salar</i> , <i>O. mykiss</i>	Norway	2.6 to 100% mortalities farmed fish
<i>Karenia brevisulcata</i>	<i>Sardinops sagax</i>	New Zealand	0.5 tonne pilchards killed
<i>Cochlodinium polykrikoides</i>	Various farmed species	Korea, Japan, China	
		Philippines	Wild reef fish
<i>Gonyaulax excavata</i>	<i>S. salar</i>	Faroe Islands	
<i>Karlodinium micrum</i>	<i>Mugil cephalus</i> , <i>Sciaenops ocellatus</i>	South Carolina	Retention pond fish kill
	<i>Morone saxatilis</i> x <i>chrysops</i>	Chesapeake Bay	15 000 2-2.75kg fish in one day
<i>Gymnodinium</i> sp	<i>Liza macrolepis</i> ,	Arabian Gulf	Farmed and wild fish
	<i>Acanthopagrus cuvieri</i>		
<i>Chaetoceros wighami</i>	<i>S. salar</i>	Scotland	550 000 fish died or were culled, 44 tonnes mortality
<i>Chaetoceros convolutus</i>	<i>Oncorhynchus kisutch</i>	British Columbia	60 000 40g fish within one week,
<i>Ceratium furca</i>	<i>S. aurata</i> , <i>Liza klunzingeri</i>	Arabian Gulf	Fish kill of wild mullet & farmed bream
<i>Ceratium furca</i>	<i>S. salar</i>	Scotland (Skye)	2.69T of 4kg fish died (670 fish)
<i>Ceratium fusus</i>	Various species	Japan	Farmed fish

<i>Skeletonema sp.</i>	<i>S. salar</i>	British Columbia	Farmed fish (4% mortality, approx. 16,000 fish, mixed bloom)
<i>Chrysochromulina polylepis</i>	<i>S. salar</i>	Scandinavia	Farmed and wild fish
<i>Heterosigma akashiwo</i>	<i>Oncorhynchus tshawytscha</i>	New Zealand, Japan	Farmed fish
<i>Alexandrium tamarense</i>	<i>S. salar</i>	Nova Scotia	Farmed fish
<i>Pseudonitschia sp.</i>	<i>S. salar</i>	British Columbia	Farmed fish

Algal blooms are naturally occurring phenomena, however, in recent decades there are suggestions that there has been an increase in the frequency and intensity of these blooms (Daranas *et al.*, 2001, Anderson, 2009). Hallegraef (1995) proposed four main explanations for this:

- 1) Eutrophication of coastal waters due to anthropogenic activities possibly in combination with unusual meteorological conditions
- 2) Increased practice of aquaculture in coastal waters
- 3) Increased awareness amongst scientists of toxic planktonic species
- 4) Movement of resting forms of toxic planktonic species between regions, for example, in ballast waters of boats (Bolch and Salas, 2007; Edwards *et al.*, 2001).

Although questions remain with regard to the suggestions that harmful algal blooms are increasing in frequency and geographic extent, there is currently much interest in such blooms in the scientific community, the general public and governments (Granéli and Turner, 2006).

The financial cost of algal blooms is a significant concern to the aquaculture industry. Taylor (1993) reported blooms of *Chaetoceros sp.*, *Heterosigma sp.* and another undescribed chloromonad that cost the salmonid farming industry in British Columbia approximately \$14 million between 1988 and 1993. In Korea, approximately \$1.31 million per year was estimated as the financial impact incurred by HABs to aquaculture (Kim, 2006). Imai (2005) reported that the average economic loss due to HABs is over \$10 million a year in Japan.

The clinical signs in farmed salmonids associated with HABs include fish swimming in an elevated position in the water column, lethargy, gasping at the surface, increased respiratory rate and mortalities. There may be excessive mucus production on the gills or gills may have small areas

of thickening which appear as pale areas or spots (Rodger, 2007). Small blood spots or petechiae are seen with some irritant phytoplankton, however, in some cases with toxic algae the gills may show no gross abnormalities. Bleeding, from the gills during examination under anaesthesia may also present as a sign. Sometimes there are associated mortalities in wild fish stocks and invertebrate benthic species associated with harmful blooms (Silke *et al.*, 2005, Mitchell and Rodger, 2007). Algal blooms can cause the affected waters to become intensely discoloured and while they have been frequently associated with 'red tides' the discolouration can in fact vary from red to white or from golden brown to emerald green (Zingone and Enevoldsen, 2000).

Histopathological observations of gill lesions caused by HAB events include acute necrosis, sloughing of epithelial cells, severe oedematous separation of the epithelium from the lamellar branchial vessels, necrosis of epithelial cells (Jones *et al.*, 1982; Fernández-Tejedor *et al.*, 2004; Mitchell and Rodger, 2007), swelling and pyknosis of primary lamellar epithelium and congestion of branchial vessels (Roberts *et al.*, 1983). In addition to the gill pathologies there may also be liver and gastrointestinal histopathologies observed (Mitchell and Rodger, 2007).

Black *et al.*, (1991) surmised that there are four main mechanisms by which fish mortalities can be caused by harmful algal blooms: (i) physical damage, (ii) asphyxiation due to oxygen depletion, (iii) gas-bubble trauma due to oxygen super-saturation caused by algal photosynthesis, and (iv) ichthyotoxin damage.

(i) Physical damage caused by HABs

Several species of marine diatom have been implicated in mortalities of farmed salmonids. These species include *Chaetoceros* sp. (Albright *et al.*, 1993; Yang and Albright, 1992), *Skeletonema* sp., *Thalassiosira* sp., *Thalassionema* sp., *Coscinodiscus* and *Pseudonitzschia* (Kent *et al.*, 1995; National Veterinary Institute 2009). Phytoplankton such as *Chaetoceros* sp. possess harmful setae with spicules (Albright *et al.*, 1993) which can cause abrasion of the epithelial surfaces. This irritation causes overproduction of mucus by the goblet cells. Physical damage to the epithelium then predisposes the fish to secondary infection with bacteria as demonstrated by Albright *et al.*, (1993), and potentially other pathogens such as protist parasites and amoebae.

(ii) Asphyxiation by HABs

Despite the fact that *Kareina mikimotoi* produces ichthyotoxins during blooms (Satake *et al.*, 2002, Satake *et al.*, 2005) mortalities due to this planktonic species are suggested to be due in part to decreased levels of oxygen saturation resulting from the decomposition of the algae in the later stages of the bloom (Silke *et al.*, 2004). In addition to this, oxygen conditions are further reduced by the respiration of the algae themselves and also of the bacteria involved during the bloom breakdown. Consequently oxygen shortages primarily occur overnight although overcast weather in daylight may incur similar effects (Bruno and Poppe, 1996).

Mechanical clogging of the gill lamellae may occur. Jones and Rhodes (1994) reported that the deaths of numerous pilchards (*Sardinops sagax*) was attributed to both this and reduced dissolved oxygen levels during a bloom of the green microalgae, *Tetraselmus* sp. in a small lagoon in New Zealand.

(iii) Gas-bubble trauma

Super-saturation is a phenomenon that is occasionally associated with algal blooms. In extreme cases dissolved oxygen levels can reach a few hundred percent and potentially cause gas bubble disease (Roberts, 2001; Speare, 1998). The main gill pathologies include oedema of the lamellae and subsequent degeneration of the overlying epithelium. Super-saturation is more likely to occur in freshwater, however, reports of extreme oxygen levels have been also recorded in marine fish. Renfro (1963) indicated that an excess of oxygen was associated with fish mortalities at Galveston Bay, Texas, with oxygen levels recorded in the region of 250-327%.

(iv) Ichthyotoxins produced by HABs

There are a variety of phytoplankton species that are of concern as toxin producers. *Karenia* species, previously known as *Gyrodinium* has been associated with fish kills for many years. Roberts and Powell (1983) demonstrated that *K. mikimotoi* (*Gyrodinium aureolum*) did not cause mechanical occlusion of the gills but did induce severe histopathological damage, including necrotic degeneration and disintegration of lamellar epithelia. Two cytotoxic polyethers, Gymnocin A (Satake *et al.*, 2002) and Gymnocin B (Satake *et al.*, 2005), have been isolated from *K. mikimoto*, and these may explain the mechanism by which this dinoflagellate damages fish species (Mitchell and Rodger, 2007).

Mitigation and control strategies for HABs in aquaculture were reviewed extensively by Kim (2006). These include precautionary measures such as monitoring for algae using turbidity and satellite imagery with actions such as early harvesting, reducing feed to fish, and use of clay, water pumps or aerators to disperse or remove the bloom. Other indirect controls such as reducing nutrient inputs and modifying water circulation have also been applied (Kim, 2006). More dramatic measures that potentially could be applied include moving pens to alternative unaffected area, enclosing the nets pens with tarpaulin or applying a top-net and sinking the pens to beneath the bloom (Kim, 2006). Direct actions on the bloom by physical (removal with skimmer or screen filter), biological (using algicidal microorganisms) or chemical means (clays and long chain polymers or surfactants such as sophorolipid) may also be used (Kim, 2006). The resources and logistics of applying some of the above listed methods mean they are currently impractical and in reality, cessation of feeding and supplementary oxygenation are the only two mitigation strategies that are applied with regularity across the finfish aquaculture industry.

Harmful zooplankton

Gelatinous zooplankton or jellyfish swarms are a significant cause of production loss to the aquaculture industry. Jellyfish-mediated damage to salmon and other species in aquaculture is considered by some workers to be increasing in frequency (Graham, 2001; Purcell *et al.*, 2007; Rodger *et al.*, 2011). The increase of reports in the literature on harmful zooplankton blooms may be related to increased scientific awareness and interest, but concerns are mounting that the marine ecosystem food chain is rapidly changing with a trend towards dominance of jellyfish (Hay and Murray, 2008; Lynam *et al.*, 2006).

A number of jellyfish species have been implicated in fish kills over the years including the hydromedusae *Phialella quadrata* (Bruno and Ellis, 1985) and *Solmaris corona* (Hay and Murray, 2008), the siphonophores *Muggiaea atlantica* (Hellberg *et al.*, 2003) and *Apolemia uvaria* (Båmstedt *et al.*, 1998) and the scyphomedusae *Cyanea capillata* (Bruno and Poppe, 1996) and *Pelagia noctiluca* (Doyle *et al.*, 2008). Hay and Murray (2008) reported that, in Scotland between the period of 1999 and 2005, approximately 60% of 4.7 million fish mortalities (9,500 tonnes) were caused by jellyfish or harmful zooplankton. The authors also observed that in Skye and the Outer Hebrides in

2002 fish losses caused by a combination of zoo- and phytoplankton accounted for greater than 10% of mortalities in numbers and approximately 17% of the biomass lost. In Ireland, Rodger (2007) observed that first sea year salmon in farms are especially vulnerable to gill pathology and gave an estimate of approximately 12% for annual mortalities due to gill disease one of the implicated aetiologies being harmful zooplankton. Species implicated in causing damage to salmonids are listed in Table 2.

Table 2. Examples of gelatinous zooplankton associated with mortalities in farmed salmonids.

Zooplankton Spp.	Salmonid Species Affected	Location	Details
<i>Aurelia aurita</i> (Scyphozoan)	<i>S. salar</i>	Norway	Farmed salmon
		Shetland	Farmed salmon
		Ireland	Farmed salmon
<i>Pelagia noctiluca</i> (Scyphozoan)	<i>S. salar</i>	Northern Ireland	Approx. 250 000 fish killed
		Ireland, Scotland	Farmed salmon
		France	Significant mortalities in farmed fish
<i>Muggiaea atlantica</i> (Siphonophore)	<i>S. salar</i>	West coast of Norway	>100 000 farmed salmon, 2000 siphonophores/m ³
		Scotland	Mortalities in farmed fish
<i>Phialella quadrata</i> (Leptomedusa)	<i>S. salar</i>	Shetland	1500 fish mortality
		Norway	
<i>Cyanea capillata</i> (Scyphozoan)	<i>S. salar</i>	Scotland, Ireland	Farmed salmon (90,000 mortalities in Ireland in 2004)
<i>Solmaris corona</i> (Narcomedusa)	<i>S. salar</i>	Scotland	Farmed salmon (650,000 mortalities in 2 days in 2002)
<i>Apolemia uvaria</i> (Siphonophore)	<i>S. salar</i>	Sweden, Norway	Farmed salmon
<i>Bolinopsis infundibulum</i> (Ctenophore)	<i>S. salar</i>	Norway	Farmed salmon
<i>Vellella vellella</i> (Athecata)	<i>S. salar</i>	Ireland	Skin and gill pathology observed

Clinical signs caused by harmful jellyfish swarms include lethargy, fish swimming close to the

surface and respiratory distress. The fish may exhibit unusual behaviours such as bursts of jumping and head shaking (Rodger, 2007). Cessation of feeding may also occur (Bruno and Ellis, 1985). There may be increased numbers of moribund fish and/or mortalities. On examination of the gill filaments excess mucus, mottling and discolouration of the gill filaments and localised, focal erosion of the gills, gill rakers and or operculum may be observed, in addition to skin, mouth and eye damage (Rodger, 2007). The most significant pathology in affected fish has been observed in the gills, mediated by the toxic effects of jellyfish venom through nematocyst discharge (Helmholz *et al.*, 2010; Rodger *et al.*, 2011). Focal necrosis of the gills, with haemorrhage, epithelial sloughing, discharge of eosinophilic granular cells and oedema have all been associated with exposure of fish to harmful gelatinous zooplankton (Roberts and Rodger, 2001). In some cases, the physical presence of high numbers of gelatinous zooplankton may result in physical clogging of gills and lack of availability of oxygen. This has been recorded with some species which do not have nematocysts, such as *Bolinopsis infundibulum* (Båmstedt *et al.*, 1998).

Besides the obvious direct loss of mortalities in aquaculture, the implications of zooplankton swarms are far reaching. A considerable cost is incurred during retrieval and disposal of these dead fish. Zooplankton swarms increase stress in affected fish populations and the incidence of disease and possible secondary bacterial (*Tenacibaculum* sp.) infections (Ferguson *et al.*, 2010). Reduced growth during and/or after exposure to swarms is possible. Costly mitigation actions may also be required. Loss of production potential may occur if emergency slaughtering is deemed necessary. Insurance premiums may increase following claims for damage due to plankton blooms and as there may be a preset limit for claims many smaller losses due to zooplankton go unreported (Rodger *et al.*, 2011).

Limited strategies for the treatment or control of zooplankton swarms exist. The presence of early warning systems for blooms may help aquaculture producers to make decisions such as whether to feed fish. Reduction or stopping feeding has been suggested as a potentially helpful strategy (Hay and Murray, 2008). Oxygenation and recirculation of deep 'bloom free' water around caged fish has also been suggested (Hay and Murray, 2008). As with the HABs, movement of cages away from the swarm area could be considered if feasible. Prophylactic antibiotic cover for possible secondary infections may also be beneficial. Employment of protective nets, booms or bubble curtains may be considered worthwhile at high risk sites (Rodger, 2007). Bubble curtain trials in a

salmon farm in Co. Donegal, Ireland where monitoring of zooplankton was undertaken, revealed that a significantly greater density (20%) of plankton was recorded on the outside of the curtain as compared with the inside (Ratcliff, 2004). However, the energy costs of operating the bubble curtain were very high, both in terms of diesel and fouling (Ratcliff, 2004).

Other environmental aetiologies of gill disease

Medicines and remedies

Several different bath treatments used in the control of infectious diseases in aquaculture have been associated with damage to the gills of the fish treated, for example, formalin (Speare *et al.*, 1997), hydrogen peroxide (Kiemer and Black, 1997) and chloramine-T (Sanchez *et al.*, 1997, Powell *et al.*, 1998).

Formalin treatments, often used to treat fungal or parasite infestations, may cause a slight dose-related increase in lamellar fusion in Atlantic salmon and to a greater extent in rainbow trout (Speare *et al.*, 1997). They found that while both species showed an increase in mucus production there was no evidence of oedema or necrosis. Hydrogen peroxide (H₂O₂) is frequently used as a bath treatment for external parasites of salmon, such as sea lice, *Lepeophthirus salmonis* and *Caligus elongatus*. Kiemer and Black (1997) showed that there is a very narrow margin between the effective treatment range and concentrations which will induce gill damage and possibly death. Increasing temperatures reduce this error margin further. The authors observed that the gill histopathology was multifocal, with areas of severe damage adjacent to healthy tissue. Secondary lamellae and the outer surfaces of gill arches were most severely affected. Pathology included hypertrophy, clubbing of secondary lamellae, mucous cell hyperplasia, hyperaemia, irregular surface of epithelial cells, haemorrhage, necrosis, fusion and lifting of the epithelial surface. The severity of pathology was consistent with length of exposure time and concentration of H₂O₂. As a result it is generally accepted that hydrogen peroxide use is safest when water temperatures are lower than 13°C.

Chloramine-T (N-sodium-Nchloro-para-toluenesulphonamide) is commonly used prophylactically and as a treatment against bacterial gill disease and ectoparasitic disease in salmonid hatcheries in freshwater. It has also been investigated as a treatment for amoebic gill disease in marine salmonids (Harris *et al.*, 2004). Several studies have been performed to determine the effects of this potentially toxic chemical on the gills of salmonids (Sanchez *et al.*, 1997, Sanchez *et al.*, 1998), the results of which did not demonstrate any conclusive detrimental effects. However chloramine-T has been shown to have acute (within twelve hours) respiratory and acid-base disturbances in fish (Powell *et al.*, 1998). Sensitivity to the agent is also reported to be species dependant, with freshwater Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*, Walbaum) having greater susceptibility than channel catfish (*Ictalurus punctatus*) (Powell and Harris, 2004). Chloramine-T has also been shown to be more toxic to Atlantic salmon in seawater than in freshwater and in extreme cases can cause acute disruption to osmoregulation (Powell and Harris, 2004).

Finally, all bath treatments regardless of the therapeutant have the potential to cause serious gill damage in Atlantic salmon. Use of sea-lice products such as pyrethroids or organophosphates has occasionally been associated with an increase in gill pathology and mortality in treated fish (Hamish Rodger, personal communication). This is partly related to inappropriate bath treatment strategies such as a failure to provide sufficient oxygenation during treatments or inadequate mixing of the product in the bath, which can result in highly toxic "hot-spots" of the treatment product.

Eutrophication and pollution

Coastal eutrophication can result in an increase in phyto-plankton leading to phyto- and zooplankton blooms (Glibert *et al.*, 2002; Anderson *et al.*, 2008; Richardson *et al.*, 2009). Factors involved in this enrichment of nutrients include sewage, agriculture runoff and discharge as well as industrial effluent and discharge. In addition to their role in plankton enrichment, these factors also play a direct role in gill disease and mortalities in farmed finfish species (Liber *et al.*, 2005; Ogundiran *et al.*, 2009). In particular, damage to gill filaments and the chloride cells on the filaments decreases the ability of the fish to carry out efficient respiration. This is important both in adult marine stage salmon and in smolts adapting to the marine environment (Clarke 1992; Lappivaara *et*

al., 1995).

Hydrogen sulphide (H₂S) may be produced naturally and under normal conditions in coastal waters but can also result from self-pollution under farm pens. Chronic exposure causes clubbing and thickening of the gill lamellae and increased numbers of mucous cells may be observed. Acute exposure cases show major irreversible damage to the gill tissues. Extensive fusion of secondary lamellae, thickening of the primary lamellae, haemorrhages and separation of the epithelium from the underlying branchial vessels have all been reported (Kiemar *et al.*, 1995).

The majority of references regarding toxicity of metals and pollutants to salmonid and non-salmonid fish and including gill pathologies relate to freshwater although Kroglund *et al.* (2007) assessed the effects of acidification and aluminium (Al) on survival of post-smolt Atlantic salmon in Norway. Exposures were of varying intensities and duration. It was found that the capacity for Atlantic salmon smolts to osmoregulate is extremely sensitive to Al concentration and that return rates of Al-exposed fish were reduced by between 20 and 50% when compared with control fish.

Nutritional aetiologies for gill disease

Nutritional deficiencies such as vitamin C, vitamin K, biotin and pantothenic acid can occasionally lead to gill pathology in farmed fish (Waagbø, 2008). Histopathology associated with such nutritional changes includes lamellar degeneration or fusion. Numerous studies have documented the gill pathology characteristic of pantothenic acid (PA) deficiency, and epithelial hyperplasia of gill lamellae has been shown to be characteristic (Wood and Yasutake, 1957; Poston and Page, 1982; Karges and Woodward, 1984).

Genetic and congenital causes for gill disease

Gill filament deformity syndrome (GFDS) is an unusual condition of diploid and triploid Tasmanian Atlantic salmon. The absence of the primary gill filaments is characteristic of the syndrome. The deformity is an autosomal condition (non-sex-linked genotype) and results in a

markedly reduced gill surface area for gas exchange and acid-base balance in triploid fish when compared to their diploid counterparts (Sadler *et al.*, 2001).

Shortening of the opercular cover of gills is a deformity that is occasionally observed in Atlantic salmon. It may be uni- or bilateral and it results in the exposure of the gill filaments to the external environment and potential damage. This frequently results in the shortening and thickening of the gill filaments. On histological analysis such gills frequently display epithelial hyperplasia and lamellar fusion and are vulnerable to secondary bacterial and parasitic infections. In addition, fish with such a defect are unable to pump water across the gill effectively and are obliged to swim continuously in order to maintain a continuous flow of water past the gills (Branson and Turnbull, 2008). The aetiology of this condition now appears, in many cases, to be related to elevated egg incubation temperature ($>8^{\circ}\text{C}$) (Baeverfjord *et al.*, 1997; Ørnsrud *et al.*, 2004).

Infectious causes of Gill Disease

Table 3 provides a summary of the main infectious pathogens of the gills of Atlantic salmon. Each group is explored in detail in the next section.

Table 3. The main salmonid pathogens associated with gill disease (modified from Mitchell and Rodger, 2011).

Group	Pathogen	Salmonid species affected	Geographical location	Associated pathology/syndrome
Virus	Atlantic salmon paramyxovirus (ASPV)	AS	Norway	PGI?
Virus	Salmon gill poxvirus (SGPV)	AS	Norway	PGI?
Parasite	<i>Neoparamoeba perurans</i>	AS, RT, CO, CS	Australia, Ireland, Norway, Scotland, USA, New Zealand, Spain, Chile	Amoebic gill disease
Parasite	<i>Loma salmonae</i>	CS, CO, RT*	Canada, Scotland	Microsporidean gill disease
Parasite	<i>Ichthyobodo</i> sp.	AS, RT	Global	Marine Costiasis
Parasite	<i>Trichodina</i> sp.	All salmonids	Global	Trichodinosis
Parasite	<i>Desmozoon lepeophtherii</i>	AS, RT	Global	PGI
Parasite	<i>Salmincola salmonea</i>	AS	Global	Mild gill inflammation
Parasite	<i>Gyrodactyloides bychowskii</i>	AS	Global	Obstructive gill damage
Bacteria	<i>Tenacibaculum maritimum</i>	AS, RT, CS	Global	Tenacibaculosis
Bacteria	' <i>Candidatus</i> Piscichlamydia salmonis'	AS, AC	Ireland, Norway , N. America, Tasmania	PGI, Epitheliocystis
Bacteria	' <i>Candidatus</i> Branchiomonas cysticola'	AS	Ireland, Norway	PGI, Epitheliocystis
Bacteria	' <i>Candidatus</i> Clavochlamydia salmonicola'	AS, BT	Ireland, Norway	Epitheliocystis

AS – Atlantic salmon, RT – rainbow trout, CS – chinook salmon, BT – brown trout, CO – coho salmon, AC - Arctic char, PGI – proliferative gill inflammation, * freshwater only

Viruses

In spite of the fact the gills are the most likely port of entry for many viruses affecting Atlantic salmon, such as Infectious salmon anemia virus ISAv (Totland *et al.*, 1996), reports of viruses acting as actual gill pathogens in this species are infrequent. Only two viruses to date have been associated with gill disease; Atlantic salmon paramyxovirus (ASPV) and salmonid gill pox virus (SGPV). In both cases the mechanism by which they negatively affect the host and their contribution to gill pathology, if any, is unclear. Atlantic salmon paramyxovirus (ASPV) was first isolated in 1995 from farmed Atlantic salmon in Norway which were suffering from proliferative gill inflammation (PGI) (Kvellestad *et al.*, 2003). To investigate the significance of ASPV an experimental challenge with a virus isolate was performed, but did not result in any mortality or pathology. Nevertheless, a synergistic role for this virus in combination with other gill pathogens was suggested (Fridell *et al.*, 2004). Immunofluorescent staining and immunohistochemistry were employed by another study attempting to uncover the aetiological significance of ASPV. The virus was found associated with diseased tissue on three different farms where fish were affected with PGI (Kvellestad *et al.*, 2005a; 2005b) suggesting it potentially may have a role to play in multifactorial disease. Through molecular studies the virus was assigned to the genus *Respirovirus* within the family *Paramyxovirus* (Falk, *et al.*, 2008). Paramyxoviruses have been associated with respiratory diseases in mammals and birds and primary replication occurs mainly in the respiratory tract (Murphy *et al.*, 1999). A common finding in pneumonia in terrestrial animals is that a virus or viruses are involved as trigger pathogens which then allow secondary bacterial infections to become established (Radostits, *et al.*, 1999). Although the biological model of virus-bacterial interaction for respiratory disease has been strongly documented in land animals, the role, if any for ASPV or other viruses in diseases of fish is not clear. Furthermore, ASPV is not consistently detected in cases of PGI in Norway (Steinum *et al.*, 2010).

In the spring of 2006, salmonid gill poxvirus (SGPV), a DNA virus was first observed by electron microscopy (Nylund, *et al.*, 2008). Immature virions were described as being 350nm in diameter and intracellular mature virions are about 300nm long. Gill epithelial cells infected with the pox virus exhibited extreme hypertrophy and degeneration of the nucleus. The virus was isolated from the gills of Atlantic salmon suffering from a proliferative gill disease which resulted in 20% mortality at a freshwater facility in northern Norway. Later in 2006, the virus was also isolated from Atlantic salmon at two marine sites in western Norway where mortalities close to 80% were recorded. In both these marine sites concurrent infections of the gills with the bacterium '*Candidatus*

Piscichlamydia salmonis and the amoeba *Neoparamoeba perurans* may have contributed to the high mortalities. As with ASPV, the significance of this virus for salmonids as a gill pathogen remains to be determined and again, multifactorial interactions may be a prerequisite for disease.

With advances in molecular and other techniques in recent years, it is becoming easier to detect micro-pathogens such as viruses. The role these have played to date in gill disease may have been underestimated. The first report to describe epitheliocystis, which is a condition of the gills characterised by intracellular cysts full of bacteria, in a bluegill (*Lepomis macrochirus*) includes an electron micrograph of affected gill tissue in which a virus was also observed. This virus had similarities to a paramyxovirus (Hoffman, *et al.*, 1969) perhaps providing the first support that the interaction of viruses with other infectious agents may be an important.

Parasites

A range of different parasites can be present on the gills of marine Atlantic salmon and can be problematic especially if present in high numbers. Parasitic infestation can also become more significant in gills that are compromised by adverse environmental conditions or concurrent disease.

Amoebic Gill Disease

Probably the most significant disease caused by gill parasites in terms of economic impacts in aquaculture is amoebic gill disease (AGD). AGD has been recognised as the most significant problem of marine farmed salmonids for many years in Tasmania (Munday *et al.*, 1990), with significant outbreaks occasionally reported in other salmon farming countries. Outbreaks have been reported in Atlantic salmon in Ireland (Rodger and McArdle, 1996), France (personal communication, Fredrick Esnault), Scotland (personal communication, Peter Southgate) Chile, Spain (Rodger and McArdle, 1996), New Zealand (Munday *et al.*, 2001) and most recently in Norway (Steinum *et al.*, 2008). The first outbreak in North America was in marine coho salmon (*Oncorhynchus kisutch*, Walbaum) where it resulted in significant losses (Kent *et al.*, 1988b). AGD is also reported to affect several other fish species cultured in the marine environment, including other salmonids, turbot (*Scophthalmus*

maximus, L.), European seabass (*Dicentrarchus labrax*, L.) and sharpsnout seabream (*Diplodus puntazzo*, Cetto) (Munday *et al.*, 2001).

Atlantic salmon appear to be the most susceptible salmonid species affected by AGD. Outbreaks in Pacific salmon have generally been minor and sporadic suggesting they may have inherent resistance to disease (Findlay *et al.*, 1995). AGD has been recorded in chinook salmon (*Oncorhynchus tshawytscha*, Walbaum) farmed in New Zealand but is insignificant in terms of mortalities and rarely warrants treatment (personal communication, Chris Kennedy).

For many years *Neoparamoeba pemaquidensis*, a free living amoeboid protist found in the marine environment was originally assumed to be the primary agent of AGD, however, in 2007, Young, *et al.* identified the true agent of AGD as a new species of amoebae, now known as *Neoparamoeba perurans*. Retrospective testing of material from outbreaks from different countries indicates this is and has been the predominant aetiological agent of AGD all along. The study confirmed that it has been the predominant agent of epizootics of AGD in Tasmania as well as cases in Ireland, North America, Scotland, New Zealand and Northwest Spain.

AGD has been an ongoing problem for Tasmanian aquaculture since its inception in 1984, with the first reports of it as a serious disease by Munday (1986). Amoebae can be found on salmon gills at temperatures of around 10°C, yet clinical disease is usually seen between 12 - 20°C and salinities approaching 35‰. Amoebae have also been detected on a range of native marine fish. Marine-farmed fish are worst affected during their first year at sea and mortality levels can vary depending on a number of host and environmental factors. In serious outbreaks up to 50% mortality can occur without treatment.

AGD accounts for 10 to 20% of production costs in Tasmania (Munday *et al.*, 2001). The disease is of lesser significance in other countries but occasionally can cause substantial morbidity or mortality, especially when associated with pre-existing disease or unusual environmental conditions. In 1995 (Rodger and McArdle, 1996) and again in 2011 (personal communication, Hamish Rodger), AGD epizootics occurred in Ireland. In the 1995 epizootic, abnormally high sea temperatures due to a very hot summer and higher than normal salinities due to lack of rainfall were considered to be significant factors (Rodger and McArdle, 1996; Palmer *et al.*, 1997). The first and only reports of AGD in Norway were in seawater farmed Atlantic salmon in autumn 2006, at four locations on the

western coast (Steinum *et al.*, 2008). The mortality attributed to infection ranged from 12 - 82%. Proliferative gill inflammation (PGI) was also present and in one of the four farms may have contributed to the unusually high mortality (82%) in this site. Mortality on the other three sites ranged from 12 to 20%. Higher than average water temperatures (+3.5 above normal) were again assumed to be responsible for precipitating the outbreaks. However, the Irish epizootic in the summer of 2011 painted a different picture. Sea temperatures were lower than average and salinity within the normal range. These conflicting findings suggest there may be other more important unidentified environmental elements involved in precipitating significant episodes of AGD. Douglas-Helders *et al.*, (2001; 2004) suggested that other stressors and environmental factors may be more important risk factors than temperature. While investigating temperature as a risk factor for AGD, the authors observed clinical disease at temperatures below 10°C. Salinity has been regarded as an important environmental factors affecting AGD and protracted infections in salmonids have frequently been associated with high salinities (Clark and Nowak, 1999, Munday *et al.*, 2001).

The potential of amoebae to survive and even multiply on the gills of dead fish was uncovered by Douglas-Helders *et al.* (2000). A steady increase in amoebal numbers on the gills of dead fish was observed to at least 30 hours post mortem. This finding clearly illustrates the importance of early and efficient mortality removal from net-pens to reduce pathogen exposure and challenge to cohabiting fish.

Clinical signs attributed to AGD include anorexia, respiratory distress, flared operculae and lethargy (Munday *et al.*, 2001). Several studies have described the pathology attributed to AGD with similar findings. Clinical examination of the fish frequently reveals multifocal patches or spots of white to grey swollen gill tissue with excess mucus surrounding the gill arches (Munday *et al.*, 1990). On histological examination hyperplasia and complete fusion of the secondary lamellae can be observed alongside fusion of the primary lamellae. The formation of small interlamellar vesicles or lacunae, subsequent to the fusion of the secondary lamellae in which amoebae were present may also be present (Munday *et al.*, 1990). High numbers of amoebae are usually observed on histology intimately attached to the eroded lamellar edges. AGD is usually diagnosed by microscopy of fresh gill mounts or histopathology. In Tasmania where AGD is endemic, presumptive diagnosis is often made by observations of gross gill pathology as described above.

Despite the fact that the gross- and histopathology of AGD has been comprehensively described,

the pathogenesis of the disease is still poorly understood (Munday *et al.*, 2001). Whether a precipitating environmental event or pre-existing gill pathology is necessary to enable colonization of the gills by *N. perurans* is ambiguous. Recent studies by Adams *et al.*, (2009) suggested that mechanical injury to gills does not increase the risk of attachment and/or colonization of the gill epithelium in salmon during experimental infection with *N. perurans*.

The current recommended treatment for AGD is a 2-3 hour freshwater bath (Parsons *et al.*, 2001, Clarke *et al.*, 2003). Use of hydrogen peroxide has produced equivocal results (Powell and Clarke, 2004). Chloramine T (a disinfectant compound) in seawater has shown to be a promising alternative to freshwater bathing (Harris *et al.*, 2004; Harris *et al.*, 2005). A number of different in-feed supplements such as bithionol, an antiprotozoal agent, have been assessed as treatments for AGD (Florent *et al.*, 2007; Florent *et al.*, 2009). Although bithionol showed promising effects, licensing of such a compound for fish entering the food chain is likely to prove difficult. This is also the case with other amoebicidal agents which have shown to have some effect *in vitro* such as nifurpirinol, mefloquine, pyrimethamine and nitrothiazole (Howard and Carson, 1995).

Some studies have linked AGD with the presence of different bacteria on the gills. Embar-Gopinath *et al.*, (2005a) looked at the relationship between salmonid gill bacteria and AGD. They found that the most severe cases of AGD occurred in fish that had high levels of co-infecting *Winogradskyella* sp.. In contrast, a cytopathological study by Roubal *et al.*, (1989), found no evidence of large bacterial loads on the gills, nor did they find any preexisting gill pathology or epithelial proliferation prior to the colonization with *N. perurans* implying that it may be a primary pathogen.

There are other conflicting reports regarding the necessity for a predisposing factor for colonization of the gills by amoebae. During a 2-year intensive study on AGD in Ireland Bermingham and Mulcahy (2004) observed colonisation of the gills with amoebae only after pathological lesions had developed. The authors proposed that environmental changes damaged the gills, predisposing them to secondary opportunistic parasites such as amoebae and other ciliates.

Bermingham and Mulcahy (2007) suggested that a number of other amoebae may be involved in AGD in addition to *Neoparamoeba* sp.. They isolated a number of other genera that have been associated with disease in other teleosts. *Platyamoeba*, *Flabellula* and *Vexillifera* sp. have all been

recorded on the gills of Atlantic salmon with AGD from both Ireland and Tasmania. In addition species such as *Mayorella*, and *Nolandella* have been observed in Ireland and *Acanthamoeba*, *Vanella* and *Heteroamoeba* in Tasmania. The pathogenic potential of these genera and their role as potential agents of AGD is not established. They may not have a primary role in precipitating an outbreak of AGD but it is plausible they can play a part once the gills are compromised.

Trichodinosis

Trichodinids are important protist parasites of both freshwater and marine fishes. By colonising both the skin and gills they can cause significant pathology. Hyperplasia of the epidermis and destruction of the normal gill structure are the most common effects of infestation. The freshwater species are commonly found on the skin while marine Trichodinids are more frequently found affecting the gills. Reports of serious mortalities in salmonids caused by trichodinids are sparse in the marine environment. McArdle (1984) reported substantial mortalities in both salmon and trout reared at sea in Ireland in the summers of 1982 and 1983. Fish were observed to be lethargic and incapable of maintaining their position in the water column. Flared operculae and a bluish film on the body surface caused by excess mucus were observed. The gills of the fish were pale and eroded. On microscopy of fresh gill mounts massive numbers of trichodinids were visible. Severe hyperplasia of the distal extremity of the primary and secondary lamellae, oedema and severe extensive erosion of some of the filaments were observed on histopathology. Bath treatments using formalin proved partially effective in controlling the infestation. The summers of 1982 and 1983 were abnormally warm in Ireland, with sea temperatures as high as 23°C being recorded in some bays. The author speculated that this, in addition to high salinity due to low rainfall that summer, may have been primary risk factors for the outbreak. In 1982, Atlantic salmon broodstock and rainbow trout were affected, while young Atlantic salmon smolts in a site adjacent to the broodstock remained unaffected. Age and sexual maturity, which have frequently been associated with heavy ectoparasite burdens, may have increased the susceptibility of the broodstock. Freshwater bathing may be effective for treating trichodinosis (Brown and Markus, 1998).

Costiasis

Ichthyobodo necator (formerly known as *Costia*) is an important flagellate parasite that infests the skin and gills of fish in both freshwater and marine environments. Reports of *Ichthyobodo* sp. as a significant problem in the marine environment are not as common as those in freshwater, where it is considered a major pathogen of salmonid fry in particular. However, a number of cases in marine salmon have been reported. Costiasis was described in post-smolts in the Faeroe Islands in the years 1986 - 1989 with substantial mortalities associated with infestation (personal communication, Andreas Strøm). Ellis and Wootten (1978) describe an outbreak of costiasis in Atlantic salmon smolts in seawater in Scotland during July 1977. Fish were emaciated, inappetent and swimming near the water's surface. On clinical examination, the gills were observed to be heavily colonised by *Ichthyobodo* sp. Acute hyperplasia, fusion of the secondary lamellae and an increase in goblet cells was observed. In heavily infested fish, large amounts of melanin granules were visible in the primary lamellae. The authors speculated that the infestation originated in freshwater and fish displayed clinical disease post-transfer, however it was not known if *Ichthyobodo* sp. adapted to freshwater were capable of surviving in the marine environment. Based on the broad host range and the presence of *Ichthyobodo* sp. on both freshwater and marine fish, Bruno (1992) speculated that the *Ichthyobodo* sp. causing disease in Atlantic salmon in saltwater was in fact a separate strain or species to that which occurs in freshwater. He examined *Ichthyobodo* parasites collected from several locations around Scotland. Gill pathology was observed in the fish from some sites but there were other factors involved that may have contributed to the pathology. Molecular evidence later corroborated his findings, indicating *Ichthyobodo* sp. are a multispecies complex with differing host preferences (Todal *et al.*, 2004; Callahan *et al.*, 2005).

Formalin baths using a tarpaulin barrier have been effective in treating clinical outbreaks of marine costiasis in Norway (personal communication, Anne-Berit Olsen) and Ireland (Hamish Rodger, personal communication).

Both *Ichthyobodo* sp. and *Trichodina* sp. have occasionally been observed in fish with PGI, but a significant role for either in this disease is considered doubtful (Kvellestad *et al.*, 2005a).

Microsporidial Gill Disease

Loma salmonae is a microsporidean parasite which infects the gills and other organs of salmonids causing microsporidial gill disease (MGD). Significant MGD occurs in farmed Pacific salmonids (*Oncorhynchus* spp.) in both freshwater and saltwater in North America. Effects of MGD include respiratory distress, increased susceptibility to secondary infections and increased mortality. The infection occurs mainly in secondary gill lamellae, with the formation of large white spore-laden xenomas within the endothelial and pillar cells (Kent and Speare, 2005).

L. salmonae infections in coho salmon were first reported as a clinical disease by Kent *et al.* (1989) in fish in seawater pens in Washington State, U.S.A. Infections were associated with some significant pathological changes in the gills. On histopathological examination, a mixed inflammatory infiltrate was observed in association with several ruptured xenomas. There was also evidence of the parasite in the heart, spleen, kidney and pseudobranch, but the majority of the pathology was observed in the lamellar gill tissue. Prevalence of infection was highest during the summer months and responsible for significant mortalities.

Ramsay *et al.* (2002) compared the susceptibility of coho salmon, chinook salmon and rainbow trout to *L. salmonae* through experimental infection. Chinook salmon were the most susceptible species, followed by coho salmon. Rainbow trout appear to have a degree of resistance to clinical disease, showing lower xenoma intensity and pathological alterations when compared with the other two species of salmonid. The significance of *L. salmonae* associated mortalities has been one of the reasons that Atlantic rather than the native Pacific chinook salmon are now being reared in some marine sites in Canada, as Atlantic salmon appear to be nearly entirely resistant to MGD (personal communication, Diane Morrison).

L. salmonae is rarely reported in Europe, however, it was isolated during an outbreak of gill disease in farmed rainbow trout in freshwater in Scotland in 1994, and appeared to cause low levels of mortality in the population (Bruno *et al.*, 1995). Other reports of the microsporidean in the UK have not been associated with disease.

Speare *et al.* (1989) report an interesting example of multifactorial gill disease in juvenile coho salmon in British Columbia which appeared to result from a combination of a diatom bloom and

infection with *L. salmonae*. Pathological changes included suppurative branchitis accompanied by extensive fusion of the gill lamellae. This pathology, attributed to the diatom bloom, was complicated by extensive infection of the endothelial cells by *L. salmonae* which exacerbated the pathology through vasculitis and thrombus formation. The fish appeared to have been infected with the microsporidean in freshwater as mature xenomas were observed in the fish one week post transfer to sea water.

The potential of *L. salmonae* infections to be compounded by co-infections with other agents was demonstrated by Mustafa *et al.* (2000). While investigating the susceptibility of rainbow trout to *L. salmonae* during a primary infection with the sea louse, *Lepeophtheirus salmonis*, the authors showed that macrophage function in the fish was impaired by the primary infection with the sea lice; therefore demonstrating that the ability of the fish to mount a cell mediated immune response was compromised. Subsequently, the trout demonstrated increased susceptibility to *L. salmonae* when challenged with the microsporidean. Multifactorial interactions such as this are likely to be a frequent occurrence during clinical outbreaks of gill disease.

There are currently no authorized effective treatments for *L. salmonae* infestations, however, work by Speare *et al.* (2007) on a whole-spore vaccine demonstrated promising results.

The microsporidean parasite, *Desmozoon lepeophtherii* (syn *Paranucleospora theridion*) was recently first reported in Atlantic salmon nearly simultaneously by two separate research groups, Freeman and Sommerville (2009), and Nylund *et al.* (2010). The parasite has a complex lifecycle involving both the sea lice *Lepeophtheirus salmonis* and Atlantic salmon. The parasite has also been detected in the louse *Caligus elongatus* and in trout. Sporoplasms produced by the microsporidean infect the gills of salmon and then spread throughout the body eventually becoming detectable in most organs. The significance of this parasite as a gill pathogen has yet to be determined but it is frequently detected in very high numbers in gills displaying PGI like pathology. It has been suggested that it may play a major role in PGI particularly in western Norway. However, the parasite can also be detected in the gills of approximately 80 % of healthy fish (Steinum *et al.*, 2010). The parasite may be opportunistic, requiring co-infections with other agents to enable it to cause disease. It is also possible that it may weaken the hosts immune system like that observed during *Nucleospora salmonis* infections, another microsporidean which infects salmonids. Early indications from preliminary challenge trials indicate this pathogen may be highly significant in terms of gill disease

(personal communication, Ayre Nyland).

Macrosopic Gill Parasites

The microscopic protist parasites discussed above are probably the most important gill parasites in salmonid aquaculture. Reports of other gill parasites becoming problematic in marine farmed salmonids are rare. *Gyrodactyloides bychowskii*, is a viviparous monogenean was first found in May 1989 on the gills of farmed marine Atlantic salmon in Norway (Mo and MacKenzie, 1991). Low numbers were observed on post smolts whereas up to hundreds of the parasites were found on larger fish over 2.5 kg. Some of the badly infested fish were found to be in poor condition with hyperplasia and cellular hypertrophy observed in the gills. Many of these fish also had concurrent infections with other species of protist ectoparasite such as *Trichodina*, *Schyphidia*, *Ichthyobodo* and *Cryptobia* spp.. *G. bychowskii* was also reported in Scotland on the gills of Atlantic salmon in the years 1999 and 2000 by Bruno *et al.* (2001). The parasites were found in high numbers on some fish; however, its impact on gill health in Scotland is not determined. Similar gill pathology to the case in Norway was observed in the fish examined, but there were also some morphological changes in the gills of fish without *G. bychowskii*. The parasites also disappeared during the summer months of the survey, supporting the suggestion that the parasite is a cryophilic Arctic genus, one that is capable of growth and reproduction between -15 – +10 °C.

Salmincola salmonea is a lernaeopodid copepod parasite of the gills, specific to Atlantic salmon, and is occasionally found in high numbers on adult returning wild salmon. It can survive and grow during the marine phase of the lifecycle, however can only reproduce successfully when its host is in freshwater. Retarded filament growth and “crypting” of the lamellae have been reported in heavy infestations.

Bacteria

Tenacibaculum maritimum

Tenacibaculum maritimum (formerly *Cytophaga marina*, *Flexibacter marinus* and *F. maritimus*) is the causative agent of tenacibaculosis (or flexibacteriosis) in marine fish. It is a Gram negative, filamentous bacterium, and was first described by Wakabayashi *et al.* in 1986. The authors examined 15 strains of gliding bacteria isolated from outbreaks of flexibacteriosis in marine fish and proposed that the bacteria should be classified as a new species, *Flexibacter maritimus* (now known as *Tenacibaculum maritimum*).

A number of different names have been used to describe tenacibaculosis based on the diversity of clinical presentations that occur, including "gliding bacterial diseases of sea fish", "eroded mouth syndrome" and "gill rot". The first report of gill lesions associated with *T. maritimum* was by Chen *et al.* (1995) on chinook salmon (*Onchorhynchus tshawytscha*, Walbaum) on the Pacific coast of North America. Body lesions associated with these bacteria were first reported in seawater Atlantic salmon by Kent *et al.* (1988a). The disease is usually more severe in juvenile fish and when water temperatures are above 15°C (Toranzo *et al.*, 2005).

Clinical signs of tenacibaculosis are variable and related to the type of infection. Fish with gill infections may be moribund, lethargic and display increased respiratory rate. Sometimes yellow or brown mats can be seen on the gills, emerging beneath the operculae or lesions may be observed on the gill rakers, following exposure to harmful zooplankton (Rodger, 2007). On clinical examination gills may have increased mucus, be pale and frank patches of severe necrosis may be visible on the lamellae. Skin lesions may also be present. Atlantic salmon and rainbow trout are both particularly susceptible to *T. maritimum* (Soltani *et al.*, 1996) compared with species such as flounder (*Platichthys flesus*, L.) which appear more resistant to infection.

The mode of transmission and the route of infection of *T. maritimum* are currently not well understood. Transmission through seawater and direct transmission from host to host have been proposed as possible routes, in addition to ingestion along with food (Avendaño-Herrera *et al.*, 2006b). A novel mode of transmission was recently suggested by Ferguson *et al.* (2010). At a marine salmon farm in Scotland high numbers of the jellyfish, *Phialella quadrata*, were observed in close proximity to fish with significant gill disease and tenacibaculosis. Using electron microscopy and molecular techniques, the authors discovered these jellyfish were carrying high loads of *T. maritimum*. They suggested the jellyfish were acting as vectors for the bacteria. The gills would have been initially damaged by nematocyst-derived toxins from the jellyfish, then the pathology

compounded by secondary bacterial infection with *T. maritimum*. Regardless whether the jellyfish are important as vectors, it is important to acknowledge that noxious zooplankton can potentially predispose fish to secondary bacterial infections through both physical and toxic mechanisms (Rodger *et al.*, 2011).

A number of studies have attempted to unravel the pathogenesis of infection with *T. maritimum*. *Tenacibaculum* sp. can affect all types of epithelium, particularly skin and gill tissues. There is consistency in the pathology observed in both salmonid and non-salmonid species. Handler *et al.* (1997) found Atlantic salmon and rainbow trout both showed a combination of erosive skin lesions and "gill rot". The gill lesions frequently involved erosion of the free edge of one or several of the primary lamellae and filaments tips were often reduced to bare, white exposed cartilage. Total necrosis of the filament tissues was observed distal to the encroaching bacterial filamentous mats. Reactions of the host to infection were generally limited to a small zone of congestion deep in the tissues adjacent to the bacteria, with little evidence of an inflammatory response. Powerful quick acting exotoxins produced by the bacteria may be responsible for disabling the normal immune reaction of the fish. The bacteria were observed either within necrotic tissue or in mats on the lamellar surface, never being observed in healthy lamellar epithelium. This finding suggests that a predisposing traumatic factor allowing the initial colonization of the gill lamellae, may be necessary for the development of disease. Outbreaks of tenacibaculosis also frequently occurred after long periods of sunny weather, leading to the speculation that UV light may potentially act as a predisposing factor.

In Atlantic salmon and rainbow trout, laboratory and field trials have found amoxycillin and trimethoprim effective against *T. maritimum* by either oral administration or bath immersion (Soltani *et al.*, 1995). Hydrogen peroxide may also be helpful as a treatment but the safety margin when using this compound is narrow, especially at higher temperatures (Avendaño-Herrera *et al.*, 2006a). Experimental vaccines trialed in Tasmania in Atlantic salmon have achieved some promising results (van Geldersen *et al.*, 2007).

In terms of synergistic interactions with other pathogens, none have been documented to date. Investigations into whether a link between underlying amoebic gill disease and tenacibaculosis yielded no significant results (Powell *et al.*, 2005).

***Aeromonas salmonicida* and other bacteria**

Infection with *Aeromonas salmonicida*, the causative agent of furunculosis, is generally a septicemic condition, but is included here as one of the features of the disease is bacterial colonies in the gills of affected fish. The gill lamellae are a common site for such bacterial colonies during infections (Roberts and Rodger, 2001). Since the widespread use of *A. salmonicida* vaccines and other management changes, clinical furunculosis is rarely observed in farmed salmonids, although it is still frequently observed in wild fish.

In a study investigating the relationship between different salmonid gill bacteria and amoebic gill disease, Bowman and Nowak (2004) found bacteria from the genus *Psychroserpens* in increased numbers in fish affected with AGD. It is not known what role these bacteria have, if any, in AGD infection, whether they play a direct role in the pathogenesis by attracting amoeba to the gills, or if an increase in bacterial numbers is secondary to the amoebic infestation. Whether there are synergistic interactions between these two agents has not been explored.

A survey of gill associated bacteria in farmed salmon with PGI in Norway (Steinum *et al.*, 2009) observed a wide distribution of γ -proteobacteria. The authors described how the bacterial communities of individual fish gills were generally quite simple and often dominated by one of two phylotypes, most commonly *Burkholderia*-like bacteria (previously *Pseudomonads*) and *Psychrobacter* spp.. Commensal and pathogenic bacteria identified in this study included members of the genera *Photobacterium*, *Flavobacterium*, *Aliivibrio*, *Shewanella*, *Tenacibaculum* and at least one species closely related to *Francisella*. This study was highly relevant, as it provided the first comprehensive insight into the normal bacterial flora of the gills in both healthy and diseased fish. Bacterial communities appeared to have a relatively consistent make-up in clinically healthy fish in individual fish farms, however, a higher degree of variation was observed in fish affected by PGI, suggesting the possibility that an imbalance in the normal bacterial flora may be linked to disease. Recent evidence dictates some of the microorganisms involved in gill disease become pathogenic through a quantitative effect (Steinum *et al.*, 2010). Certain species of bacteria may be present at low numbers in healthy fish, even part of the normal gill flora. When certain unknown criteria are affected, these bacteria get the opportunity to multiply resulting in significant pathology for the

host.

The Chlamydiae and epitheliocystis

The Chlamydiae - history, life cycle and clinical significance

Members of the phylum *Chlamydiae* are all obligate intracellular bacteria. Until recently their diversity, host-range and occurrence in the environment has been grossly underestimated. The term "chlamydia-like-organisms" (CLO) has historically been used to refer to any microorganisms that exhibit a two-stage developmental cycle, such as members of the family *Chlamydiaceae*. Recent phylogenetic analyses on some of these CLOs has shown that the group is polyphyletic, therefore the term "chlamydia-like-organism" is descriptive as opposed to having any taxonomic value (Corsaro and Greub, 2006). Other authors have proposed the name "environmental chlamydiae" (Horn and Wagner, 2001) or "novel chlamydiae" (Corsaro and Greub, 2006) to refer to chlamydial organisms that fall outside the family *Chlamydiaceae*. In reality these terms are all used interchangeably to describe this expanding group of novel bacteria.

Chlamydiae are parasites of eukaryotic cells and infect mainly epithelial mucosa (Bavoli *et al.*, 1996). Whether infecting human, other animals or amoebae, all the Chlamydiae form intracellular inclusions, unique vacuole-type organelles that facilitate intracellular bacterial replication and survival by avoiding fusion with host cell lysosomes (Bavoli *et al.*, 2000). Chlamydiae are highly adapted to entering host cells, and form these inclusions using both host cell factors and proteins secreted by the bacteria themselves (Dautry-Varsat *et al.*, 2004). The acute infectious developmental cycle consists of infectious and non-infectious stages with different morphologies. The elementary body (EB) is small, about 0.3µm and metabolically inactive. After entering host cells the EBs differentiate into larger reticulate bodies (RBs), about 1 µm, which are more pleomorphic and are metabolically active. These RBs then proliferate and give rise to 1000 or more progeny per host cell. The infectious cycle is completed within 2 - 3 days depending on the species of bacteria and the bacteria differentiate back into EBs where they are released into the extracellular medium by unknown mechanisms (Dautry-Varsat *et al.*, 2004) where they can begin a new cycle of

replication (Figure 2). Host cell death or apoptosis controlled by the bacteria towards the end of the infection cycle may be the main mechanism of release of EBs (Perfettini *et al.*, 2003). Not all cycles end with cell death and release of infectious EBs however. Chlamydiae also have the ability to persist in a viable but non-infectious state as enlarged, non-dividing RBs that do not differentiate into the infectious EBs, unless provided with an appropriate environmental trigger (Hackstadt *et al.*, 1997).

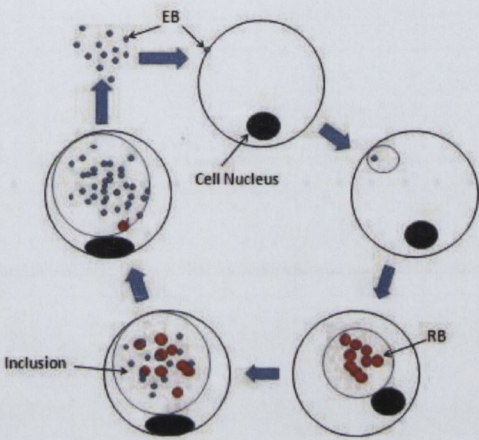


Figure 2. The Chlamydial developmental cycle. Elementary bodies (EBs) invade host cells and develop into reticulate bodies (RBs) that divide within a specialised compartment called the inclusion. The RBs then mature into EBs and are released from the host to infect new cells.

Many new members of the order Chlamydiales have been identified as disease causing in animals and humans in recent years. *Parachlamydia acanthamoebae* and *Simkania negevensis* have been associated with respiratory tract infections in humans, and *Waddlia chondrophila* is considered a potential abortive agent in ruminants. All these species thrive in free living amoebae and can use them as alternative hosts (Thomas *et al.*, 2006). Co-culture with axenic amoebae is a useful means of isolating new species of *Chlamydia* and may be more useful than cell-culture (Thomas *et al.*, 2006). To date successful use of this technique has not been reported for the Chlamydiae responsible for epitheliocystis in fish but it may be potentially used in the future.

Despite the clinical significance of chlamydial infections, there is still a lack of satisfactory approaches for the diagnosis of Chlamydiae. Fluorescence *in situ* hybridization (FISH) using fluorescently labelled oligonucleotide probes targeting ribosomal RNA has been shown to be a very effective method of identification of microorganisms without culture (Amann *et al.*, 1990b), and is particularly useful for those organisms that are slow growing or difficult to cultivate. It is increasingly becoming one of the diagnostics of choice for Chlamydial-infections in both human and animal clinical specimens. Not only is FISH fast and sensitive but it can stain chlamydial inclusions as little as 12 hours post infection (Poppert *et al.*, 2002). Further to this, the application of multiple probe hybridizations at different taxonomic levels with different dyes minimises the risk of any false positive signals caused by unspecific binding of dyes to non target organisms or host cell structures (Poppert *et al.*, 2002).

Epitheliocystis and the Chlamydiae

Chlamydia-like bacteria in fish have been recognised since Hoffman *et al.*, (1969), however the molecular characterisations only began in more recent years. The first agent to be associated with epitheliocystis in Atlantic salmon was '*Candidatus* Piscichlamydia salmonis' (Draghi *et al.*, 2004). The term '*Candidatus*' indicates an organism has not yet been cultured *in vitro*. A number of different CLOs from fish have since been characterized to date based on 16s ribosomal RNA gene sequences. The results indicate the Chlamydiae which infect fish are a highly divergent group of intracellular organisms, only distantly related to each other and to other members of the order Chlamydiales. It is highly likely many more new species of bacteria from this group of intracellular symbionts that infect fish will be discovered in the future.

Epitheliocystis has been recognised and studied in fish for many years, but the information available on the epidemiology or pathogenicity of this organism has not been expanded greatly since the early investigations. Advances have been made however in the development of molecular tools to identify the bacteria involved. There is a substantial gap in the knowledge when it comes to epitheliocystis and its impact on fish health, particularly in salmonids. The relationship between the cysts and gill pathology needs to be explored. Whether it is a primary pathogen, a secondary opportunistic invader or merely an incidental finding remains to be determined. In particular its

interactions, synergistic or otherwise, with both other gill pathogens of salmonids and adverse environmental factors need to be clarified.

Epitheliocystis is an infectious condition that affects the gills and less commonly the skin of fish. It has been reported from more than 50 freshwater and marine species (Nowak and LaPatra, 2006). The disease was first described in Germany by Plehn in 1920 in common carp, *Cyprinus carpio* and named *Mucophilus cyprini* as it was attributed at that time to unicellular algae (Plehn, 1920). Later it was considered to be a disease of protist aetiology. However, while investigating the disease and associated mortalities in bluegills (*Lepomis macrochirus*), Hoffman *et al.* (1969) eventually confirmed the causative agents were *Bedsonia* spp. (now known as Chlamydiae) and the condition was coined as “epitheliocystis”.

Epitheliocystis in fish is caused by a cosmopolitan group of intracellular Gram-negative bacteria, many of which remain to be characterized. To date, most have been members of the phylum Chlamydiae. The diversity, host range and occurrence in the environment of different Chlamydiae are considered to have been largely underestimated (Horn, 2008). Investigating chlamydia-like bacteria and their role as potential pathogens has been a challenge for both human and animal medicine due to their ubiquitous nature and inconsistent associations with various diseases. The same ambiguity exists as to their role as pathogens of fish. In general, it is widely suggested that these bacteria are more frequently opportunistic rather than primary pathogens of vertebrates (Horn, 2008). Further molecular analysis is required to characterize isolates from other fish species so the phylogenetic relationships of these chlamydia-like-organisms (CLOs) can be elucidated.

Epitheliocystis has long been a focus for attention in farmed Atlantic salmon and debate persists as to its significance. The bacterial agents currently associated with epitheliocystis in salmonids are summarised in Table 4.

Table 4. The occurrence of gill pathology and mortality in farmed and wild salmonid fish with epitheliocystis (pathology scored in accordance with severity, + = mild, ++ = moderate, +++ = severe, - = no pathology, R – recorded disease, NR – not recorded).

Host Species	Bacterial species	Farmed	Wild	Pathology	Mortality	Reference
Atlantic salmon Marine	' <i>Candidatus</i> Piscichlamydia salmonis'	R	NR	-/++	-/+	Draghi <i>et al.</i> (2004)
Atlantic salmon Marine	' <i>Candidatus</i> Branchiomonas cysticola'	R	NR	+++	+++	Tönshoff <i>et al.</i> (submitted for pub.)
Atlantic salmon Freshwater	' <i>Candidatus</i> Clavochlamydia salmonicola'	R	NR	-	-	Karlsen <i>et al.</i> (2008) Mitchell <i>et al.</i> (2010)
Brown trout	' <i>Candidatus</i> Clavochlamydia salmonicola'	NR	R	-	-	Karlsen <i>et al.</i> (2008)
Arctic char	' <i>Candidatus</i> Piscichlamydia salmonis'	R	NR	+++	+++	Draghi <i>et al.</i> (2010)

Opinions in the literature vary on the significance of the condition with some authors regarding it as merely an incidental finding (Nowak and Clarke, 1999) while others have observed epitheliocystis associated with cases of gill-related mortality and disease (Kvellingstad *et al.*, 2005; Nylund *et al.*, 1998; Steinum, *et al.*, 2010). Mortalities of up to 80% in some farm sites in Norway in addition to loss of production in terms of growth, have been attributed to proliferative gill inflammation (PGI) which is frequently associated with epitheliocystis.

Proliferative gill inflammation (PGI) is a disease that causes significant losses in farmed Atlantic salmon in Norway (National Veterinary Institute, 2009). Similar proliferative gill diseases have been diagnosed in Scotland and Ireland. The most severe losses occur in salmon transferred the sea in spring (called 'S1s') during their first autumn. The disease has been a challenge for Norwegian aquaculture since the 1980s. In the 1998-1999 production cycle, 18.8% of farms in Norway were affected by PGI (Nygaard, 2004). In 2002-2003 that figure rose to approximately 35%, or 250 sites (Nygaard, 2004, Kvellingstad *et al.*, 2005b) The number of cases of PGI appear to be declining again, with 182 outbreaks recorded in the 2008-2009 fish. The apparent recent decrease in the number of PGI cases may also be as a result of decreased reporting (PGI is not notifiable).

In salmonids, at least two different bacterial species from the phylum Chlamydiae have so far been associated with epitheliocystis. These are '*Candidatus* Piscichlamydia salmonis' from marine stage Atlantic salmon (Draghi, *et al.*, 2004) in Norway and Ireland and freshwater Arctic char (*Salvelinus alpinus*) in North America (Draghi *et al.*, 2010) and '*Candidatus* Clavochlamydia salmonicola' from freshwater Atlantic salmon and wild brown trout (*Salmo trutta* L.) in Norway (Karlsen *et al.*, 2008) and freshwater Atlantic salmon in Ireland (Mitchell *et al.*, 2010). Epitheliocystis caused by '*Ca. C. salmonicola*' has been observed in freshwater salmonids with mild gill inflammation and respiratory problems in Norway (Karlsen *et al.*, 2008). Mitchell *et al.*, (2010) studied salmon infected with '*Ca. C. salmonicola*' from fresh water through to seawater and observed that the epitheliocystis and all trace of the bacteria disappeared after approximately six weeks of the fish being at sea.

The relationship between '*Ca. P. salmonis*' and PGI was investigated by Steinum *et al.*, (2010) in 10 geographically distinct salmon farming zones in Norway. A positive association existed between the presence of '*Ca. P. salmonis*' and the disease in fish affected in all areas. However, the occurrence of epitheliocystis and the levels of it observed showed no association with molecular detection of '*Ca. P. salmonis*', suggesting that at least one other organism was likely to be responsible for many of the observed bacterial inclusions. This organism has been recently identified as the first non-chlamydial agent of epitheliocystis, '*Candidatus* Branchiomonas cysticola' (Tönshoff *et al.*, 2012).

Epitheliocystis has been described in a number of different farmed salmonids but was first described in Atlantic salmon in Scotland by Turnbull *et al.* (1989). Using literature available from wild and farmed fish disease surveys, Nowak and LaPatra (2006) reported that the prevalence of epitheliocystis is generally greater in farmed (mean 73%, range 20 - 100%) than in wild fish (mean 28%, range 1 - 91%), and mortalities attributed to epitheliocystis in farmed fish can be very high, ranging from 4 - 100%. During a study of cardiomyopathy syndrome (CMS) and other diseases in Norway, 132 out of 174 farms (75.9%) reported the occurrence of epitheliocystis during the study period (1998 to 2001). In 12 of these farms there was significant mortality attributed to the condition (Brun *et al.*, 2003) or at least to gill disease in which it was a factor. A prevalence study in Tasmania (Nowak and Clarke, 1999) found much lower numbers of fish were generally affected by epitheliocystis, in the region of 20%, with no significant mortality reported. There appears to be a seasonal pattern to epitheliocystis infections. In Tasmania the highest prevalence of infection

occurred during the summer months. In Ireland, infection levels are highest in summer / autumn (Susie Mitchell, personal observation) and the peak of infection in Norway (National Veterinary Institute, 2009) and Scotland (personal communication, David Cockerill) occurs in late autumn. This seasonality may indicate water temperature is an important risk factor in infections. Risk factors associated with the development of PGI remain to be fully determined although trends indicate there is an increased risk of PGI with a high number of fish on site and a reduced risk of disease when the pens are located in areas with good water exchange and where regular net cleaning is practiced (Grøntvedt *et al.*, 2006). The study showed feeding of immunostimulants may also be beneficial.

The transmission and mode of infection of the epitheliocystis agent remains undetermined. Given the ubiquitous nature of the pathogen and infection patterns there is strong evidence for one or perhaps several different environmental reservoirs. Many chlamydia are known symbionts of amoeba. The amoeba may serve as reservoirs for transmission and be vehicles of dispersal (Horn, 2008). However, despite several attempts, isolation of '*Ca. P. salmonis*' or any other fish chlamydial agent of epitheliocystis from different species of amoeba has so far been unsuccessful.

Transmission of the disease is not clearly understood, hampered by the fact that no challenge model has, as yet, been developed. Anecdotal reporting of horizontal transmission has been demonstrated in some species. Wolf (1988), in his chapter on epitheliocystis, describes how a colleague, D.S. Wyand, conducted successful transmission experiments in goldfish (*Carassius auratus* L.) by adding infective gill tissue to aquaria. Disease developed in naïve fish 3-4 weeks post exposure to infective material. Similar transmission experiments have allegedly been carried out in the bluegill, as reported again anecdotally by Lannan *et al.* (1999). Until the pathogen can be isolated and cultured, the development of robust reproducible challenge models will be difficult.

Epitheliocystis infections are frequently described without any associated pathology or clinical effects. In infections where there is a proliferative host response to the cysts, affected fish have been described as lethargic and displaying signs of respiratory distress such as increased ventilation and gasping at the water surface. Deformation of the opercular cover, increased mucus production and distortion of the lamellar structure has also been described. The gills of moribund or dead fish may be covered densely in white or mucoid spheres (Nylund *et al.*, 1998).

Under light microscopy large cysts can sometimes be seen in wet preparations of gill tissue (Nowak and LaPatra 2006), but more frequently the epitheliocystis are only visible on slides stained for histopathology. Infected gill lamellar epithelial cells become spherical basophilic cysts

surrounded by an eosinophilic hyaline capsule which are proposed to be the remnants of the host cell membrane and cytoplasm (Molnar and Boros, 1981). The cysts contain basophilic granular material which represents the bacteria. A complete developmental cycle has been described for the agents of epitheliocystis from a range of fish species using transmission electron microscopy. In each case the bacteria precede from small rigid infectious forms to larger pleomorphic non-infectious forms that divide by fission to produce daughter cells (Paperna *et al.*, 1981).

Despite the fact that a number of different molecular techniques have been developed recently to enable the identification and characterization of the agents of epitheliocystis, histopathology remains the mainstay of diagnosis of the condition. This enables the significance of the infection to be quantified by observations on the relationship between any gill pathology and the location of the cysts (Nowak and LaPatra, 2006).

Due to their intracellular nature, the Chlamydiae cannot be isolated through regular microbiological methods such as agar or broth culture. Culture of many of the pathogenic Chlamydiae from terrestrial species of animal and humans is usually carried out by growth and isolation of the bacteria using appropriate cell lines. However, despite the fact that numerous attempts have been made by different researchers using different fish cell lines and conditions to culture the bacteria, no progress with culturing the agents of epitheliocystis has been made to date (personal communications, Matthias Horn, Terje Steinum). This lack of any type of *in vitro* culture techniques hampers the development of experimental challenge models making the transmission and pathogenesis of the disease difficult to elucidate (Nowak and LaPatra 2006).

Several studies have identified the target host cell of epitheliocystis in Atlantic salmon as the lamellar epithelial cell (Turnbull, 1993; Nowak and Clarke, 1999). As mentioned earlier, epitheliocystis can occur as a benign or proliferative disease characterized by the presence of cysts in the lamellar epithelium. The two presentations of infection have been observed in many fish species, including Atlantic salmon. The infective cysts usually appear as hypertrophied epithelial cells ranging in size from 10 - 400 micrometers (Lannan *et al.*, 1999). In benign infections, cysts are surrounded by a layer of squamous or cuboidal epithelial cells. No host response may be apparent even when there are a large number of cysts. The number, size and location of the cysts vary with infection, species of fish affected and possibly the immune status and age of the host. In certain cases however the organism induces an extensive host response with proliferation of host epithelia and excessive mucus production, sometimes referred to as hyperinfection (Lannan *et al.*, 1999). The cause for this variation is unclear. Factors such as the age of the fish, stress or environmental

conditions rather than the type of cell infected may be responsible for switching on the proliferative cycle. Mortality in older fish is usually associated with proliferative epitheliocystis; however non-proliferative responses are rarely observed when early life stages of fish succumb to the disease (Nowak and LaPatra 2006).

The first study describing the molecular characterization of a causative agent of epitheliocystis in marine Atlantic salmon was by Draghi *et al.* (2004), using DNA sequence analysis and *in situ* hybridization. They studied outbreaks of epitheliocystis that occurred in Atlantic salmon in Ireland and Norway in 1999 and 2000 respectively. DNA sequence analysis revealed a chlamydia-like agent in affected gill tissues and the name '*Candidatus* Piscichlamydia salmonis' was proposed. Gills from both outbreaks had proliferative lesions. Pathological changes included multifocal diffuse epithelial hyperplasia, lamellar fusion, bridging across tips of adjacent lamellae, focal necrosis of epithelial cells, and infiltrates of macrophages. The bacterial inclusions were not always associated with epithelial hyperplasia, and were occasionally located on lamellae with no lesions. During the study, gill tissues from another Irish outbreak in 1995 were also examined and processed. These gills had no proliferative changes and fewer more widespread bacterial inclusions, usually just located on the distal tips of non-lesioned lamellae. Ultrastructural differences between the developmental stages of the 2000 Norwegian sample and the 1995 Irish sample suggest two different chlamydia-like developmental cycles exist in farmed Atlantic salmon; one associated with proliferative lesions and the other with non-proliferative infections. The possibility that the two types of infection may have been caused by different species of chlamydia was suggested by the authors. Crespo *et al.* (1999) also found evidence for two distinct chlamydia-like developmental cycles in sea bream (*Sparus aurata* L.), using light and electron microscopy. As observed in Atlantic salmon, one type was associated with a proliferative response in the gills whereas the other elicited no host response.

A number of studies have discussed treatment methods for epitheliocystis in different species. Treatment of largemouth bass (*Micropterus salmoides*, Lacépède) with oxytetracycline (25ppm) twice a day for three days was reported to be effective in fish with hyperinfection (Goodwin *et al.*, 2005). This particular antibiotic has been used with equivocal results in farmed Atlantic salmon undergoing infection with '*Ca. P. salmonis*' in Scotland (personal communications, Peter Southgate, Richard Collins). Rodger and Giron (2008) reported successful treatment of epitheliocystis in farmed perch (*Perca fluviatilis*, L.) using oral oxytetracycline at 75mg/kg for 10 days. In the past chlramphenicol was used successfully to treat cultured sea bream (Paperna, 1977). This drug is not currently licensed for fish entering the food chain. However, florfenicol, an analog of chlramphenicol, is currently licensed for use in salmonids. Use of this antibiotic to treat

epitheliocystis has not yet been reported, but it may potentially be very effective.

Finally, the use of compounds other than antibiotics to treat epitheliocystis have rarely been reported. The disinfectant compound 'Chloramine T' was used as a bath treatment for Atlantic salmon during an outbreak of epitheliocystis in Norway but proved unsuccessful (personal communication, Duncan Colquhoun). UV treatment of seawater during the rearing of marine juvenile species of amberjack (*Seriola dumerili*, Risso) and leopard coral trout (*Plectropomus leopardus*, Lacépède) has been reported as an effective means of controlling outbreaks of the disease (Nowak and LaPatra, 2006). There is much scope for further research in this area.

1.3 Objectives of this thesis

The work detailed in this thesis was undertaken to further our knowledge of epitheliocystis and its role in gill disease in Atlantic salmon.

Experiments and studies were undertaken to fulfill the following aims:

- To perform an extensive review of all the available knowledge on gill disorders of both infectious and non-infectious aetiologies.
- To establish which species of bacteria were responsible for epitheliocystis in freshwater Atlantic salmon in Ireland in a number of different geographical locations, to assess if there were any pathological effects of infection and finally to characterise the epidemiology of infections.
- To develop a novel histopathological gill scoring system for use as a monitoring tool to both identify a threshold for pathology of clinical significance and enable comparisons of pathology over time and between different cases of gill disease.
- To investigate the prevalence, geographical distribution and role of the newly discovered first non-chlamydial agent of epitheliocystis, "*Candidatus* Branchiomonas cysticola" in gill disease.
- To develop a challenge model for epitheliocystis infections using "*Candidatus* Clavochlamydia salmonis".

2 Epitheliocystis in Atlantic salmon, *Salmo salar* L., farmed in fresh water in Ireland is associated with 'Candidatus Clavochlamydia salmonicola' infection.

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Statement of Contribution: Dr. Terje Steinum (Veterinary Institute, Oslo, Norway) assisted in the design of the primers and probe for Real-Time PCR analysis and gave instruction on how to carry out all molecular techniques described in this chapter. All remaining work was carried out by the author of this thesis.

2.1 Introduction

The impact of gill disease in marine-farmed Atlantic salmon, *Salmo salar* L., has increased in Europe in the last decade, with Ireland, Norway and Scotland all reporting substantial losses (Nylund *et al.*, 1998; Rodger, 2007; David Cockerill, personal communication). In Ireland alone, it was the leading cause of mortality in farmed Atlantic salmon from 2003 – 2006 (Rodger, 2007). Records indicate that gill disease was directly responsible for approximately 15% mortality over the 2007 – 2008 culture cycle, in addition to substantial production losses associated with sub-clinical disease (Hamish Rodger, pers. comm.).

Gill diseases of proliferative types are being increasingly recognised. In Norway, proliferative gill inflammation (PGI), a disease syndrome of multifactorial aetiology has emerged as a serious threat to fish health. PGI occurs typically during the first autumn following spring seawater transfer in marine stage Atlantic salmon and mortality levels can be in the region of 15-20%. (Kvellestad *et al.*, 2005). Diagnosis of PGI is based on the presence of four pathological changes: gill epithelial hyperplasia, infiltration of inflammatory cells, the presence of necrosis in the affected tissues and circulatory disturbances in areas of pathological change (Kvellestad *et al.*, 2005). A disease with a similar clinical picture has been also diagnosed during autumn in spring Atlantic salmon smolts in both Scotland (David Cockerill, pers. comm.) and Ireland (pers. obs.). Epithelial inclusions (epitheliocysts) containing Chlamydia-like organisms (CLO) are commonly associated with these proliferative conditions.

Epitheliocystis is an infectious condition affecting the gills and, less commonly, the skin of fish. It has been reported from more than 50 freshwater and marine species throughout the world (Turnbull, 1993; Fryer and Lannan, 1994; Nowak and LaPatra, 2006). CLO infection may result in varying host responses in cultured fish species, from no observable change to severe gill lamellar hyperplasia and cellular degeneration with associated mortality (Paperna, 1977; Paperna and Sabnai, 1980; Crespo *et al.*, 1990; Venizelos and Benneti 1996; Crespo *et al.*, 1999; Draghi *et al.*, 2004; Kim *et al.*, 2004; Goodwin, *et al.*, 2005; Katharios *et al.*, 2008).

While the lack of culture systems for these bacteria makes detailed characterization, and fulfillment of Kochs postulates difficult, molecular data (exclusively based on 16S rRNA) from CLOs infecting fish has recently become available (Draghi *et al.*, 2004; Meijer *et al.*, 2006; Draghi, *et al.*, 2007; Karlsen *et al.*, 2008). CLOs identified in piscine hosts to date appear to comprise a genetically diverse group of bacteria that are only distantly related members of the order *Chlamydiales* (Meijer

et al., 2006; Karlsen *et al.*, 2008). Epitheliocystis in fish is, therefore, now generally accepted as being caused by a relatively diverse group of intracellular Gram-negative bacteria within the Chlamydiaceae.

Histologically, epitheliocysts caused by bacteria in sea water Atlantic salmon, can be generally distinguished from those caused by '*Candidatus Clavochlamydia salmonicola*' ('*Ca. C. salmonicola*'), the agent of epitheliocystis identified in freshwater Atlantic salmon. (Karlsen *et al.*, 2008). There are frequently marked differences in the morphology of the cysts (pers. obs.). Those caused by '*Ca. C. salmonicola*' are frequently found in the interlamellar spaces and are large and voluminous, whereas those observed in seawater Atlantic salmon are frequently found at the apical tips of the lamellae and are smaller, circular and more densely packed. Until recently all seawater epitheliocysts in Atlantic salmon were assumed to be caused by '*Candidatus Piscichlamydia salmonis*.'

The purpose of studying freshwater epitheliocyst infections was many-fold. Firstly, we wanted to investigate the identity of the main CLO in freshwater salmon in Ireland from a variety of different geographical locations. Secondly, we wanted to explore the epidemiology of this bacterium by looking at factors such as prevalence and infection abundance and how these varied both geographically and after seawater transfer. Finally, the potential of these freshwater infections to impact on gill health both prior to and post seawater transfer was also considered.

2.2 Materials and methods

Sample collection

For the freshwater study, twenty Atlantic salmon pre-smolts were randomly sampled from each of four sites in Ireland, two in the northwest (Site A and Site D) and two in the west (Site B and Site C). The exact location of the farms will remain anonymous for commercial reasons. The sampling sites included three commercial farms (Sites A, B & D) and one salmon-ranching facility (Site C). Two of the commercial farms (Sites A & D) and the ranching facility were land-based in which the fish were held in tanks and water supplied from local rivers, while the remaining site consisted of lake-based net pens. No seawater was used in any of the farms. Fish were sampled between November and December 2008 (Table 1).

Smolts supplied by Site D were followed in the sea water longitudinal study. Prior to seawater transfer, samples were taken from site D in February 2009 and fish were transferred to Site X

at sea within a two-week period. Fish were then sampled at regular intervals following seawater transfer (approximately four, six, ten and twelve weeks post - transfer). Ten to fifteen fish were sampled from a single pen at each sampling (Table 1).

All fish sampled were from the same cohort and were between 40 – 60g body weight in freshwater and 50 – 90g in seawater. No gross clinical abnormalities or pathological changes were observed.

Table 1. Freshwater and marine study timetable including farm sites, date of sampling and number of fish sampled in each case.

Site	Date of sampling	Number of fish sampled
A	12 th November 2008	20
B	30 th November 2008	20
C	3 rd December 2008	20
D	12 th November 2008	20
D	22 nd February 2009	20
X	18 th March 2009	15
X	4 th April 2009	10
X	29 th April 2009	15
X	14 th May 2009	15

16S rRNA gene amplification and sequence analysis

Tissue samples were collected from the first gill arch on the left side of each fish, transferred to RNeasyTM (Ambion Inc., Texas, U.S.) and stored at -20°C. DNA was extracted from gill tissues using the High Pure PCR Template Preparation Kit (Roche Ltd., Basel, Switzerland) while RNA was isolated using the TRIzol[®] Reagent (Life Technologies Inc., Gaithersburg, MD, USA). Nearly full-length 16S rRNA gene sequences were amplified and sequenced from cDNA of three random samples from each of the four freshwater sites using primers detailed in Table 3. Negative controls incorporating DEPC-treated water (Invitrogen) were included in all experiments. The majority of sequencing was performed using DYEnamicTM ET terminator reagents (GE Healthcare Bio-Sciences, Little Chalfont,

UK), and a MegaBace 1000 sequencing system (GE Healthcare Bio-Sciences) Additional sequencing of purified PCR products to verify results was performed by Macrogen Inc., Seoul, Korea. Raw sequence data was edited and assembled in Vector NTI Suite 11 software (Invitrogen (InforMax Inc.)) and contiguous sequences submitted to BLAST search (Altschul *et al.*, 1990).

Real Time PCR

An assay was designed targeting a 178 bp sequence of the ‘*Ca. C. salmonicola*’ 16S rRNA gene utilizing primers ClavoRTf, ClavoRTr and probe ClavoRTp (TAG Copenhagen AS, Copenhagen, Denmark. Table 3). Assay specificity was initially evaluated *in silico* using BLAST analysis. No potential cross reactions with other bacteria available in the GenBank database (NCBI) were identified. Comparison with sequences from other epitheliocystis-causing agents revealed extensive mismatches in both primers and probe (Table 4). Actual specificity of the PCR was further tested against a taxonomically broad panel of bacterial isolates (Table 2). All strains tested were either reference strains or clinical isolates identified by the National Veterinary Institute (Norway).

Table 2. Taxonomically broad bacterial isolates used to test specificity of PCR for ‘*Ca. C. salmonicola*’.

Bacterial Isolates	
<i>Arthrobacter globi</i>	<i>Photobacterium phosphoreum</i>
<i>Aeromonas. salmonicida</i> subsp. <i>salmonicida</i>	<i>Piscirickettsia salmonis</i>
<i>A. salmonicida</i> subsp. <i>smithia</i>	<i>Proteus mirabilis</i>
<i>A. salmonicida</i> subsp. <i>achromogenes</i>	<i>Pseudomonas fluorescens</i>
<i>Bacillus cereus</i>	<i>Rhodococcus erythropolis</i>
<i>Brochothric thermosphacta</i>	<i>R. equi</i>
<i>Carnobacterium piscicola</i>	<i>Serratia marcescens</i>
<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>
<i>Francisella noatuensis</i>	<i>Streptococcus agalactiae</i>
<i>Micrococcus luteus</i>	<i>Tenacibaculum maritimus</i>
<i>Moritella viscosa</i>	<i>Vibrio salmonicida</i>
<i>Mycobacterium marinum</i>	<i>V. splendidus</i>
<i>Nocardia asteroides</i>	<i>V. anguillarum</i>
<i>Pasteurella skyensis</i>	<i>Yersinia ruckeri</i>

Following optimization, the final assay conditions were: 10 pmol of each primer and 5 pmol of probe were used in 20 µl reactions with Taqman Universal PCR Mastermix (Roche molecular systems Inc. Branchburg, USA) according to recommendations. The reaction protocol, run on the Mx3005P™ real time PCR system (Stratagene, Texas, USA), was as follows: 2 minutes at 50°C, 10 minutes at 95°C, followed by 50 cycles of 95°C for 30 seconds and 60°C for 1 min. Analysis of RT-PCR data and

standard curve construction, from a 10X dilution series, was done with MxPro software (Stratagene, Texas, USA). Calculations suggested the efficiency of the '*Ca. C. salmonicola*' assay to be 103.3% (slope -3.245, y-intercept 40.97). No amplification was observed after ~ cycle 38 during evaluation or analysis of the material. Any sample producing a lower Ct value than 38 and a log phase amplification curve was considered positive for '*Ca. C. salmonicola*' by Real Time PCR. DNA extraction efficiency and suitability of the extracted DNA for PCR was evaluated using a PCR directed at the Atlantic salmon Elongation factor alpha 1 gene as described by Bruno *et al.*, 2007, Table 3). Five random samples from each of the four freshwater sites, investigated with this assay revealed essentially identical Ct values (within ~ one Ct value). This indicates very similar total DNA quantities and extraction efficiency for individual samples, allowing meaningful comparisons of Ct values between samples for the '*Ca. C. salmonicola*' assay. Negative and positive controls using DEPC-treated water (Invitrogen) and DNA from a Norwegian gill tissue sample, confirmed positive by sequencing, were included in all testing.

Table 3. Primers and probes used. Primer ClavoRTf contains R (A or G) since previously published ‘*Ca. Clavochlamydia salmonicola*’ sequences EF577391 and EF577392 (Karlsen *et al.* 2008) differ in this position.

Primer/Probe	Sequence (5' – 3')	Specificity	Reference
Primer pairs used to produce 16S rRNA gene amplicons for sequencing			
1. EcF1 (for.)	5'-ATTGAATGCTGACGGCGTGG-3'	Known Chlamydiae	Karlsen <i>et al.</i> 2008
1. ClavoR	5'-CGATATCTCTATCGCTGTCACG-3'	' <i>Ca. C. salmonicola</i> '	This study
2. ClavoF2	5'-CATACTTGGTGTGAGTGGACTC-3'	' <i>Ca. C. salmonicola</i> '	This study
2.CHR11 (rev.)	5'-CGCCATTACTAGCAATTCC-3'	Known Chlamydiae	Karlsen <i>et al.</i> 2008
3. 16SIGF	5'-CGGCGTGGATGAGGCAT-3'	Known Chlamydiae	Everett <i>et al.</i> 1999
3. 16SIGR	5'-TCAGTCCCAGTGTTGGC-3'	Known Chlamydiae	Everett <i>et al.</i> 1999
Additional primers for 16S rRNA gene amplicon sequencing			
ClavoF3	5'-GGTAAAGAAGCACCGGCTAAC-3'		This study
ClavoR2	5'-CTACGCATTTCACCGCTACAC-3'		This study
Real Time PCR assay for '<i>Ca. C. salmonicola</i>'			
ClavoRTf	5'-GGAACGATRACTTCGGTTGTTG-3'	' <i>Ca. C. salmonicola</i> '	This study
ClavoRTr	5'-CATAGACTCTTCATCAACCACG-3'	' <i>Ca. C. salmonicola</i> '	This study
ClavoRTp	5'-Fam-CGAACTCCAGATGCCTGGAGCCG-BHQ-3'	' <i>Ca. C. salmonicola</i> '	This study
Real Time PCR assay for Elongation factor alpha 1 gene			
AS_ELFSTD_F	5'-GGCCAGATCTCCAGGGCTAT-3'	Atlantic salmon	Bruno <i>et al.</i> 2007
AS_ELFSTD_R	5'-TGAAGTTGCAGGCGATGTGA-3'	Atlantic salmon	Bruno <i>et al.</i> 2007
AS_ELFSTD_P	5'-Fam-CCTGTGCTGGATTGCCATACTG-BHQ-3'	Atlantic salmon	Bruno <i>et al.</i> 2007

Table 4. *In silico* mismatches between ‘*Ca. C. salmonicola*’ and other CLOs causing epitheliocystis in fish hosts or present in water. The *Neochlamydia*-like bacterium in Artic charr (Draghi *et al.*, 2007) has a sequence identical to AY225593.

Identity (Accession no.)	Source / Host	<i>In silico</i> mismatches		
		Forward Primer ClavoRTf (22 bp)	Reverse Primer ClavoRTr (22 bp)	Probe ClavoRTp (23 bp)
<i>Criblamydia sequanensis</i> (DQ124300)	River water	16	6	11
<i>Neochlamydia</i> sp. (AY225593)	Cat (Artic charr)	18	8	9
‘ <i>Ca. P. salmonis</i> ’ (AY462244)	Atlantic salmon	14	8	17
Uncult. Chlamydiales (AY013394)	Silver perch	15	8	10
Uncult. Chlamydiales (AY013396)	Leafy seadragon	14	10	12
Uncult. Chlamydiales (AY013474)	Barramundi	13	6	7

Histopathology

155 branchial tissue samples were collected in total from randomly selected fish on each of the sites (Table 1). The second gill arch from both sides of each fish was chosen for histopathological examination. Samples were fixed in 10% neutral buffered formalin; 5µm sections from paraffin embedded tissue were cut and stained with hematoxylin and eosin. Representative slides were also stained with giemsa and gram stains. Samples were then scanned at X100 magnification and the number and morphology of bacterial cysts recorded (“cysts” are defined aggregates of basophilic, host membrane-bound, bacterial organisms, readily visible on histopathology).

Fluorescence in-situ Hybridisation

Bacterial inclusions were visualized using fluorescence in-situ hybridisation (FISH). Five fish with high Ct values by real time PCR were selected. FISH was carried out on both deparaffinised slides and fresh frozen gill tissues. Three fluorescently labeled rRNA-targeted oligonucleotide probes for hybridisation were designed using ProbeBase (Loy *et al.*, 2007), a general eubacterial probe (EUB-Mix-Cy5) that targets most bacteria, a general Chlamydial probe (Chls-523-FLUOS) that targets most members of the order Chlamydiales and finally a probe specific for ‘*Ca. C. salmonicola*’ (Clavo-16S-652-Cy3). The frozen gill tissues was thawed, gently squashed and fixed in 4% paraformaldehyde for 1 hour at 4°C. The squashed tissues were transferred to Teflon-coated slides and dried for 15

minutes at 46°C. Slides were then dehydrated using an ethanol series (50%, 80% & 96%) and dried again at 46°C for five minutes. Both UV light and proteinase K digestion techniques were applied to the deparaffinised slides in an attempt to reduce autofluorescence in these tissues. Hybridisations were performed on the fresh tissues and the deparaffinised sections according to the protocol described by Daims *et al.* (2005), in addition to control hybridisations using nonsense probes with all three fluorescent dyes. DAPI (4',6-diamidino-2-phenylindole), a fluorescent *stain* that binds strongly to DNA, was applied to all deparaffinised sections and fresh frozen tissues to establish viable bacterial organisms were present within the cysts. Slides were visualized by confocal laser scanning microscopy.

Epidemiology

The prevalence of infection was calculated using histopathology based on visual inspection of two hematoxylin and eosin-stained gill arches from each fish. Abundance of infection was calculated by examining 10 fields of view (x100 magnification), and an absolute count of cysts per 10 fields of view (X100 magnification) for each fish was recorded. A categorical measure of abundance using a low-medium-high scale was applied (0 cysts = Negative, 1-20 cysts = Low, 21-100 cysts = Medium, 100+ cysts = High). Prevalence of infection was also calculated using RT-PCR and the Ct values used as a measure of abundance.

Statistical Analysis

Prevalence and abundance were used as descriptive statistics for infections. Confidence intervals and standard errors were reported alongside these where appropriate. Abundance was used instead of infection intensity as we wished to consider the whole of the fish population sampled and not just infected individuals (Bush *et al.*, 1997). In this case abundance would provide a good indication of infection intensity as prevalence figures were generally very high. As there were a proportion of fish with no cysts visible on histopathology, this data was transformed by addition of 1 plus log transformation prior to analysis. The non-parametric Kendall's Tau was then used to check for associations between the infection abundance as reflected by the Ct values and the number of infective cysts per fish observed on histopathology. The two diagnostic methods of real-time PCR and histopathology were compared using Chi square analysis.

2.3 Results

PCR and sequencing

Alignment and BLAST analysis of eight contiguous cDNA sequences (~1.25-kb long) from sites revealed 100% sequence identity to '*Ca. C. salmonicola*' (EF577391). The CLO associated with epitheliocysts in freshwater farmed Atlantic salmon at four distinct geographical locations in Ireland is therefore identified as very closely related to, and most probably identical with, '*Ca. C. salmonicola*'. Four 16S cDNA sequences were submitted to the EMBL database (GenBank) and assigned accession numbers FN545849 - FN545852. Furthermore, only '*Ca. C. salmonicola*' 16S rDNA sequences were obtained from all farms studied using the "Chlamydia" signature sequence specific primers, thus confirming '*Ca. C. salmonicola*' to be the most prevalent, if not the only, CLO present in these fish.

Histopathology

Branchia inclusions consisting of densely packed coccobacillary basophilic particles were present throughout the gill filaments (Figure 1). The majority of these inclusions were located between the bases of two adjacent lamellae (Figure 2). The inclusions were unevenly distributed throughout the filaments, with clusters of inclusions spanning two to three filaments commonly observed (Figure 3). The inclusions were usually located in the proximal half or middle of each filament, with fewer observed near the tips. Lamellar epithelial cells appeared to be the target host cells for infection.

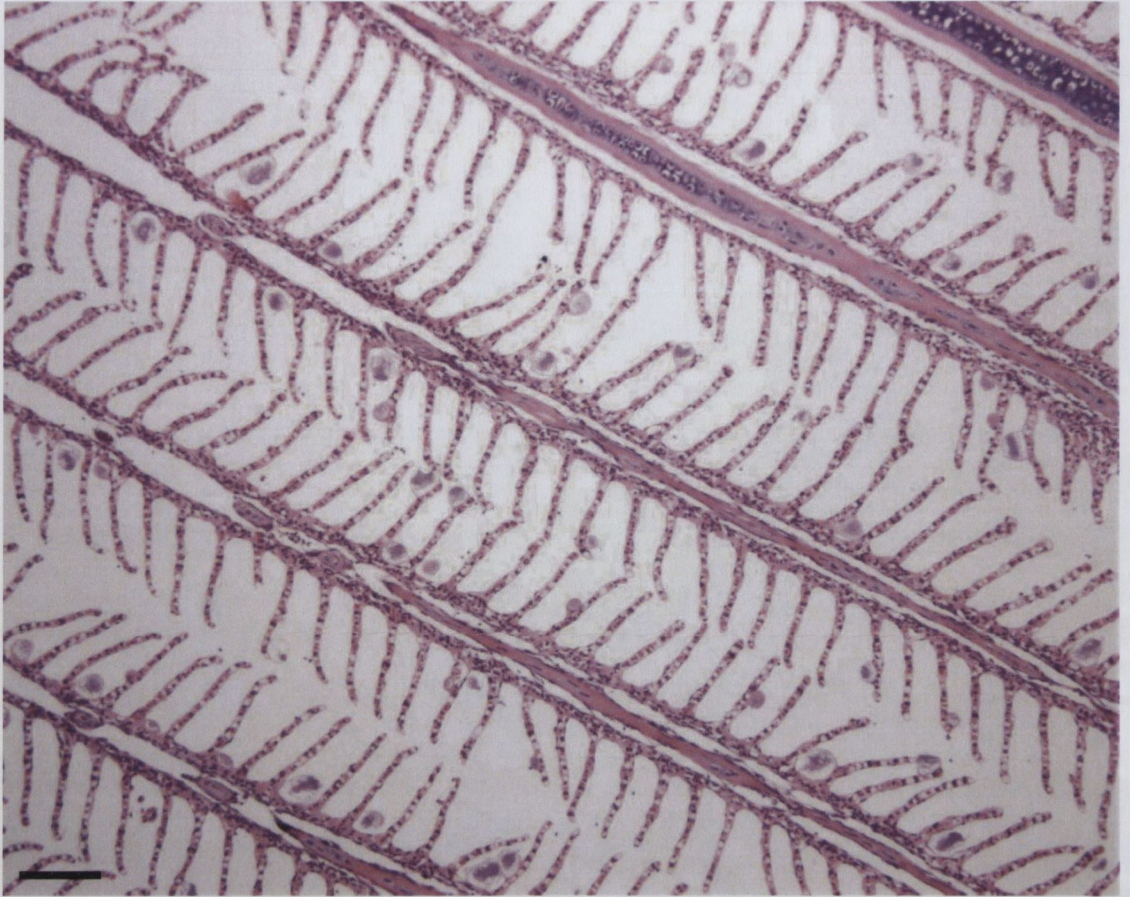


Figure 1. Atlantic salmon gill showing moderate number of '*Ca. Clavochlamydia salmonicola*' cysts (H & E) (bar = 100 μ m)

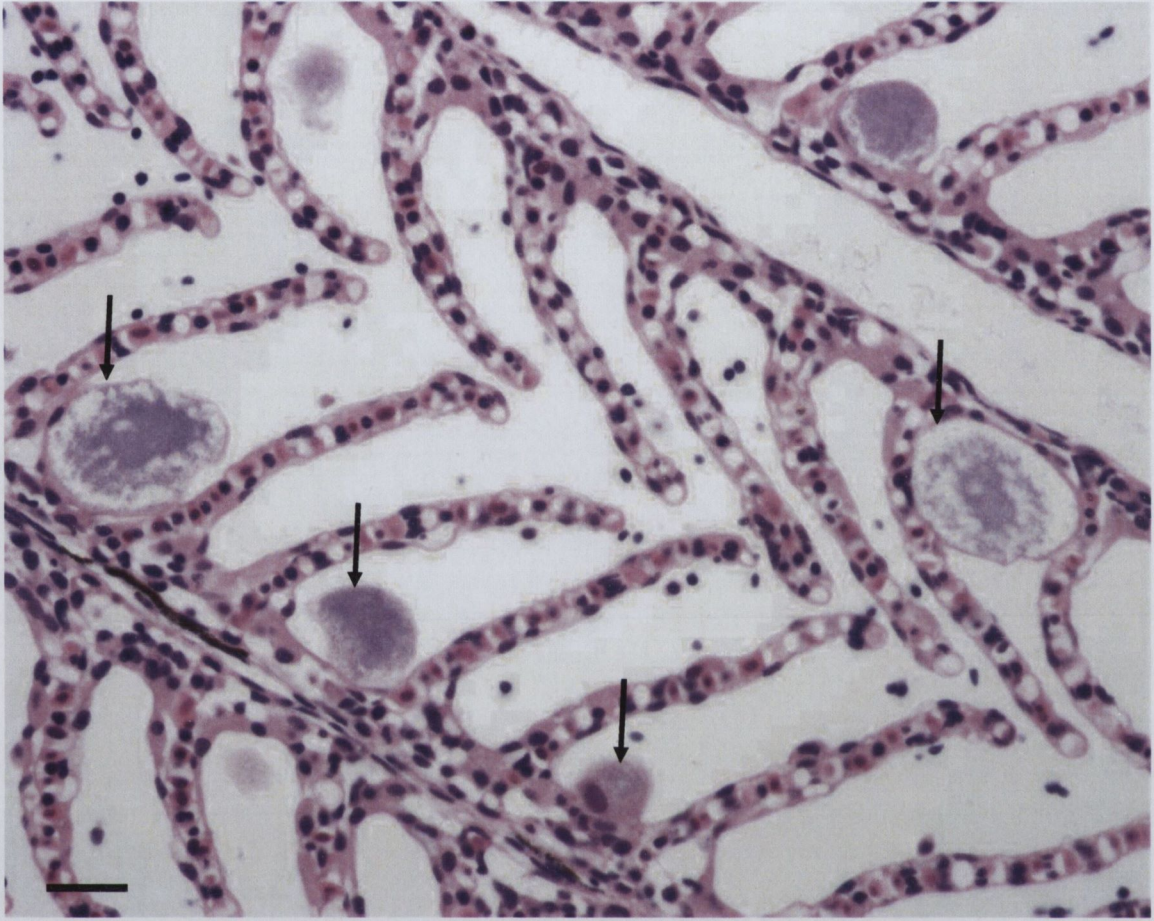


Figure 2. Cysts located between the bases of adjacent lamellae (black arrows) (H & E) (bar = 30μm)

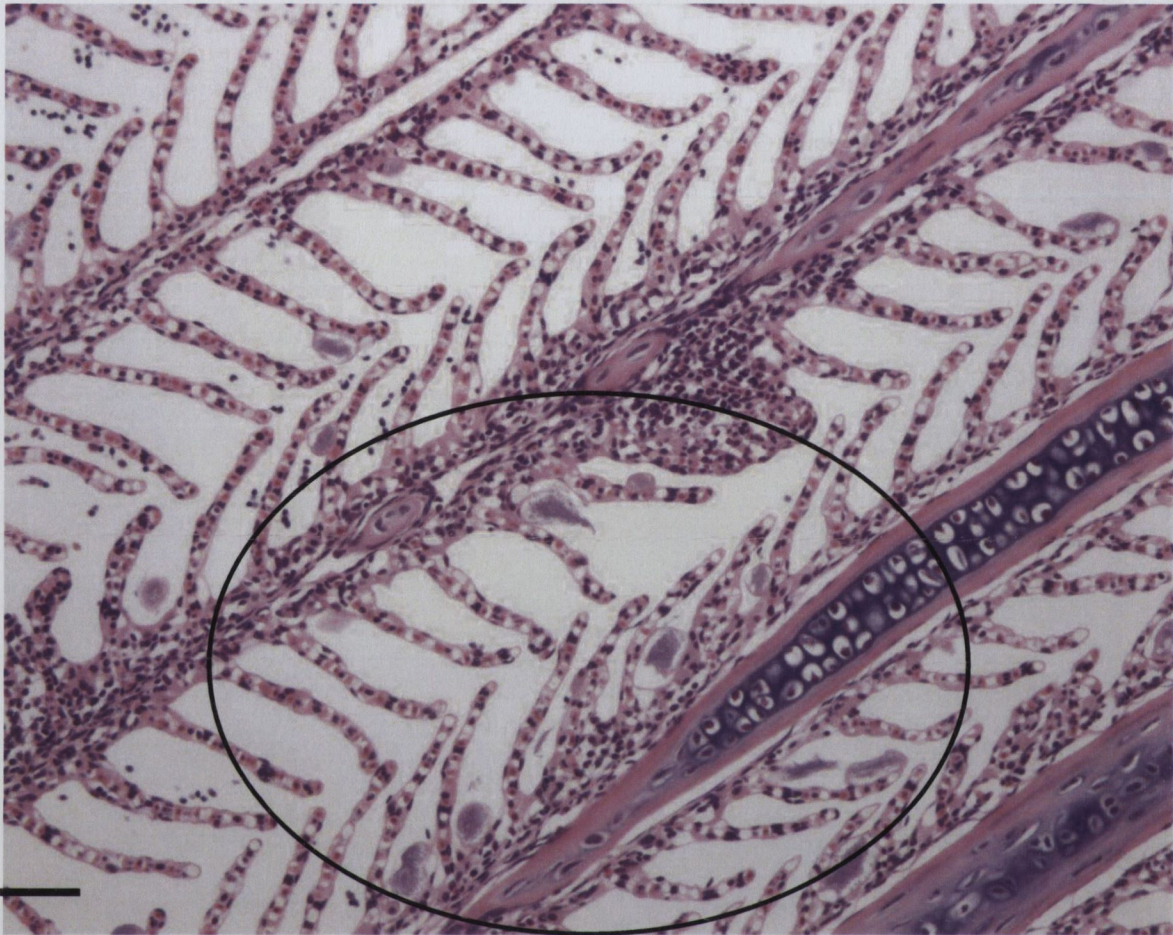


Figure 3. Picture showing an “infection cluster” spanning three filaments of Atlantic salmon gills. This picture also illustrates the large variation in cyst morphology in terms of size and shape (H & E) (bar = 75µm)

Large variations in inclusion morphology were apparent, both in individual fish, and also between fish. Small oval inclusions with densely packed organisms were observed alongside expanded and voluminous cysts with considerable space apparent between the coccobacilli (Figures 3 & 4). Regardless of inclusion morphology, the nucleus of the infected cell was usually marginalized and the cell cytoplasm reduced to a fine strip around the periphery of the cyst. Only slight pathological changes were observed adjacent to the infective cysts on light microscopy. However, many of the fish examined displayed minor focal change elsewhere on the lamellae, typically consisting of mild, multifocal hyperplasia with occasional focal lamellar fusion (Figure 4).

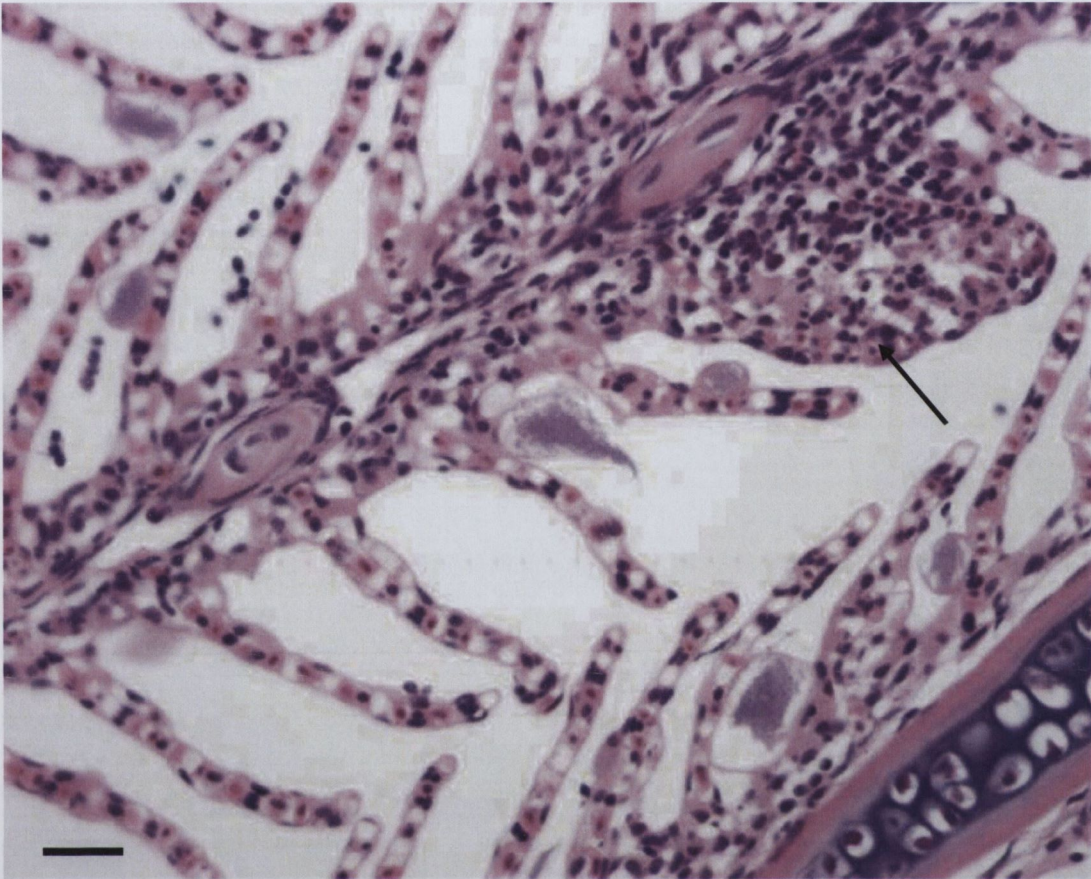


Figure 4. Close up of Figure 3 showing an area of hyperplasia not associated directly with any of the cysts (black arrow). Again, cysts of different sizes and morphologies are visible (H & E) (bar = 50 μ m)

In site D, low levels of *Tricophyra*, a sessile, filter-feeding, protozoan parasite, were observed on approximately 50% of the fish. There was no apparent correlation between the occurrence of or the levels of this parasite and epitheliocysts. No other pathogens were histologically detected.

Gimsa and Gram staining were applied to representative slides. *Ca. C. salmonicola* was strongly Gram negative and stained with Giemsa (Figure 5).

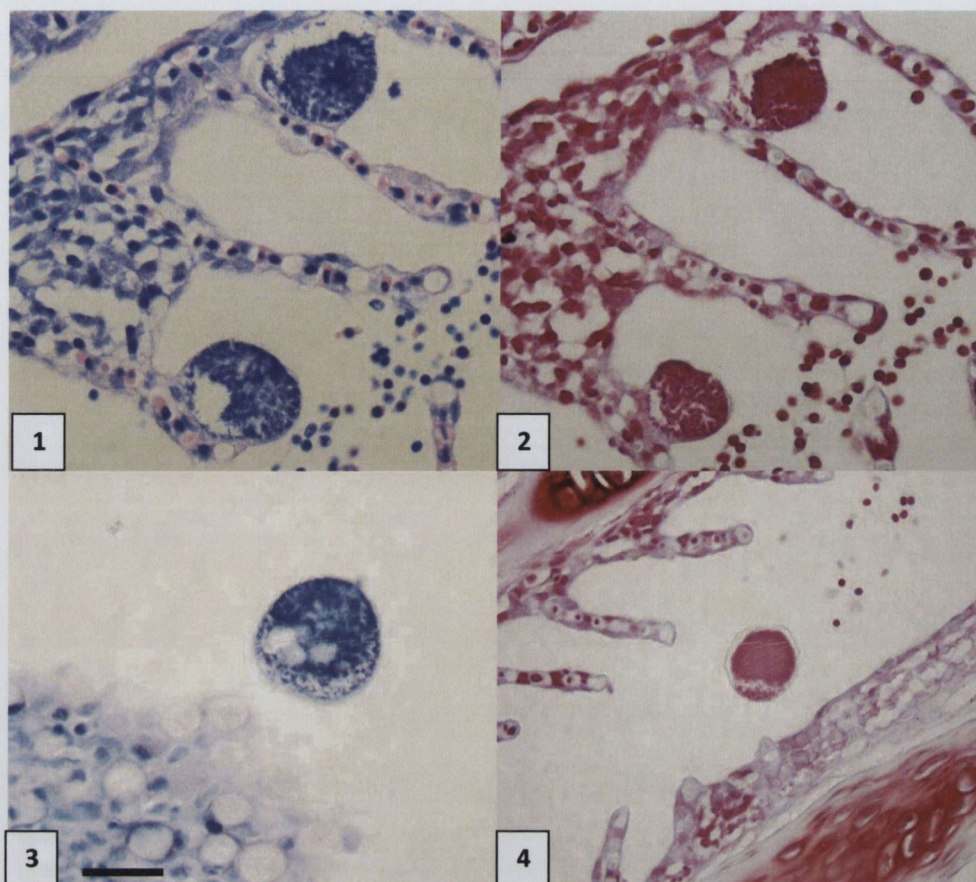


Figure 5. '*Ca. C. salmonicola*' cysts in situ in the gills, stained with Giemsa and Gram stains. Cysts clearly stain with Giemsa (1 & 2) and are Gram negative (3 & 4). (bar = 50 μ m)

Epidemiology

The prevalence and intensity of infection were calculated for all freshwater sites including site D at two different time points. Prevalence of infection ranged from 20 – 100% as estimated by histology and 75– 100% by RT-PCR (Table 5) Infection abundance differed significantly by site based on both histological observation ($F = 15.07$, $p \leq 0.0001$, $df = 2$) (Table 4) and RT-PCR Ct Value ($F = 26.56$, $p \leq 0.0001$, $df = 4.85$) (Table 5) The variation in abundance is reflected by the broad range of Ct values from 22.0 to 38.2.

Table 5. Prevalence and Infection abundance as measured by histopathology and RT-PCR. Confidence intervals included where appropriate.

Site I.D	Real Time PCR – Prevalence and Infection		Histopathology – Prevalence and Infection	
	Abundance		Abundance	
	Prevalence (± C.I.s)	Abundance – Mean Ct. Value (± S.D.) (Range)	Prevalence (± C.I.s)	Abundance – Mean cyst number (± SD) (Range)
Site A	75%	33.81 (± 3.91)	20%	0.75 (± 1.83)
	(±19.0%)	(27.14 - 38.21)	(±17.5%)	(0 – 7)
Site B	100%	27.98 (±1.94)	75%	15.76 (± 54.51)
		(23.31 – 31.68)	(±19.0%)	(0 – 252)
Site C	100%	27.16 (± 2.41)	80%	28.25 (± 48.49)
		(23.84 – 32.86)	(17.5%)	(0 – 203)
Site D	100%	27.62 (± 2.33)	85%	10.20 (± 10.49)
		(23.99 – 33.61)	(15.6%)	(0 – 37)
Site D	100%	23.90 (± 1.36)	100%	79.80 (±104.00)
		(22.00 -25.34)		(9 – 333)

Table 6. Prevalence and infection abundance using both histopathology and RT-PCR pre and post- seawater transfer. Confidence intervals included where appropriate.

Site I.D.	Real Time PCR – Prevalence and Infection		Histopathology – Prevalence and Infection	
	Abundance		Abundance	
	Prevalence (± C.I.s)	Abundance – Mean Ct. Value (± SD) (Range)	Prevalence (± C.I.s)	Abundance – Mean cyst number (± SD) (Range)
Pre- Transfer	100%	23.90 (± 1.36) (22.00 - 25.34)	100%	79.80 (± 104.00) (9 – 333)
4 weeks post- transfer	100%	28.38 (±4.74) (21.26 – 34.93)	73% (±22.5%)	42 (± 78.54) (0 – 294)
6 weeks post- transfer	10% (±18.6%)	35.92 (±1.00) (35.92 – 35.92)	0%	0
10 weeks post- transfer	0%	0	0%	0
12 weeks post- transfer	0%	0	0%	0

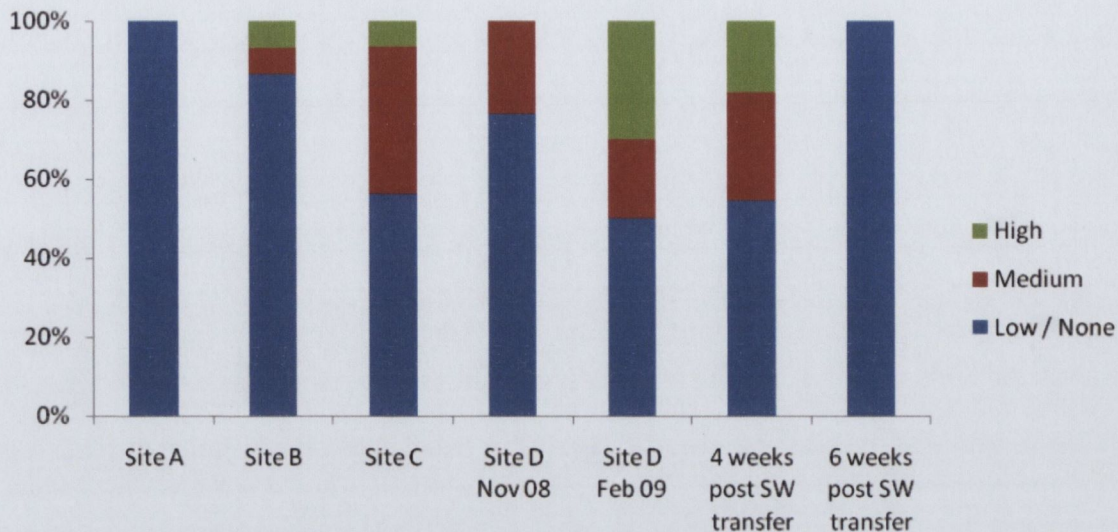


Figure 6. Histologically observed cyst levels in fish at each sampling point. Fish from site D Feb 09 were cohort followed post seawater transfer

RT-PCR appeared to be more sensitive than histopathology in detection of infection. However, as there is no “gold-standard” diagnostic test for ‘*Ca. C. salmonicola*’ these methods cannot be directly compared in terms of sensitivity and specificity, nevertheless trends can be observed. For infected fish, the percentage detection using RT-PCR was 94.6% ($\pm 4.9\%$) in contrast to 69% ($\pm 10.1\%$) for histopathology (Chi sq. = 19.63, $p \leq 0.0001$, $df = 1$). Fish positive by RT-PCR but negative on histopathology had an average Ct value of 29.5. A significant correlation was present between decreasing Ct values and the number of infective cysts per fish (Kendall’s tau = -0.724 $p \leq 0.0001$) (Figure 7). During the marine longitudinal study, histopathology and RT-PCR was used to obtain a semi-quantitative indication of the levels of infection in the fish at different time points post-seawater transfer (Table 6). The results indicate that ‘*Ca. C. salmonicola*’ disappears between 4 – 6 weeks in the marine environment.

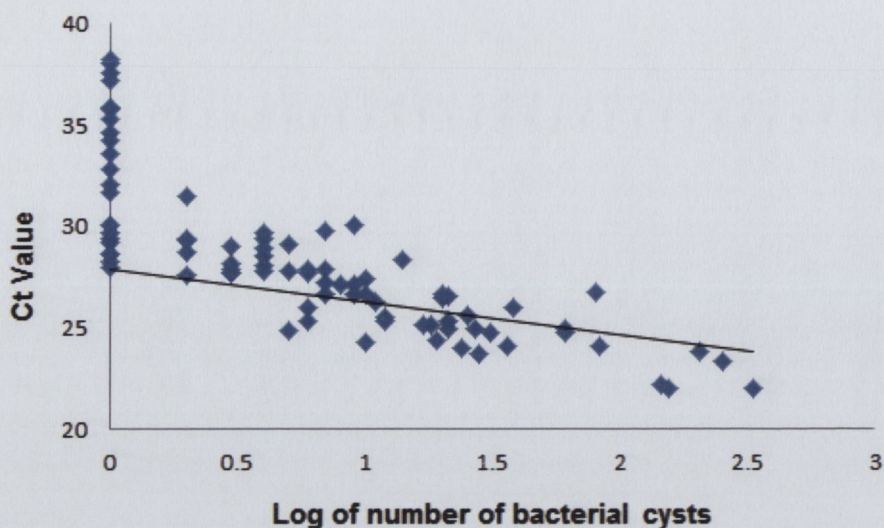


Figure 7. Graph of Ct Value vs Intensity of Infection based on histopathology (log of number of infected cysts per fish in 10 fields of view of gill tissue (quantified at X100 magnification). Line is just to show trend through non-zero points for illustration purposes only

Fluorescence in-situ Hybridisation

Positive signals were obtained in the fresh tissues using FISH in each case, with numerous squashed cysts being visible on microscopy (Figure 8). Despite the use of UV light and protenase K, moderate levels of autofluoresence were observed in the deparaffinised sections. By comparison however, strong signals were achieved within the cysts (Figure 8). DAPI staining indicated the signals within the cysts were being produced by viable bacteria. No bacteria were localised in areas of pathology distant from the cysts in the deparaffinised sections.

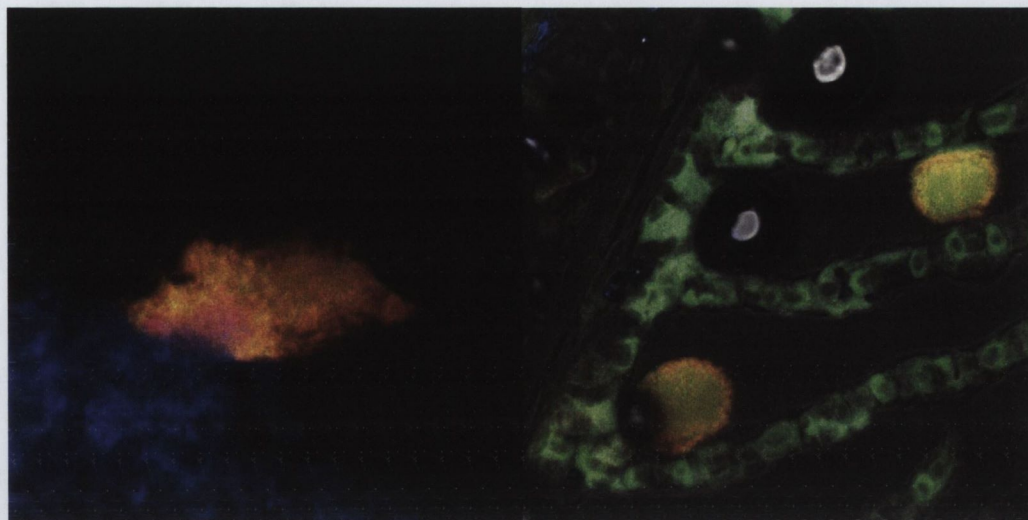


Figure 8. FISH of '*Ca. C. salmonis*' in fresh squeezed gill tissue (left) and deparaffinized slides of gill tissue (right). General bacterial probe EUB-Mix labelled in Cy5, General Chlamydial probe labelled in Fluos; competitor, '*Ca. C. salmonicola*' specific probe labelled in Cy5. (Artefactual air bubbles visible in deparaffinized section)

2.4 Discussion

'*Candidatus* Clavochlamydia salmonicola' was identified as the agent responsible for epitheliocystis in Atlantic salmon in four different freshwater sites in Ireland. This is the first description of any freshwater agent of epitheliocystis in Ireland and only the second ever report of this bacterium affecting Atlantic salmon. It is also the first report to explore and investigate the epidemiology of CLO infections in freshwater salmonids, and to establish the existence and fate of '*Ca. C. salmonicola*' in marine stage Atlantic salmon.

The identity level (100%) of 16S rRNA gene sequences obtained from the infected Atlantic salmon from the four different freshwater sites, with the sequences described by Karlsten *et al.* (2008), confirm the organism identified in the present study to be highly related to, and most probably identical with "*Ca. C. salmonicola*," (Karlsten *et al.*, 2008) from salmonids in Norway. The fact that this bacterium was causing the observed cysts was supported by a number of factors. Firstly, '*Ca. C. salmonicola*' related sequences alone were retrieved following 16S rRNA gene

'signature sequence' PCR. Secondly, there was a strong correlation between the number of histologically observed cysts and number of bacteria as estimated by RT-PCR. Finally, fluorescence *in situ* hybridization using highly specific oligonucleotide rRNA probes was used to localise '*Ca. C. salmonicola*' within the cysts in whole mount gill sections, directly relating the obtained sequences to the epitheliocysts in the fish.

As observed by Karlsen *et al.* (2008), no significant pathological changes were observed in most fish and epithelial hyperplasia did not appear to be associated with the cysts in either the fish sampled from freshwater or during the marine longitudinal study. The target cell for infection appeared to be the epithelial cell, in line with that found by Nowak and Clarke (1999) and Turnbull (1993). Low levels of focal hyperplasia were observed in some fish but these were almost always distant from observed cysts. Nylund *et al.* (1998) also observed a similar phenomenon, with hyperplasia and fusion of the lamellae not always associated with the epitheliocysts in sea farmed Atlantic salmon. Whether these mild pathological changes may arise subsequent to cyst rupture or some other effect of infection remains to be determined. There was no evidence of '*Ca. C. salmonicola*' by Fluorescence *in situ* hybridization in these focal areas of pathology. The clinical effects of '*Ca. C. salmonicola*' were not discussed at length by Karlsen *et al.* (2008) however, the authors describe at least some of the fish as having mild gill inflammation and others as having respiratory problems. With the exception of site D, which experienced an increase in mortality during September 2008, all fish in the current freshwater study were sampled from apparently healthy populations. The mortality episode on site D was eventually attributed to gill pathology of unknown aetiology. Approximately 60% of these fish displayed epitheliocystis infection at the time of sampling. Whether this contributed to the mortality remains undetermined.

The phylum *Chlamydiae* contains a diverse range of human and mammalian pathogens, in addition to bacteria that can infect free living amoebae, fish, invertebrate and insect species (Kalayoglu and Byrne, 2006). The ubiquitous nature of the *Chlamydiae* can make it difficult to investigate their role as pathogens in disease and to demonstrate causal relationships (Horn, 2008). Since infection does not always result in pathology, it has always been a challenge to categorise many *Chlamydiae*, especially those infecting fish as either significant disease-causing agents or benign symbiont bacteria. In terms of the clinical effects of infection with '*Ca. C. salmonicola*' and its impact on gill health pre and post seawater transfer, no adverse pathology of significance was observed in the fish during any stage. It is feasible to consider, that rupture of some of the larger '*Ca. C. salmonicola*' cysts may have adverse effects on epithelial integrity through mechanical damage. A similar mechanism of damage was suggested as a predisposing factor for bacterial gill disease in

striped bass fingerling mortality (Herman and Wolf, 1987).

Many human *Chlamydiae* of medical importance have been found to cause latent infections without any clear clinical symptoms, and acute cases of disease are only induced by stress factors which influence the latency of these organisms (Bazala and Randa, 2005). In addition, the role of *Chlamydiae* in complex diseases of multifactorial origin is frequently documented. *Chlamydophila pneumonia* is widely recognised as a significant contributor to community-acquired pneumonia in humans (Fang *et al.*, 1990). It induces ciliostasis in the lung bronchial system and can contribute to initiation and pathogenesis of respiratory infections, acting as a pathogen promoter (Sherner-Avni and Liberman, 1995). It seems reasonable to consider that infection with '*Ca. C. salmonicola*' may have a similar effect to that played by *C. pneumonia* in humans, by affecting the normal immune mechanisms of the gills in either the freshwater or marine environment. Either way, mechanical damage or impaired immunity could both feasibly culminate in increased vulnerability of the gills leaving them susceptible to both environmental and infectious challenges.

As previously reported by Karlsen *et al.* (2008) cysts were commonly observed as clusters spanning adjacent lamellae, as opposed to being evenly distributed. Such clustering may reflect the spread of infection from an initial focal infection to closely situated lamellae (Karlsen *et al.*, 2008). Little is understood relating to transmission of CLO infections in fish, but this pattern might suggest that when a cyst ruptures, adjacent epithelial cells become infected.

Prevalence of infection was high in most sites but infection abundance varied significantly. The reason for this variation is not clear. It may be as a result of one or a number of factors, such as water quality parameters, host immune status, life stage or cohort of fish. The existence or occurrence of environmental reservoirs or intermediate hosts for the *Chlamydiae* (if any), may also have a significant effect on infections. High prevalence, variation in abundance and broad geographical distribution are characteristics of the epidemiology of this bacterium, at least in Ireland. To date no other studies have investigated the epidemiology of '*Ca. C. salmonicola*', so whether this pattern is a common phenomenon remains to be elucidated.

A significant relationship was identified between the number of observed cysts detected by histopathology and RT-PCR Ct. values, although this correlation becomes tenuous when intensity of infection is very low. In site A, which had the lowest intensity of infection, the prevalence by histology was only 20%, whereas using RT-PCR it was calculated to be 75%. Not surprisingly, RT-PCR is more sensitive for detection of '*Ca. C. salmonicola*'. The cut off for histological detection of cysts appears to mirror an equivalent Ct value of approximately 29 in infected fish.

To the best of our knowledge '*Ca. C. salmonicola*' or the presence of cysts consistent with '*Ca. C. salmonicola*' infection have never been reported from marine-stage fish. It was therefore postulated that infections might be eliminated upon seawater transfer. During the marine longitudinal study, it emerged that '*Ca. C. salmonicola*' did not disappear from the fish until 4 – 6 weeks post-seawater transfer. This opens a number of topics for consideration. Normal turnover of lamellar epithelial cells from differentiation to apoptosis and sloughing at the lamellar tip has been estimated in juvenile trout (*Oncorhynchus mykiss*, Walbaum) to be in the region of 8 days (Zenker *et al.*, 1987). There is no comparable data available for Atlantic salmon. Assuming the rate of epithelial turnover would be similar, persistence of infections beyond this time frame may indicate one of the following, either a) new infections are occurring at sea, b) infected epithelial cells undergo a change in their maturation cycle or delayed apoptosis and cell development becomes arrested upon infection or, finally; c) undifferentiated progenitor cells are targeted for infection by '*Ca. C. salmonicola*' which have a longer life span than epithelial cells. This idea would be supported by the observations from histopathology that the majority of cysts were found to be localised in the crypt between two adjacent lamellae, where most of the epithelial progenitor cells reside. Further research and tracking of infected cells is required to deduce the actual events that take place during cyst development and the effects on host cell life cycle. Based on the observations at this study site, sustained, viable infections with '*Ca. C. salmonicola*' do not appear to occur in the marine phase. Further studies with increased sampling frequency at different sites would help qualify these findings.

The high level of sequence similarity between Irish and Norwegian strains of '*Ca. C. salmonicola*' indicates a broad geographical distribution for this bacterium. However, as is the case with many CLOs in fish, the significance of '*Ca. C. salmonicola*' infections will remain difficult to quantify until effective culture systems are established. The development of challenge trials for any fish CLOs would greatly enhance our understanding of the organisms involved, their infectivity and mode of transmission. Continued focus on molecular characterizations of the different CLOs involved in epitheliocystis will help to better define these pathogens and their relationships to other *Chlamydiae* known to be of clinical significance in both mammalian and fish hosts.

3 Longitudinal studies on gill health in marine farmed Atlantic salmon (*Salmo salar*) using a novel histopathological gill scoring protocol

Published in part in the journal *Aquaculture International* under the title “A novel gill scoring protocol to assess gill disease” by Mitchell et al. (2011).

Statement of Contribution: All gelatinous zooplankton in the samples were identified and enumerated to the lowest possible taxonomic level by Dr. Emily Baxter, University College Cork, Ireland. The gill scoring protocol was devised in conjunction with Dr. Hamish Rodger, Vet Aqua International, Galway, Ireland. All remaining work was carried out by the author of this thesis.

3.1 Introduction

The aetiology of salmonid gill disease in the marine environment is frequently complex and multifactorial. In many cases, an episode of gill disease may be initiated when fish are exposed to a water-borne irritant such as a harmful algal bloom or zooplankton swarm, which is then followed by secondary bacterial infection or parasitic infestation (Rodger *et al.*, 2011). To date, few longitudinal studies monitoring gill health have been documented and most data and samples are collected by reactive sampling in the face of clinical outbreaks. Moribund fish, or fish displaying clear clinical signs of disease, are usually at an advanced stage when they are sampled for diagnostic work, by which time the precipitating cause of disease is frequently obscure. Consequently, there is often insufficient information to define the progression of pathological development that occurs in gill tissues, particularly in cases with multifactorial aetiologies.

The aim of the current study is to describe gill health and disease in farmed Atlantic salmon at a marine site in the west of Ireland through frequent sampling during a six month continuous period. Investigations into the contribution of different infectious agents to gill disease over the study period will be carried out and the significance to gill health of environmental factors, zooplankton and phytoplankton will be quantified where appropriate.

From a clinical perspective, gill disease can be assessed through a combination of behaviour, clinical signs and gross- and histopathology. The latter is generally the most definitive, as in many cases significant lesions are visible only by microscopy. Histopathological scoring systems, although frequently used to quantify disease in many areas of human and veterinary medicine, are rarely used in fish. Bernet *et al.* (1999) has described some useful histopathological alterations in the gills as part of a multi-organ scoring protocol for aquatic pollution. One of the main objectives during this longitudinal study was to devise and implement a practical gill-specific system, to enable histopathological changes from both environmental and infectious challenges to be easily quantified and compared, in both this case and for future investigations.

3.2 Materials and Methods

Study site and sampling details

The longitudinal study was carried out on a marine Atlantic salmon fish farm on the west coast of Ireland in 2009. Fish were transferred to sea as smolts during the period January–March and sampling was initiated at monthly intervals (May to July) and increased to twice monthly during

August, September and October. On each sampling occasion, five fish were randomly selected using a hand net from each of four fixed pens on site (total $n = 20$ at each sampling episode).

The second gill arch on the left hand side was excised from each fish and immediately fixed in 10% neutral buffered formalin for histopathology. Sections (5- μ m) from paraffin-embedded tissue were stained with haematoxylin and eosin. Samples were then scanned microscopically at 40x, 100x and 400x magnifications. Gills were assigned an appropriate score using the gill scoring protocol described below. During each sampling, one of the five fish was randomly selected from each pen to screen for systemic diseases, such as common salmonid diseases caused by viruses (e.g. pancreas disease) and bacteria (e.g. furunculosis, rickettsiosis). In these fish, organs sampled included heart, brain, kidney, liver, pyloric caecae, pancreas, muscle, skin and spleen. Blood samples to screen for salmonid alpha virus (SAV), the aetiological agent of pancreas disease (PD) were taken where indicated by clinical presentation (Munro *et al.*, 1984). The signs of PD commonly include lethargy, anorexia, the presence of faecal casts, characteristic behavioural changes and high mortality.

Gills were inspected for signs of gross pathology such as excess mucus, pale gill filaments, swelling, necrosis and the presence of macro- parasites. Gill filament tips were excised and examined as wet mounts under light microscopy to identify and quantify microscopic marine protist parasites such as species of *Trichodina*, *Ichthyobodo* and *Neoparamoeba*.

Histopathological gill scoring protocol

A semi-quantitative histopathological scoring system was developed containing both index and ancillary criteria. The index criteria were selected as common pathological changes that occur in response to a gill insult. These criteria would provide the root of the score. The ancillary criteria were selected to reflect the occurrence of minor less frequent indicators of gill pathology. The score for the index parameters was based on the presence and extent of the following: lamellar hyperplasia, lamellar fusion, cellular anomalies (including degeneration, necrosis and sloughing) and lamellar oedema with a score ranging from 0 to 3 being assigned. A number of ancillary parameters were then scored based solely on their presence/absence, with a score of 0 or 1: cellular hypertrophy, inflammation, excessive numbers of eosinophilic granular cells, circulatory disturbances (haemorrhage, telangiectasis, congestion), and finally, the presence of both known and unidentifiable gill pathogens both bacterial (epitheliocystis, *Tenacibaculum* sp., other bacteria) and parasitic (for example the protists *Trichodina* sp., *Ichthyobodo* sp. and *Neoparamoeba* sp.) (Table 1).

Table 1. Description of index and ancillary criteria and relevant scores for the gill scoring protocol

Index Criteria				
Pathology (score)	Lamellar Hyperplasia	Lamellar Fusion	Cellular Anomalies	Lamellar Oedema
None (0)	Not significant – none or very minor	Not significant – none or very minor	None	None
Mild (1)	Mild increase in epithelial cells (low-level of focal or widespread hyperplasia, affecting < 10% of gill tissue)	Occasional focal fusion of filaments (< 10% of gill tissue affected)	Scattered occasional degenerating or necrotic cells or focal areas of sloughing	Epithelio – capillary separation with proteinaceous fluid in the epithelio-capillary space: < 10% of gill tissue affected
Moderate (2)	Moderate widespread or multifocal hyperplasia, affecting 10–50% of the tissue	Multifocal areas of fusion (moderate widespread hyperplasia affecting 10–50% of gill tissue) interspersed with normal gill tissue	Degenerating cells, necrotic cells and/or cell sloughing in multifocal areas throughout the tissue	10-50% of gill tissue affected
Severe (3)	Extensive lamellar hyperplasia, > 50% of gill tissue affected	Extensive fusion and loss of normal architecture, > 50% of gill tissue affected	Widespread necrosis, degeneration and/or sloughing visible throughout section	> 50% of gill tissue affected
Ancillary Criteria – Score for presence (1) or absence (0)				
– Inflammation (presence of inflammatory cells outside the blood vessels)				
– Eosinophilic granular cells (EGCs) (higher than normal numbers of EGCs in gill filament)				
– Circulatory disturbances (thrombi, telangiectasis, congestion), score = 1 if > 10% tissue is affected				
– Cellular hypertrophy (hydropic degeneration of lamellar cells)				
– Bacteria – <i>Tenacibaculum</i> spp. (mats of filamentous bacteria on lamellar surfaces)				
– Bacteria – Epitheliocystis				
– Other bacteria				
– Protist parasites – <i>Neoparamoeba</i>				
– Protist parasites – <i>Costia</i>				
– Protist parasites – <i>Trichodina</i>				
– Other parasites				

The score is quoted in the format 'index.ancillary', for example a fish with severe hyperplasia and the presence of the parasite *Trichodina* would receive a score of '3.1', producing a combined score of four. The index.ancillary format was chosen to provide extra descriptive information, as opposed to just the total score which is provided by most scoring systems. This was important in the unusual event that a single physiologically severe lesion would generate a low score, for example, widespread lamellar fusion might be assigned a score of 3.0 and in the absence of other pathological changes the total score would be low. Alternatively, a fish with a suite of minor lesions that were not physiologically significant might receive an abnormally high score of 0.7. Neither scenario occurred in practice when using the scoring system for this or subsequent studies. The total score can range from 0 to a possible maximum of 24. When using the system to assess fish with gill disease of varying severity, a score of 0–3 usually reflected no significant pathology, a score of 4–6, mild gill pathology, probably of minor clinical significance, a score of 7–9, moderate gill pathology of clinical significance, and finally a score greater than 10 was associated with severe gill pathology of high clinical significance. A selection of the histopathological criteria identified in the gill scoring protocol is illustrated in Figure 1.

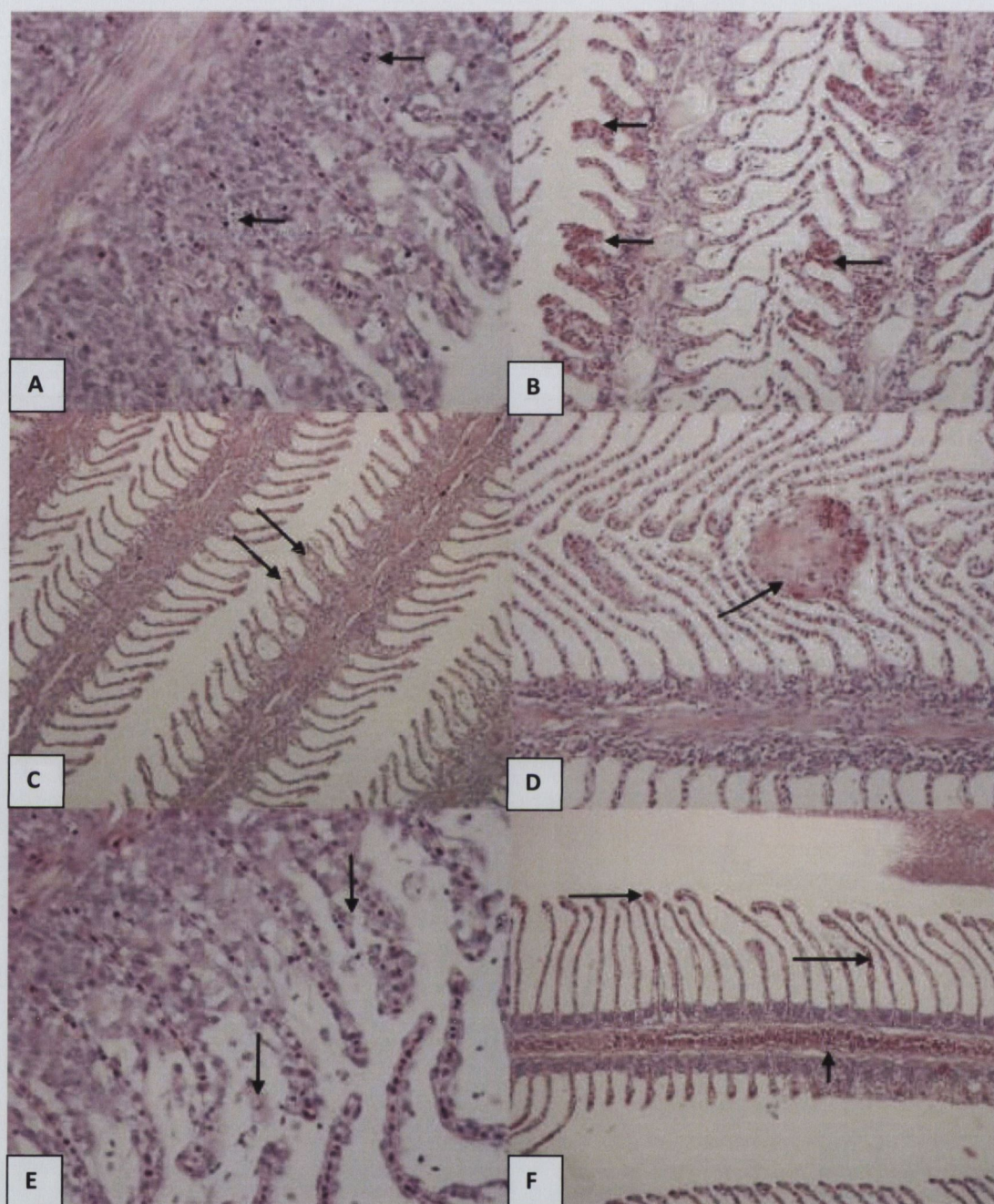


Figure 1. A selection of common pathological conditions affecting the gills of Atlantic salmon (*Salmo salar*) observed during the longitudinal study:

- A) Diffuse lamellar hyperplasia with scattered degenerating and necrotic cells (arrows)
- B) Circulatory disturbance in the form of widespread multifocal haemorrhage (arrows) in the secondary lamellae
- C) Mild focal fusion of the secondary lamellae (arrows)
- D) Circulatory disturbance in the form of telangiectasis (arrow)
- E) Sloughing of lamellar epithelial cells (arrows)
- F) Circulatory disturbance in the form of widespread congestion in both the primary (short arrow) and lamellae (long arrows)

Haematoxylin and eosin, 400x and 200x

The gill scoring system was tested for intra-observer agreement (see statistical methods below). As recommended by Desmet (2003), the different criteria were discussed and defined in detail by the observers prior to assigning scores to the slides to improve agreement and reduce inter-observer variability.

Presence of epitheliocystis

The prevalence of epitheliocystis in fish from all pens was monitored over the study period using histopathological examination. If a fish was affected by epitheliocystis, the number of cysts per 10 fields of view (10x) was counted. A simple three-tiered measure was used to quantify infection (low levels of infection: < 10 cysts/10 fields of view, medium levels: 10-99 cysts/10 fields of view and high: ≥ 100 cysts/10 fields of view) as previously described by Mitchell *et al.* (2010).

Zooplankton sampling

Zooplankton samples were collected from the end of May to early October. Samples were collected fortnightly at high tide and coinciding with the neap phase of the tidal cycle. Sampling was performed using a 0.4 m ring net (200- μ m mesh) and a non-filtering cod-end. On each sampling occasion, three vertical net hauls were conducted: inside Pen 1, in the area between Pens 1 and 2 and approximately 200 m away from the pens towards the mouth of the bay to depths of approximately 9, 16 and 8 m respectively. Samples were immediately preserved in 4% seawater formalin.

All gelatinous zooplankton of the phylum Cnidaria (those with stinging cells) in the samples were identified and enumerated to the lowest possible taxonomic level. The abundance (individuals m^{-3}) of cnidarian zooplankton was calculated using the area of the net opening multiplied by the depth sampled in metres (assuming 100 % filtration efficiency of the net due to vertical haul over a short distance) (Baxter *et al.*, 2011).

The individuals of *P. noctiluca*, although only relatively small compared with most scyphozoans, are much larger than the other species mentioned above, e.g. 4 cm compared with 7 mm. Abundance of *P. noctiluca* was adjusted based on observations of the site diver who reported that individuals of this species were aggregated only within the top 1 m of the water column.

Temperature, oxygen and phytoplankton data

Temperature (°C) and oxygen values (mg L⁻¹) were recorded using a Polaris Oxyguard probe. Phytoplankton data for the site was obtained from the Marine Institute Phytoplankton Monitoring Programme (Marine Institute, 2009).

Statistical Methods

Twenty histopathology slides displaying a range of gill pathologies from previous clinical cases of gill disease were selected, assigned random numbers and screened by two observers. A weighted kappa statistic was used to compare actual agreement against that which may be expected by chance. A kappa statistic of 1 represents perfect agreement and 0 represents the level of agreement expected by chance alone. Arbitrary divisions have been made to categorise the level of agreement as follows: poor, < 0.2; fair, 0.21–0.40; moderate, 0.41–0.60; good, 0.61–0.80; and very good, 0.81–1.0 (Altman 1991).

A two way analysis of variance (ANOVA) was initially applied to assess average gill score with respect to sampling date and pen.

The factors that were considered to be potentially affecting the gill score were then analysed by maximum likelihood techniques based on log linear analysis of contingency tables using the software package PASW (Version 18.0.0.). An initial full factorial model incorporated the following factors: individual pen (4 levels, Pens 1, 2, 3 & 4) epitheliocystis (2 levels, presence or absence), zooplankton densities (3 levels, none, low and high density) and gill pathology (4 levels, none, low, moderate and severe). Then, using a backward selection procedure beginning with the most complex model, all possible interactions were incorporated and those combinations that did not contribute significantly to explaining variation in the data were eliminated in a stepwise fashion beginning with the highest-level interaction. This resulted in a minimum sufficient model for which the likelihood ratio of χ^2 was not significant, indicating that the model was sufficient in explaining the data. The remaining terms in the final model are summarised together with the likelihood ratio for the final model in the text.

3.3 Results

Clinical signs, gross pathology and parasitology

Clinical signs of gill disease were first apparent in the fish sampled on the 7th October. These included swimming near the water surface and exaggerated opercular movements reflecting an increase in respiratory effort. Gross gill pathology was also apparent in some of these fish with pale gill filaments and excess mucus. Occasional focal necrotic patches of tissue were also visible on the gills. Fish sampled on the 9th September had dermal pathology in the form of raised scales and oedema. No other gross pathology of significance was evident on external or internal examination of the fish at any stage. No macro- or microscopic gill parasites were observed on any occasion.

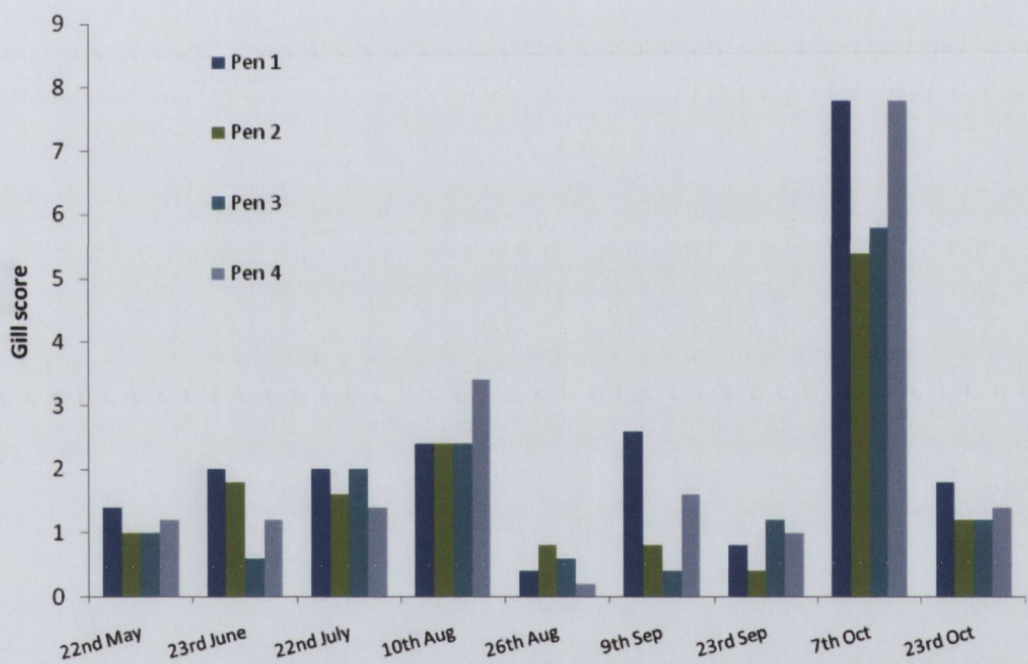


Figure 2. Mean gill scores from each pen sampled during the longitudinal study

Histopathology and gill scoring

Total histopathological gill scores ranged from 0–9 over the study period with a mean value of 2 (+/- S.E. 0.26) (Figure 2). On most sampling dates, mild gill pathology in the form of epithelial hyperplasia and occasional necrotic epithelial cells was observed. A two way analysis of variance (ANOVA) uncovered a highly significant interaction between the average gill score and sampling date

and pen ($F_{8,144} = 24, p < 0.0001$) on 7th October. Gill lesions observed on this date included moderate lamellar hyperplasia, low levels of fusion, epithelial cell necrosis, cell sloughing and congestion, with most fish having a combination of the above. The average total gill score on this sampling date was 7 (+/- S.E. 0.64).

Epitheliocystis

The average prevalence of epitheliocystis in fish in each pen varied between 0 and 40% at each sampling point, however the culminative prevalence was very low overall (see Table 2). Figure 3 illustrates the percentage of fish affected by epitheliocystis at each sampling point. The numbers of epitheliocysts observed in infected fish were also consistently very low, with only 1-6 cysts per fish observed in total, indicating a low intensity of infection.

Table 2. Culminative prevalence and infection intensity of epitheliocystis by pen

Pen ID (n=45 in each case with fish from 9 sampling time points)	% Prevalence of Infection over time ($\pm$ C.I., % Range)	Infection Intensity (Low, Medium, High)
Pen 1	1.78 ($\pm$ 1.68, 0,20)	Low
Pen 2	0.89 ($\pm$ 0.22, 0,20)	Low
Pen 3	1.78 ($\pm$ 1.68, 0,20)	Low
Pen 4	2.22 ($\pm$ 1.86, 0,40)	Low

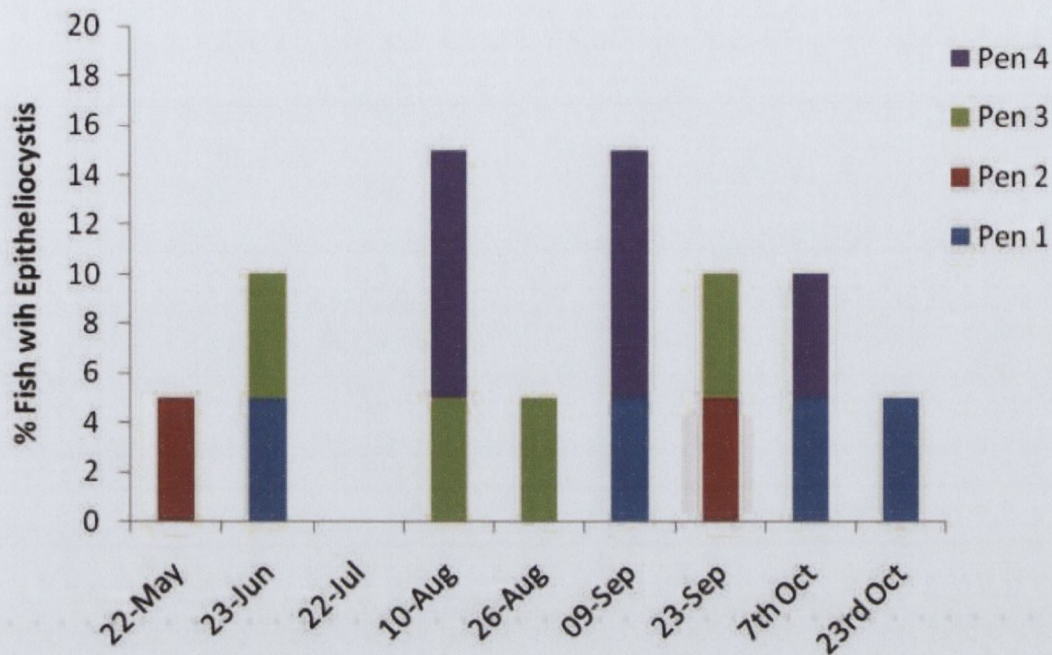


Figure 3. Percentage of fish at each sampling point affected by epitheliocystis by pen

Zooplankton sampling

From a total of eight tow samples, 12 species of Cnidaria were identified: nine species of hydromedusae, one species of siphonophore, one species of scyphomedusae and the larval stages of hydroids (the sessile form of hydromedusae). Four species previously implicated in fish kills or gill disorders were identified throughout the study: *Muggiaea atlantica*, *Pelagia noctiluca*, *Solmaris corona* and *Phialella quadrata*. Low levels of cnidaria were present throughout the beginning of the longitudinal study with a sharp peak in late August. A second peak occurred in late September with high abundances of the siphonophore *M. atlantica* abundance and the occurrence of the scyphozoan *P. noctiluca* (Figure 4).

At the beginning of October the oceanic hydromedusa *S. corona* appeared in the plankton at low densities. Whilst *P. quadrata* was recorded on almost all sampling occasions, the abundance of these small hydromedusae remained low throughout the study not reaching densities of more than 24.8 individuals m^{-3} . The relationship between gill score and cnidarian abundance is illustrated in Figure 5.

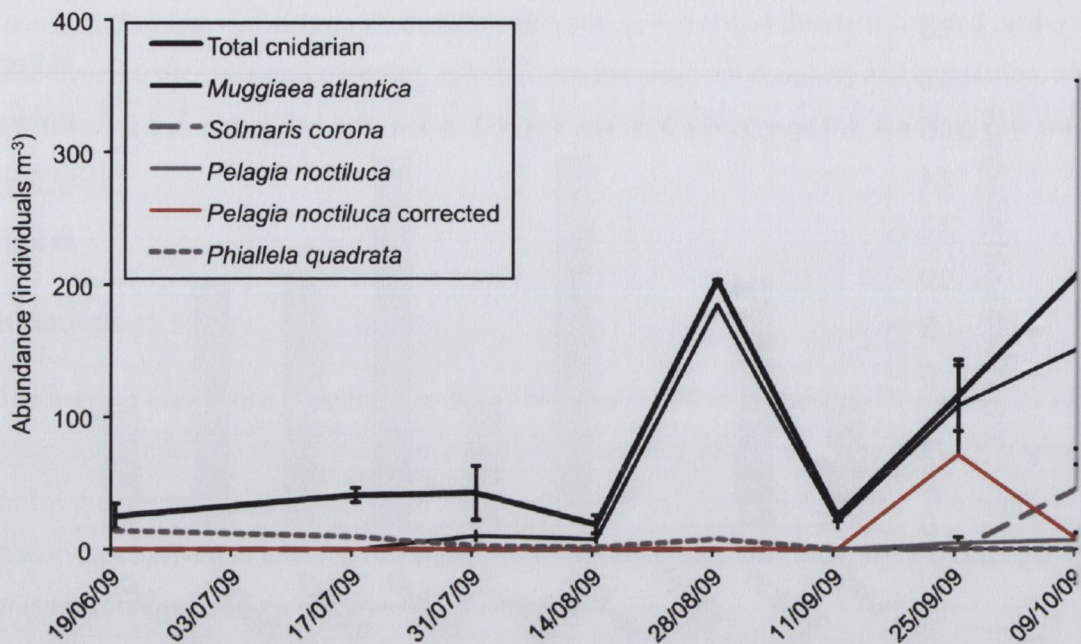


Figure 4. Mean total cnidarian abundance (± 1 S.E.) over the duration of the study with component abundances of species previously implicated in fish kills

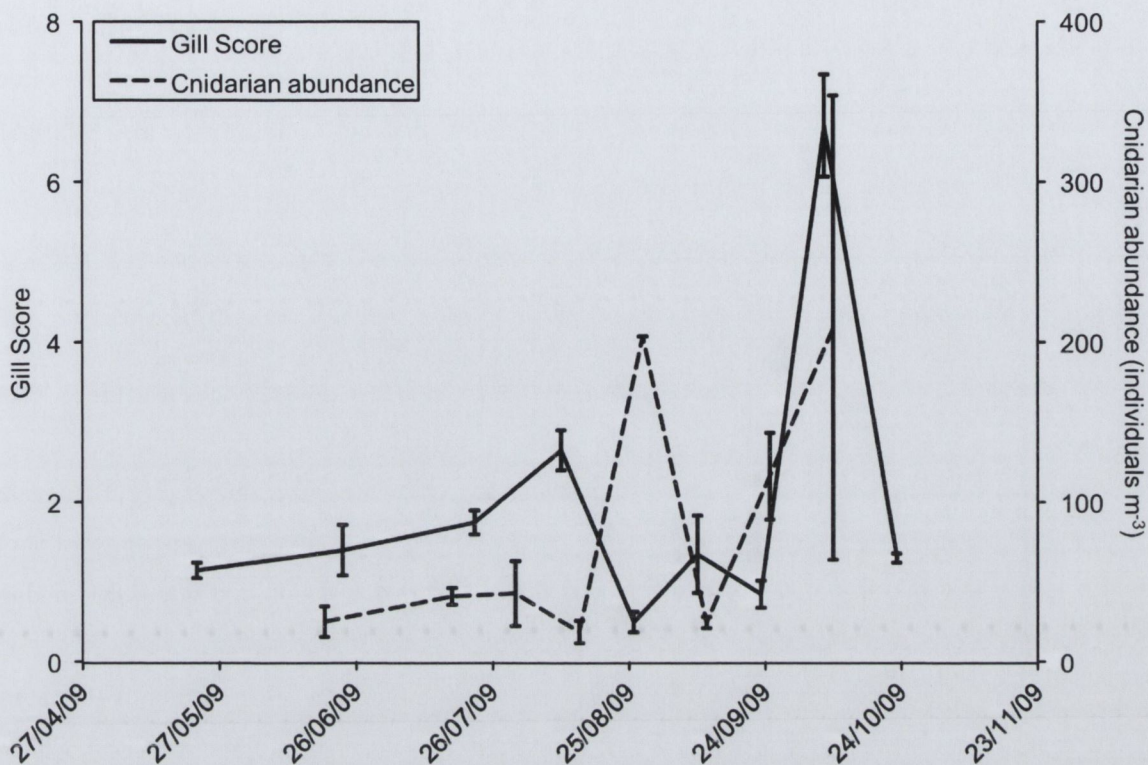


Figure 5. Average gill score and mean cnidarian abundance over the duration of the study

Environmental Data

Sea temperature ranged from 11.6 °C in May when monitoring commenced, to 13.9 °C in October with a peak in late June of 16.9 °C. Oxygen levels ranged from 8.9 mg L⁻¹ in May to 7.5 mg L⁻¹ in October with a minimum of 5.9 mg L⁻¹ in August. No extreme environmental fluctuations were observed during the study in terms of oxygen levels or temperature. Although phytoplankton was sampled frequently during the study, no harmful algae species were recorded in significant numbers in samples taken in the vicinity of the farm (Marine Institute, 2009).

Mortalities

Clinical signs characteristic of PD, as caused by SAV, were first observed on the 29th July in fish from Pen 1. Serum samples confirmed the presence of SAV antibodies (Graham *et al.*, 2003) and histopathology, RT-PCR and virus isolation later confirmed the diagnosis in fish from all pens on site. Mortality attributed to PD reached approximately 20% on site over the following 8 weeks. During this period there was some minor gill pathology observed in the fish in the form of epithelial

hyperplasia and low levels of lamellar fusion. This was replaced by more significant gill pathology in early October (described above) and a dramatic increase in mortality. Significantly elevated gill scores were observed (up to 9.0 in some fish) coincident with the *P. noctiluca* swarm (Figure 5) and 25% of the remaining fish on site died subsequent to this event.

Statistical Methods

The value of the weighted kappa calculated was 0.68, indicating good agreement between observers while reading slides using the gill scoring protocol.

Log linear analysis revealed that there was a highly significant effect of the density of zooplankton on the levels of gill pathology observed ($\chi^2 = 39.96$, $p < .0001$). The goodness of fit of the overall model was given by the likelihood ratio $\chi^2 = 32.64$, ($p = 0.998$), indicating the model was sufficient in explaining the data.

Initially, there also appeared to be a significant interaction between the level of gill pathology and the presence of epitheliocystis ($\chi^2 = 10.45$, $p = 0.015$). However as one of the ancillary gill scoring parameters was the actual presence of epitheliocystis, this result had to be interpreted with caution. To investigate if there was a true effect of epitheliocystis on the level of gill pathology any scores allocated for epitheliocystis were removed from the overall scores for each fish. The log linear analysis was repeated using these new values. In this case the interaction was not significant ($\chi^2 = 3.06$, $p = 0.38$).

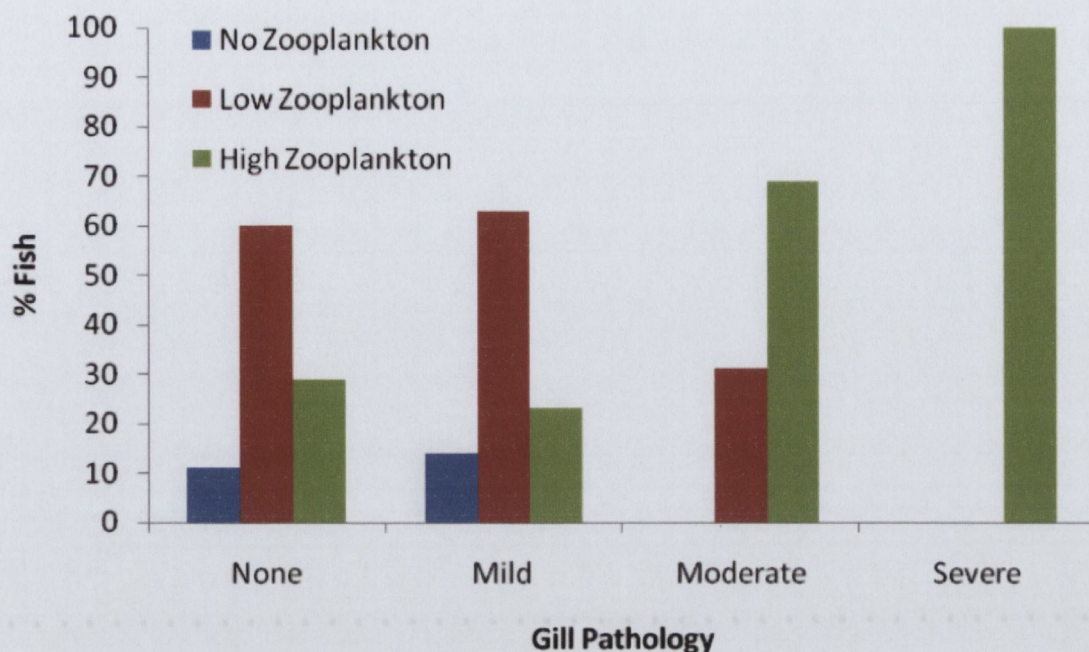


Figure 6. The relationship between gill pathology and the different levels of zooplankton. A significant association between high levels of zooplankton and gill pathology was uncovered (Chi square = 39.96, $p < .0001$)

3.4 Discussion

In the present study, a novel histopathological scoring system was used effectively to reflect changes in gill health and disease in marine-farmed Atlantic salmon. There are many examples of grading and scoring systems in both veterinary and human histopathology (Brink *et al.*, 2010; Knodell *et al.*, 1981; Westin *et al.*, 1999; Zeldin *et al.*, 2001). Few such systems have been employed in fish to date, with the exception of studies on gonadal alterations, post-exposure to endocrine disrupting chemicals, in the fathead minnow (*Pimephales promelas*); Japanese medaka (*Oryzias latipes*); and the zebrafish (*Danio rerio*) (Johnson *et al.*, 2009), the MacConnell-Baldwin numerical scale for scoring cartilaginous lesions in fish with whirling disease (Andree *et al.*, 2002), a scoring methodology for lesions induced with experimental infection of salmonid alpha virus (Graham *et al.*, 2011) and a multi-organ protocol to assess aquatic pollution (Bernet *et al.* 1999). In contrast to this,

gross pathology scoring systems are frequently used in Atlantic salmon. Visceral-adhesion scoring post-vaccination (Midtlyng *et al.*, 1996), cataract scoring to quantify lens opacities (Wall and Bjerkås, 1999) and gross gill changes associated with amoebic gill disease (Adams and Nowak, 2001) are three scoring systems regularly used to make clinical judgements in the field. Gross gill pathology was observed on the occasional fish (excess mucus and focal necrosis), however many more fish had significant gill pathology when examined histologically, indicating it is a more sensitive indicator of gill health.

When designing the scoring system, a number of factors were taken into consideration. Firstly, the parameters measured needed to be easy to identify in order to optimise reproducibility and reliability between observations. It was important that the system developed be easy to apply with rudimentary fish histopathology skills, thus ensuring accessibility to most aquatic animal clinicians. The index criteria (Table 2) were selected as factors that are usually affected in response to a gill insult, be it infectious, environmental or otherwise in nature. As the gills have a relatively small number of component cells and tissues, the range of pathological responses they display is quite limited. The most frequently observed changes in the gills after an insult include three responses: lamellar oedema, lamellar hyperplasia and lamellar fusion (Roberts and Rodger, 2001). Cellular anomalies (necrotic, degenerating or sloughing cells) are also commonly observed on histopathology so were included as a forth index criteria. The ancillary criteria (Table 2) allowed an additional score to be added to the index value to account for other pathological responses that occur less frequently, such as the presence of circulatory disturbances, or factors that may contribute to gill disease as a whole such as the presence of parasites.

Secondly, in order to arrive at the best description in the text of what was visible histopathologically (Table 2) and to minimise inter-observer variation, the different criteria were discussed in detail between the observers prior to the study, as suggested by Desmet (2003).

Finally, one of the main issues with using histopathological scoring systems is obtaining reproducible results and agreement among observers. This stems from the subjective nature of histopathological interpretation which generally represents a judgement and not a measurement. Consequently, scoring systems often are a compromise between complexity and reproducibility (Desmet 2003). A weighted kappa coefficient was used to ensure the scoring system produced reproducible results and had acceptable levels of inter-observer variation. In this case, the value calculated for Kappa was 0.68, indicating relatively good agreement between observers. The Kappa coefficient is frequently used as a statistical method to evaluate reproducibility when designing

medical diagnostic scoring systems. Kappa measures pair-wise agreement among observers who are making category judgements while correcting for expected chance agreement. Kappa is generally considered a useful tool (Svanholm *et al.*, 1989), however opinion is divided on its usefulness. On the negative side Kappa does not distinguish between random and systematic variation, can compare only two observers at one time and does not distinguish between small and large disagreements between observers (Westin *et al.*, 1999). This latter fact can be negated somewhat by the use of the "Weighted Kappa", as used in this study, which considers the size of the disagreement between observers and is a better reflection of inter-observer variation. As an alternative, a simple percentage agreement has been suggested as a good measure of reliability (Westin *et al.*, 1999).

The results of the log linear analysis suggest there is a significant effect of the density of cnidarian zooplankton on gill pathology. The peak gill scores were observed coincident with exposure of the fish to a swarm predominantly made up of the scyphozoan jellyfish, *P. noctiluca*. Literature detailing the species of zooplankton harmful to fish is limited, however a number of species have been implicated in fish kills over the years. These include the hydromedusae *P. quadrata* (Bruno and Ellis 1985) and *S. corona* (Hay and Murray 2008), the siphonophores *M. atlantica* (Hellberg *et al.*, 2003) and *Apolemia uvaria* (Båmstedt *et al.*, 1998) and the scyphomedusae *Cyanea Capillata* (Bruno and Poppe, 1996) and the aforementioned *P. noctiluca* (Doyle *et al.*, 2008). Most of these records have been sporadic and in the form of one-off clinical case reports. For instance, *P. noctiluca* was associated with mortality of Atlantic salmon at a fish farm in Northern Ireland in 2007 (Doyle *et al.*, 2008).

The gelatinous zooplankton longitudinal sampling indicated that a large diversity of cnidarian zooplankton were present (up to 12 different species in total) in the vicinity of the salmon pens. The significance of many of these different species for farmed salmon remains to be determined. Further longitudinal sampling and monitoring of the levels of different species of zooplankton would be useful to facilitate differentiation of the noxious species from less harmful ones. In terms of zooplankton detection, one of the shortfalls is the interrupted nature of sampling regimes. Many zooplankton swarms are transient in nature and in particular, oceanic species such as *P. noctiluca*, can arrive on one tide and be gone on the next. An understanding of the depth distributions of such swarms would be useful and how this will ultimately affect the densities faced by the fish and their ability to avoid them while within the net-pens. Gelatinous zooplankton can also have detrimental effects other fish tissues. Mild chronic dermal pathology was observed on some of the fish sampled on the 9th September. This may have been related to the peak in cnidarian abundance that occurred around the end of August (Figure 4), which was largely composed of *M. atlantica*. This species has been implicated in causing severe damage to and death of farmed fish from stings to the skin and

gills (Fosså *et al.*, 2003).

The impact of the other measured factors to gill health remained obscure. No extreme environmental fluctuations or phytoplankton blooms occurred during the study period, nor were there any protist parasites observed on microscopic examination of wet mounts or histopathology. The results of the log linear analysis initially suggested there was some association between the presence of epitheliocystis and the level of gill pathology observed, however, as the presence of epitheliocystis was also contributing numerically to the value of the gill score, this was discounted. The prevalence and intensity of epitheliocystis remained very low throughout the study, the average prevalence being less than 20% on any sampling occasion. This was similar to the findings of Nowak and Clarke (1999) in Australia where epitheliocystis was recorded at low levels and no notable gill pathology was associated with the cysts. It has been postulated that epitheliocystis is an important component of some multifactorial gill diseases such as proliferative gill inflammation (PGI) (Kvellestad *et al.*, 2005; Steinum *et al.*, 2010).

In conclusion, a holistic approach to investigating gill disease is essential to uncover the aetiologies of gill disorders as there can be significant interactions between environmental factors and infectious agents. Noxious algal blooms aside, the impact of environmental challenges for farmed fish especially in the form of harmful zooplankton may have been overlooked in the past (Mitchell and Rodger, 2011). Controlled challenge trials with different zooplankton species would be helpful to identify those responsible for gill disease.

Grading and scoring in histopathology is a much used valuable tool in both research and for making treatment or management decisions in many areas of medical and veterinary science (Cross, 1998). The scoring system developed here was effective in terms of quantifying pathological observations and it has subsequently been used for such in a recent study by Baxter *et al.* (2011). It appears to work well for marine Atlantic salmon, and may be useful for both clinicians and researchers in the future by providing a tool to describe and compare different types of gill disease.

4 'Candidatus Branchiomonas cysticola' an important non-Chlamydial agent of epitheliocystis in seawater farmed Atlantic salmon (*Salmo salar*)

Published in part in *PlosOne* (online) as "Branchiomonas cysticola, a novel bacterium associated with epitheliocystis" by Tönshoff et al. (2012).

Statement of Contribution: Elena Tönshoff (Department of Microbial Ecology, University of Vienna, Austria) carried out the initial FISH that localised 'Ca. B. cysticola' in the epitheliocysts for the 1st time and also designed the FISH probes for 'Ca. B. cysticola' that I used to produce the images from the fish sampled in Ireland. She also performed the sequencing analysis on the Irish and Norwegian isolates of 'Ca. B. cysticola'. Dr. Terje Steinum supplied the samples from the Norwegian longitudinal studies and the Real-Time PCR results for 'Ca. P. salmonis'. Agnar Kvellestad (Norwegian Veterinary School, Oslo, Norway) performed and interpreted the transmission electron microscopy of the bacterial cysts and supplied the images. Agnar also assessed and quantified the gill pathology on the samples from the Norwegian longitudinal study. All remaining work was carried out by the author of this thesis.

4.1 Introduction

The term 'epitheliocystis' has been widely used to describe a condition characterised by cytoplasmic membrane-bound inclusions containing Gram-negative bacteria found in gill and less commonly skin epithelial cells of fish. Research over the last decades has provided both ultrastructural (Molnar and Boros, 1981; Paperna *et al.*, 1981; Rourke *et al.*, 1984; Groff *et al.*, 1996; Nylund *et al.*, 1998; Szakolczai *et al.*, 1999) and more recently molecular (Draghi *et al.*, 2004; Meijer *et al.*, 2006; Draghi *et al.*, 2007; Karlsen *et al.*, 2008; Polkinghorne *et al.*, 2010) evidence that the causative agents of epitheliocystis belong exclusively to the Order Chlamydiales. A number of phylogenetically distinct Chlamydia-like organisms (CLOs) have been identified associated with the condition in a diversity of fish species. Meijer and colleagues (2006) identified three genetically distinct Chlamydiae from the leafy seadragon (*Phycodurus eques*), silver perch (*Bidanus bidyanus*) and barramundi (*Lates calcarifer*), Draghi *et al.* (2007) identified a *Neochlamydia*-like species in Arctic charr and more recently, molecular evidence for a novel member of the Order Chlamydiales in a non-teleost species, the leopard shark (*Triakis semifasciata*) has emerged (Polkinghorne *et al.*, 2010).

In Atlantic salmon (*Salmo salar*) two CLOs have been associated with epitheliocystis, '*Candidatus* Clavochlamydia salmonicola' in freshwater (Karlsen, *et al.*, 2008) and '*Candidatus* Piscichlamydia salmonis' mostly in seawater (Draghi *et al.*, 2004). '*Ca. P. salmonis*' and epitheliocystis have been associated with the disease proliferative gill inflammation (PGI) (Steinum *et al.*, 2010). Disease outbreaks with a similar picture to PGI involving epitheliocystis have also been reported in Scotland and Ireland (Rodger, 2007). Estimation of economic losses is difficult, but PGI and gill disorders have been considered among the most important diseases in farmed Atlantic salmon in both Norway and Ireland (Skjelstad *et al.*, 2007; Rodger, 2007).

Recently, during a study of farmed Atlantic salmon populations displaying PGI in Norway (Steinum *et al.*, 2010), a significant discrepancy between the number of histologically observed epitheliocysts and the number of genomic units of '*Ca. P. salmonis*' estimated by qPCR was registered. As samples were also negative for '*Ca. C. salmonicola*', it was concluded that another as yet unidentified bacterium must be responsible for many of the observed inclusions. As a previous study on the gill flora of Atlantic salmon (Steinum *et al.*, 2009) had identified an undescribed betaproteobacterium as one of the dominant members of the gill-associated microbiota, its relationship with the occurrence and levels of epitheliocysts observed in the fish was investigated.

Fish were selected from one farm in Norway and through electron microscopy, molecular studies and fluorescence *in situ* hybridization it emerged that the undescribed betaproteobacterium appeared to be responsible for many if not all of the observed cysts. The name '*Candidatus* Branchiomonas cysticola' was proposed for this novel cyst-forming agent of epitheliocysts in seawater Atlantic salmon (Tönshoff *et al.*, submitted for publication).

In the present report, the main objectives were to investigate the prevalence and geographical distribution of '*Ca. B. cysticola*' and the homogeneity of this novel cyst-forming bacterium in a number of geographically distinct populations of Atlantic salmon through real-time PCR, 16S rRNA sequencing analysis and fluorescence *in situ* hybridization. Furthermore, the importance of this bacterium both in terms of gill pathology and the occurrence of epitheliocystis in fish is explored.

4.2 Materials and Methods

Tissue sampling and storage

Retrospective material from 9 Norwegian and 3 Irish marine Atlantic salmon farms, sampled during different longitudinal studies, were collected for analysis (Table 1).

Table 1. Details of fish sampled during different longitudinal studies including location, year of sampling and number of fish sampled from each site

Farm ID	Year Sampled	Number of fish	Farm ID	Year Sampled	Number of fish
Ireland – 1	2010	20	Norway – 567	2004	11
Ireland – 2	2009	25	Norway – 571	2004	8
Ireland – 3	2009	10	Norway – 588	2004	10
Norway – 443	2004	15	Norway – 476	2007	10
Norway – 469	2004	15	Norway – 483	2007	15
Norway – 525	2004	8	Norway – 518	2007	15

The Norwegian farms were located in the mid and south-western part of the country while the Irish farms were in the west and north west. Fish from both countries had been sampled during late autumn. In each case, a section of the second left gill arch was fixed in 10% buffered formalin for later processing and a piece of the third left gill arch was collected in *RNAlater*TM (Ambion) for PCR screening. Molecular samples from four Norwegian and two Irish freshwater hatcheries were also collected to screen for '*Ca. B. cysticola*'.

Histological examination

Paraffin embedded formalin-fixed gills were sectioned (5µm), stained with haematoxylin and eosin (HE) according to standard histological techniques (Bancroft and Stevens, 1996) and one section per fish examined by light microscopy. Out of the total sample of 162 fish a range of gill pathologies were observed and according to the severity of the lesions, were graded into pathological categories. These were based on the fraction of the filament length affected with proliferative type pathology: None (Grade 0), slight (Grade 1), where approximately < 10% of the filament was affected, moderate (Grade 2), 10-50% affected and finally extensive (Grade 3) > 50% affected. Epitheliocyst numbers were categorised into not detected, few, some or many, as previously described (Steinum *et al.*, 2010).

DNA extraction and efficiency control

After homogenization of approximately 17.5 mg of gill soft tissues with the Roche MagNA lyser (Roche Ltd., Basel, Switzerland), DNA was extracted from half the homogenate volume using the Roche High Pure PCR Template Preparation kit (Roche) according to manufacturer's instructions. Consistency of DNA extraction was checked in 29 random samples from both the Irish and Norwegian fish with the Atlantic salmon elongation factor alpha 1 gene assay (Bruno *et al.*, 2007). DNA extraction efficiency was found to be essentially identical (mean $\pm$ SD cycle threshold, Ct, value: 20.6 ± 0.8), which allowed meaningful comparisons of Ct-values between individual assays.

Real-time PCR

The '*Ca. B. cysticola*' 16S rRNA gene assay was performed in a universal real-time PCR master mix (Applied Biosystems Inc., Foster City, US) with primers BPf2 and BPr2 (final concentration 0.5 μ M, Table 2) which defines a 95 bp amplicon and probe BPP2 (final concentration 0.25 μ M, Table 2). The amplification was run on the Mx3005PTM real-time PCR system using the default quantitative 2 step program with annealing and synthesis at 60°C for 1 min. Negative controls using DEPC-treated water (Invitrogen) and a positive sample, confirmed by sequencing, were included in each run. The '*Ca. P. salmonis*' 16S rRNA gene assay (Steinum *et al.*, 2010) was performed using the Faststart DNA Master Hybridization Probes kit (Roche) according to the manufacturers recommendations with primers 16S RTfor and 16S RTrev (Table 2), defining a 150bp amplicon, and fluorescence resonance energy transfer (FRET) probes (Tib-molbiol, Table 2) at a final concentration of 0.5 and 0.2 μ M respectively. The amplification program (Lightcycler) was denaturation at 95°C for 5s, annealing at 59°C for 15s, and extension at 72°C for 13s.

In the case of each bacterium, '*Ca. B. Cysticola*' and '*Ca. P. Salmonis*,' the range of the observed Ct-values were divided into three equally large intervals and the grading scale for load for each was as follows: '*Ca. B. Cysticola*' 0 = not detected, 1 = low load (Ct-values 29.1-36.3), 2 = moderate load (Ct-values 24.2-29.0) and 3 = high load (Ct-values 17.2 – 24.1). '*Ca. P. Salmonis*': 0 = not detected, 1 = low load (Ct-values 32.8-38.4), 2 = moderate load (Ct-values 29.5-32.7) and high load (Ct-values 25.7-29.1).

Table 2. PCR primers and probes used in the present study. Lightcycler and Taqman probe labelling and modification are shown: 5'-Fluorescein amidite (FAM) and 3'-Black berry quencher (BBQ), 3'-Fluorescein, 5'-LC640 dye and 3'-phosphate

Real-time assay	Sequence (5'→3')	Reference
BPf2	AATACATCGGAACGTGTCTAGTG	This study
BPr2	GCCATCAGCCGCTCATGTG	This study
BPp2	Fam-CTCGGTCCCAGGCTTTCCTCTCCA-BBQ	This study
16SRTfor	CCGCAAGGACAACTACAC	Steinum <i>et al.</i> (2010)
16S Rtrev	ATCGACTTAGGCAGTCTCG	Steinum <i>et al.</i> (2010)
16S RTFL	AACCCAACACCTCACGGCACGA-FL	Steinum <i>et al.</i> (2010)
16S RTLC	640-CTGACGACAGCCATGCAGCAC-PH	Steinum <i>et al.</i> (2010)
Sequencing primers	Sequence (5'→3')	Reference
A1_14F	TGC TTG CAC TAT GTT GGC	Tönshoff <i>et al.</i> 2011
A1_1420R	CTG CTT CTG GTC AAC CCC	Tönshoff <i>et al.</i> 2011
reverse primer 3	ACTGCTGCCTCCCGT	Dewhirst <i>et al.</i> 1992
reverse primer 5	CTACCAGGGTATCTAATC	Dewhirst <i>et al.</i> 1992
538C	GTAGTCCACGCCGTAAACG	Petterson <i>et al.</i> 1996
V4 (modified)	GATTGTGACCGGCAGTATC	This study
V5	GTCCACACTCCTACGGGAGGC	Lunder <i>et al.</i> 2000
V6	GGGGAYGACGTCAAGTC	Lunder <i>et al.</i> 2000

Standard curve construction and assay evaluation

An *E. coli* Topo Cloning vector was used to clone ‘*Ca. B. cysticola*’. The real-time PCR amplicon was linearized by HindIII digestion. A ten-fold dilution series with the linearized vector-insert construct and a starting concentration of 99.85 ng DNA/μl (equal to 1.7 x 10¹⁰ copies/μl) was

analysed to make a standard curve. Template quality had been confirmed in samples selected arbitrarily during a previous study (Steinum *et al.*, 2010) using universal bacterial 16S rRNA primers A-18 and S-20 (Suau *et al.*, 1999). The ‘*Ca. B. cysticola*’ assay was tested for specificity against a taxonomically broad panel of 31 bacterial species (Table 3).

Table 3. Bacterial templates representing a broad taxonomic range of species used to test the ‘*Ca. B. cysticola*’ real-time PCR assay for specificity

Bacterial Isolates	
<i>Arthrobacter globi</i>	<i>B. pseudomallei</i>
<i>Aeromonas. salmonicida</i> subsp. <i>salmonicida</i>	<i>Photobacterium phosphoreum</i>
<i>A. salmonicida</i> subsp. <i>smithia</i>	<i>Piscirickettsia salmonis</i>
<i>A. salmonicida</i> subsp. <i>achromogenes</i>	<i>Proteus mirabilis</i>
<i>Bacillus cereus</i>	<i>Pseudomonas fluorescens</i>
<i>Brochothric thermosphacta</i>	<i>Rhodococcus erythropolis</i>
<i>Carnobacterium piscicola</i>	<i>R. equi</i>
<i>Escherichia coli</i>	<i>Serratia marcescens</i>
<i>Francisella noatuensis</i>	<i>Staphylococcus aureus</i>
<i>Micrococcus luteus</i>	<i>Streptococcus agalactiae</i>
<i>Moritella viscosa</i>	<i>Tenacibaculum maritimus</i>
<i>Mycobacterium marinum</i>	<i>Vibrio salmonicida</i>
<i>Nocardia asteroides</i>	<i>V. splendidus</i>
<i>Pasteurella skyensis</i>	<i>V. anguillarum</i>
<i>Burkholderia mallei</i>	<i>Yersinia ruckeri</i>
<i>B. thailandensis</i>	

16S rDNA sequencing and sequence analysis

After purification using the Nucleospin^R purification kit (Macherey-Nagel, Düren, Germany), the ‘*Ca. B. cysticola*’ real-time PCR amplicon was cloned into the TA TOPO dual promoter vector (Invitrogen). Plasmid DNA was extracted with Nucleospin^R Plasmid kit (Macherey-Nagel) and this insert sequenced alongside samples from both an Irish and Norwegian site. Almost full-length 16S rRNA amplicons were obtained by standard PCR with primers A1_14F and A1_1420R and sequenced with six additional primers (Table 1). All sequencing was performed using DYEnamicTM ET terminator reagents (GE Healthcare Bio-Sciences, Little Chalfont, UK) and the MegaBace 1000 sequencing system (GE Healthcare Bio-Sciences). Subsequent sequence analysis was performed with the Vector NTI Suite 11 software (InforMax Inc.) and NCBI blastn searches (Altschul *et al.*, 1990).

Transmission electron microscopy

Gills were examined from three seawater farmed Atlantic salmon affected by PGI, from one of the Norwegian farms in the longitudinal study. Tissues were washed in 0.1 M sodium cacodylate buffer at pH 7.4, postfixed in a mixture of 2 % (w/v) osmium tetroxide and 1.5 % (w/v) potassium ferri hexacyanide in buffer, washed, passed through graded ethanols and propylene oxide, and embedded in Lx-112 medium (Ladd Research Industries, Inc., Burlington, Vermont, UK). Semi-thin sections were stained with toluidine blue for light microscopy, and ultrathin sections were contrasted with uranyl and lead for electron microscopy. Samples were also taken from these fish for histology and molecular studies and processed as described above.

Florescence in-situ hybridization

Gills from fish with high levels of epitheliocysts from one of the Irish farms and three Norwegian farms were selected for fluorescence *in situ* hybridization (FISH). Specific fluorescently labelled oligonucleotide probes for '*Ca. B. cysticola*' (A1/A4-129) and '*Ca P salmonis*' (Psc-523, Psc 16s-197) (ProbeBase, Daims *et al.*, 2005) were designed and applied to deparaffinised slides of gill tissue. Probes were fluorescently labelled, and targeted to appropriate areas of the sequence in question according to Tönshoff *et al.* (unpublished results, under review). Probes for the beginning and the end of the target sequences were developed to check for chimera-sequences. A general bacterial probe EUB-Mix-Fluos (Amann *et al.*, 1990) and 4', 6-diamidino-2-phenylindole (DAPI), a fluorescent *stain* that binds strongly to DNA, were also used for bacterial localisation during hybridization. The efficacy of the FISH probes was tested on clones with inserts of the target bacterial sequences, prior to application to the test gill tissues. Non-sense probes were used as negative controls. The appropriate formamide concentration was established for each probe to be 35% and samples were hybridized at 56°C for 45 minutes. Samples were then washed, allowed to dry and examined under oil using confocal microscopy.

Statistical analysis

Using a student's T-test, there was no significant differences between the mean Ct-values for '*Ca. B. cysticola*' ($P = 0.80, \pm \text{S.E. } 0.79$) when fish from Ireland and Norway were compared. This was also the case for values for '*Ca. P. salmonis*' ($P = 0.66, \pm \text{S.E. } 0.55$). Thereafter, these two populations

were amalgamated for all further analyses.

For all fish, cyst levels were quantified categorically at the ordinal level on histopathology using the same grading system described by Steinum *et al.* (2010). In order to investigate the relationship between bacterial load and cyst level using contingency tables it was necessary to convert the Ct-values to ordinal measures prior to commencing the analyses (categories 0-3, as described above). There are a number of different statistical measures of association. Goodman and Kruskal's gamma test was selected for analysis of the data in this case as all variables were measured at the ordinal level. The gamma test measures the strength of association of cross tabulated ordinal data and was performed using the software package PASW (Version 18.0.0.). Gamma can be used to measure both the strength and the direction of an association between two ordinal level variables. The value of gamma can vary from + 1.00 for a perfect positive relationship to -1.00 for a perfect negative relationship. When gamma = 0 it means there is no relationship or association between the two variables. After the gamma statistics were calculated for each pair of variables, a Z-test and z-scores were calculated to test for significance. Finally, the level of pathology was also quantified in each fish using categorical measures (see above) so the relationship between gill pathology and '*Ca. B. cysticola*' could also be explored for positive significant associations.

4.3 Results

Real-time PCR evaluation and sequence analysis

The results of the standard curve construction (slope = -3.3, amplification efficiency (E) = 98.7% and y-intercept ~ 38.0) suggest the assay to be highly sensitive. The real-time PCR assay primers and probe were thoroughly examined for *in silico* specificity by extensive alignment and blastn searches against known sequences of public databases. Further, no cross hybridization and amplification were identified with the '*Ca. B. cysticola*' assay on testing of the taxonomically broad panel of 34 bacterial species. Alignment and BLAST analysis of the plasmid DNA insert and the 2 contiguous cDNA sequences from Irish and Norwegian fish revealed 100% sequence identity to '*Ca. B. cysticola*' (Tönshoff *et al.*, unpublished results, under review) indicating the bacteria is highly conserved across different geographical locations.

Histopathological findings

Of the total sample of 162 fish, a range of gill pathologies were observed and according to the severity of the lesions, were graded into pathological categories as described above. Figure 1a illustrates the numbers of fish in each category. The histopathological findings in the fish ranged from mild lamellar tip hyperplasia and occasional fusion of filaments, to extensive hyperplastic inflammatory lesions obliterating the normal lamellar architecture and widespread fusion and necrosis of epithelial cells. Even though the pathology was nearly exclusively proliferative in character, not all of the cases met the exact criteria for PGI (Kvellestad *et al.*, 2005). Epitheliocysts were observed in fish from all pathology grades, however the highest levels were observed in fish with moderate to extensive pathology (Figure 1b). In these fish the epitheliocysts appeared to be generally scattered throughout the gill epithelium. Alternatively, in fish with few epitheliocysts, the cysts were usually observed near the apical tips of the secondary lamellae, occasionally adjacent to mild epithelial proliferation but frequently with very little host reaction. There was a significant association between the number of cysts and the severity of the pathology ($\gamma = 0.63$, $z\text{-score} = 5.25$), with the highest levels of cysts mostly observed in fish with the most severe changes (Figure 1b).

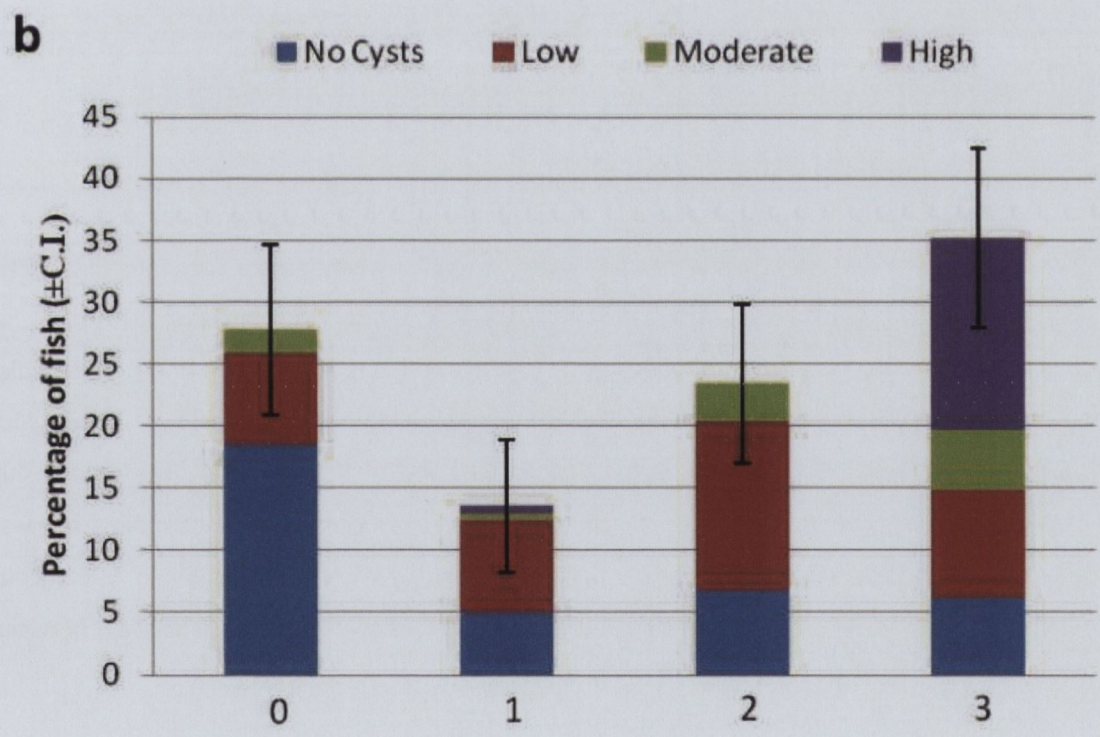
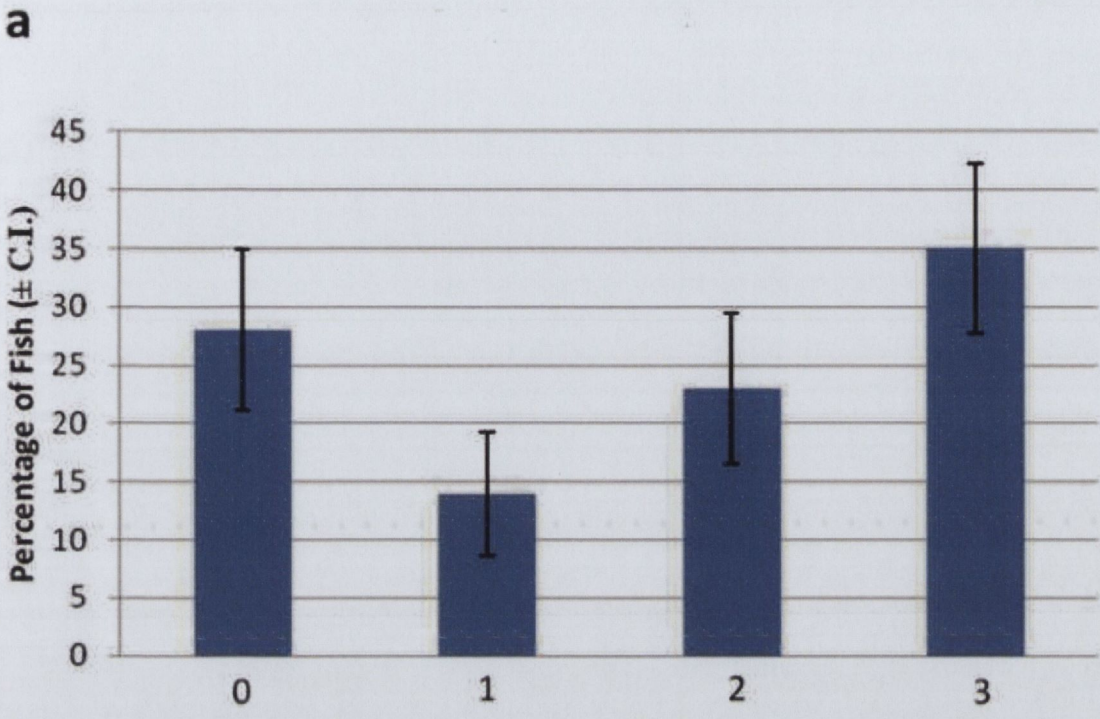


Figure 1. Percentage of fish in each of the pathological categories 0 – 3 (a) (±C.I.). The percentage of fish with each level of epitheliocysts in each of the pathological categories is illustrated in (b)

‘*Candidatus Branchiomonas cysticola*’ detection and comparison with levels of ‘*Candidatus Piscichlamydia salmonis*’

‘*Ca. B. cysticola*’ was detected in 147 / 162 fish in total in the study with a mean Ct-value of 26.4 (S.E.±0.4). ‘*Ca P. salmonis*’ was detected in 113/162 fish in total with a mean Ct-value of 31.2 (S.E. ± 0.3). Table 4 details the percentage of fish affected and the levels of each bacteria in farms from Ireland and Norway.

Table 4. Percentage fish affected by ‘*Ca. B. cysticola*’ and ‘*Ca. P. salmonis*’ in each country and average levels of bacteria present in the tissues, denoted by semi-quantitative PCR (Ct. values)

Country	‘ <i>Ca. B. cysticola</i> ’			‘ <i>Ca. P. salmonis</i> ’		
	% Positive Farms	% Fish affected (±C.I.)	Mean Ct Value (±S.E.)	% Positive Farms	% Fish affected (±C.I.)	Mean Ct Value (±S.E.)
Ireland	100	78.1(±10.9)	26.4 (±1.1)	100	89.1(±8.2)	30.5 (±0.8)
Norway	100	97.2(±3.1)	26.1 (±0.5)	67	59.8(±9.2)	31.2 (±0.4)

Microbial and histological findings compared

The categorical data were cross-tabulated to enable the relationship between different variables to be explored (Table 5) using Goodman and Kruskal’s gamma test. The critical z-score value, which indicates if associations are significant, was ±2.58 (at 0.01 level of significance). The relationship between the load of each bacterium and the numbers of epitheliocysts detected histologically was investigated. A significant association was identified between cyst level and levels of ‘*Ca. B. cysticola*’ (gamma = 0.60, z-score= 4.92). Although there were indications of a positive association between cyst level and ‘*Ca P salmonis*,’ this was not significant (gamma = 0.29, z-score= 2.08).

Both the relationships between the levels of ‘*Ca. B. cysticola*’ and the severity of pathological changes (gamma = 0.59, z-score = 4.85) and the number of epitheliocysts and the severity of the pathological changes (gamma = 0.63, z-score = 5.25) were also significant.

Table 5. (following page) Results from Atlantic salmon post-smolts from the Irish and Norwegian farms analyzed for associations with the Goodman and Kruskal's gamma test. **A)** '*Candidatus* *Piscichlamydia* *salmonis*' load (low, moderate and high) versus epitheliocysts number (few, some, many), gamma = 0.29, z-score = 2.08, **B)** '*Candidatus* *Branchiomonas* *cysticola*' load versus epitheliocysts number, gamma = 0.60, z-score = 4.92, **C)** '*Candidatus* *Branchiomonas* *cysticola*' load versus severity of pathology (slight, moderate, extensive), gamma = 0.59, z-score = 4.85 and finally **D)** epitheliocyst number versus severity of pathology, gamma = 0.63, z-score = 5.25. The positive associations for B), C) and D) can be considered statistically significant as the critical z-score value (at 0.01 level of significance) is ± 2.58

a) Epitheliocysts				
' <i>Ca. P. salmonis</i> '	None	Few	Some	Many
Not detected	24	18	5	2
Low load	19	11	1	7
Moderate load	13	12	5	8
High load	3	19	6	9
b) Epitheliocysts				
' <i>Ca. B. cysticola</i> '	None	Few	Some	Many
Not detected	13	2	0	0
Low load	24	24	1	0
Moderate load	19	23	3	4
High load	3	11	13	22
c) Gill Pathology				
' <i>Ca. B. cysticola</i> '	None	Slight	Moderate	Extensive
Not detected	12	2	0	1
Low load	16	15	9	9
Moderate load	12	3	19	15
High load	5	2	10	32
d) Gill Pathology				
Epitheliocysts	None	Slight	Moderate	Extensive
None	30	8	11	10
Few	12	12	22	14
Some	3	1	5	8
Many	0	1	0	25

Observations in freshwater hatcheries and farms without PGI outbreaks

Sixty pre-smolts from six freshwater hatcheries in Norway and Ireland were also screened for '*Ca. B. cysticola*' using the real-time PCR. The bacterium was not detected in any of the fish. There was no significant gill pathology observed in any of these fish. Some of the Irish fish had epitheliocysts consistent in appearance with those caused by '*Ca. C. salmonicola*' (Mitchell *et al.*, 2010).

Transmission electron microscopy

Electron microscopy was performed on gill tissues from two '*Ca. P. salmonis*' qPCR negative fish with low- and high- numbers of epitheliocysts respectively. Large membrane-bound cytoplasmic inclusions containing many pleomorphic bacterial cells with double tri-laminar membranes were seen in the epithelial cysts of both fish. Both elongated and rounded forms were seen, with a number of the latter type containing small rounded vacuoles, some of which appeared to be artefactual. While the observed forms were similar to reticulate and intermediate bodies of the Chlamydial developmental cycle, elementary bodies were not observed (Figure 2).

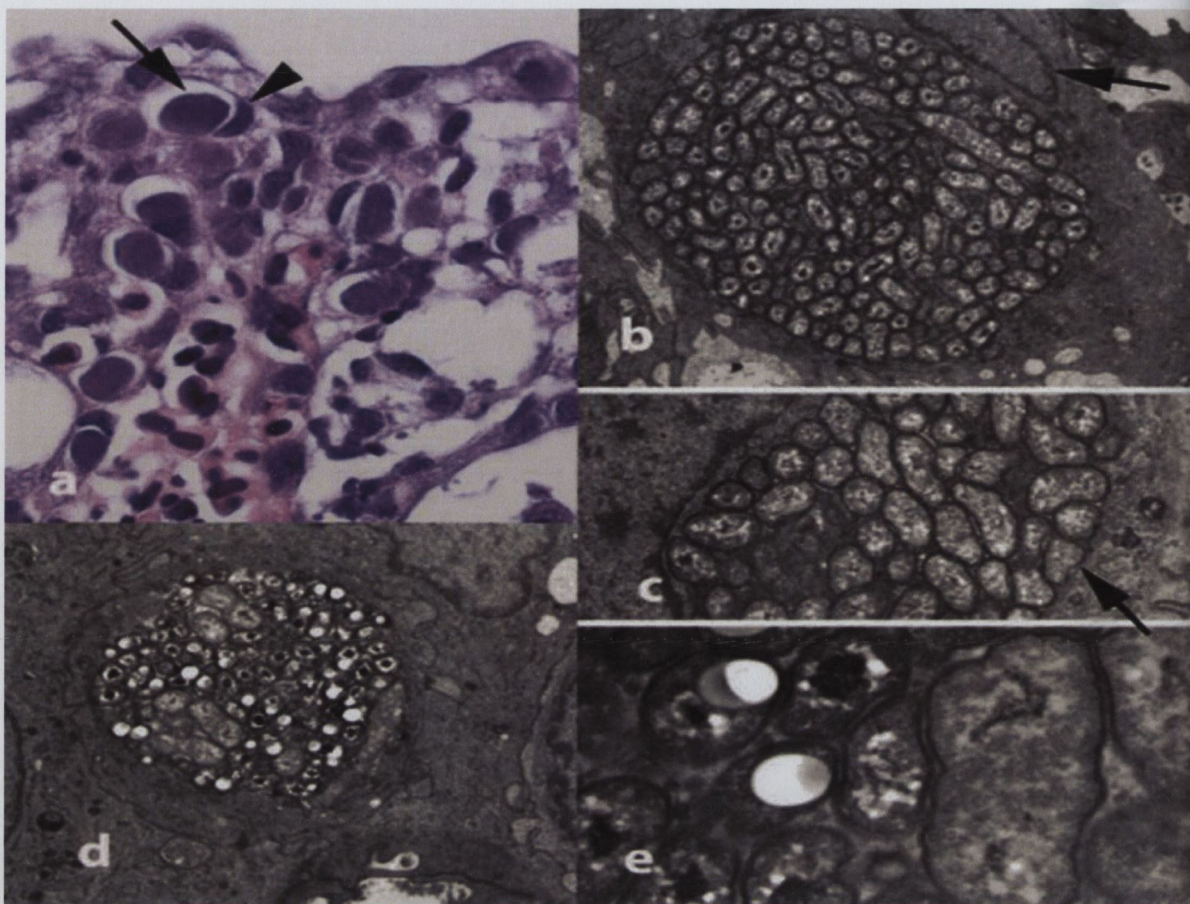


Figure 2. (Morphological examination of) Epitheliocysts in gills of Norwegian seawater farmed Atlantic salmon suffering from proliferative gill inflammation. a) sections of formalin-fixed and paraffin-embedded tissue, (b-e) ultrathin sections from glutaraldehyde-fixed tissue examined by transmission electron microscopy

(a) The epitheliocysts (arrow) appeared in epithelial cells as regular rounded to oval and well-circumscribed cytoplasmic inclusions that were granular and basophilic and that occupied most of the cell volume. The host cell nuclei were displaced to one side (arrowhead). Haematoxylin and eosin staining, 1000x

(b) Each epitheliocyst was membrane-bound (arrow in c) and contained many highly pleomorphic bacteria with a double trilaminar membrane. Elongated and shorter or rounded forms with or without nucleoids were seen.

(c) A cyst dominated by reticulate body-like bacterial cells and cells with small nucleoids

(d-e) Some cysts contained many bacterial cells with distinct nucleoids, being intermediate body-like, and/or vacuoles. Structures surrounding some of these vacuoles appeared compressed indicating an expansion of vacuoles during tissue processing

Fluorescence *in situ* hybridization

Prior to testing any of the gill tissues with FISH, probes A1 / A4-129 for '*Ca. B. cysticola*' and Psc 16s-197 and Psc-523 for '*Ca. P. salmonis*' were tested successfully on cloned PCR products containing the target sequences of interest. In the deparaffinised gill sections, fluorescence *in situ* hybridization in all fish tested from both Norway and Ireland produced strong signals within the cysts using both EUB-Mix (a general eubacterial probe) and the specific '*Ca. B. cysticola*' probe, A1 / A4-129 (Figure 3, 1). In all cases the signals were homogenous in appearance, indicating the cysts were most likely occupied by one bacterium, '*Ca. B. cysticola*'. Piscichlaymydial probes Psc 16s-197 and Psc-523 did not generate any signals within the cysts. Faint signals were apparent diffusely distributed throughout the tissue however, which may have represented single cells or small clusters of the bacteria outside the cysts. Similar results were observed when hybridisations were carried out on fresh, squeezed gill tissues, with clear signals from cysts using the '*Ca. B. cysticola*' probes (Figure 3, 2), and ambiguous results using the Psc 16s-197 and Psc-523 probes (Figure 3, 3). DAPI staining techniques were also used on all samples indicating no additional cysts other than those staining positive for '*Ca. B. cysticola*' were present in the tissues.

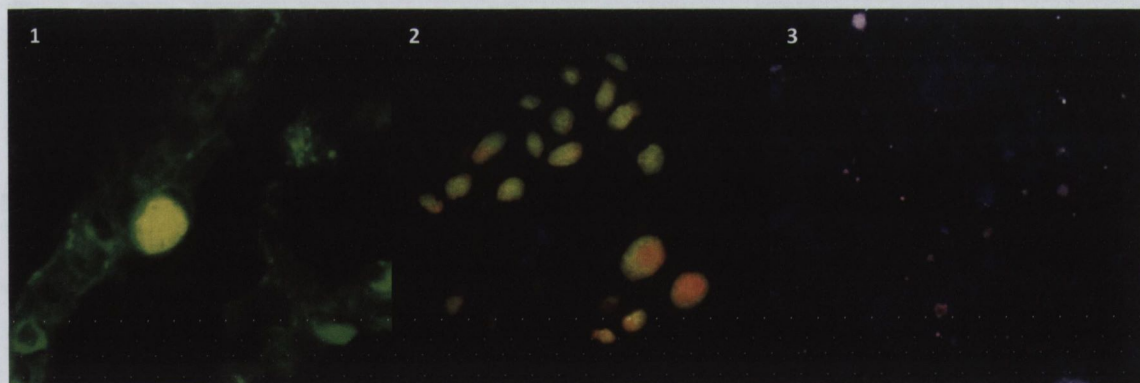


Figure 3. FISH of '*Ca. B. cysticola*' in Atlantic salmon from Ireland, deparaffinized slides of gill tissue (1), fresh squeezed gill tissue (2) and FISH of '*Ca. P. salmonis*' in the same Atlantic salmon, fresh squeezed gill tissue (3). General bacterial probe EUB-Mix labelled in Cy5, Betaproteobacterial-specific probe Btwo23A labelled in Fluos; competitor, *Ca. B. cysticola* specific probe A1/A4-129 labelled in Cy3, '*Ca. P. salmonis*' specific probes Psc-523 and Psc-16s-197 labelled in Cy5

4.4 Discussion

We have in the present study shown that '*Ca. B. cysticola*', recently identified as the only non-Chlamydial bacterium to be associated with epitheliocystis (Tönshoff *et al.*, 2012), to be highly prevalent, widely distributed and genetically conserved at the 16S rRNA level. '*Ca. B. cysticola*' appears to be a common endosymbiont of Atlantic salmon gill epithelial cells. Although highly prevalent, it is found in significantly greater quantities in fish with high levels of epitheliocystis and severe gill disease. The statistical analyses suggest not only is it strongly associated with epitheliocystis, but there is a moderate association between this bacterium and the levels of gill pathology observed, indicating that it may be an important gill pathogen and one that has to date been overlooked.

Although the bacterium is present at low levels in many fish without gill pathology, there is a significant correlation between increasing levels of the bacterium and adverse pathological changes in the gills suggest the negative effects of '*Ca. B. cysticola*' may be related to the quantity of bacteria present. Such a 'dose-dependant' pathological effect has been suggested for other microbial gill pathogens associated with PGI such as the microsporidean, *Desmozoon lepeophtherii* (Steinum *et al.*, 2010). However, in the absence of infectious challenge evidence, whether high tissue levels of '*Ca. B. cysticola*' are the cause of, or secondary to severe proliferative gill diseases remains unclear.

The significance of epitheliocystis in Atlantic salmon as a disease-causing agent has been frequently investigated. Despite this, studies have failed to consistently demonstrate a clear link between the presence of the condition and gill disease (Adams and Nowak 2006; Nylund *et al.*, 1998; Mitchell *et al.*, 2010), with it only occasionally been observed in conjunction with gill pathology (Draghi *et al.*, 2004; Nylund *et al.*, 1998). Steinum *et al.* (2010) recently found a marked significant association between the presence of high levels of epitheliocysts and the severity of PGI, a finding supported by the present study. However, whether the cysts are primary or proliferate secondarily to other pathologies remains to be elucidated. A strongly significant association between the levels of '*Ca. B. cysticola*' and the numbers of epitheliocysts enumerated on histology was observed but in contrast, this was not the case with '*Ca. P. salmonis*'. This further supports the finding that '*Ca. B. cysticola*' is the main if not the only cyst-forming bacterium in fish from this study, and potentially in Atlantic salmon in general.

While the TEM micrographs showed cellular morphology similar to that associated with '*Ca. P. salmonis*' infection (Draghi *et al.*, 2004; Nylund *et al.*, 1998), we found no molecular evidence of '*Ca.*

P. salmonis' within the TEM study material. Furthermore no evidence of '*Ca. P. salmonis*' was detected within epitheliocysts using FISH. Even though both light- and electron- microscopy revealed epitheliocyst morphology similar to that previously reported from seawater farmed Atlantic salmon (Nylund *et al.*, 1998; Draghi *et al.*, 2004), differences in bacterial cell morphology were noted. Essentially, while many pleomorphic, reticulate and intermediate body-like cells similar to those reported by Nylund *et al.* (1998) and Draghi *et al.* (2004) were detected, the electron-dense elementary bodies reported by Nylund *et al.* (1998), were not. Elementary body formation is not documented outside the *Chlamydiae* (AbdelRahman and Belland, 2005), nor have they been consistently observed within epitheliocysts in other salmonids (Draghi *et al.*, 2007; 2010). Even though microscopic morphology alone can be of limited value for *in situ* classification of bacteria, the lack of such cell forms within epitheliocysts in the present study further supports that the cysts are of non-chlamydial aetiology. The morphology in conjunction with the FISH results and the sequence retrieval data all support that '*Ca. B. cysticola*' is the main aetiological agent of epitheliocysts in this case.

Strong positive signals with FISH utilising '*Ca. B. cysticola*' probes were achieved in fish analysed from both Ireland and Norway. The signals in the cysts were clearly homogenous indicating there is likely to be only '*Ca. B. cysticola*' within the cysts, as opposed to a mixed bacterial population. The failure of FISH to demonstrate '*Ca. P. salmonis*' in gill tissues where molecular techniques detect the bacterium may be attributed to a number of factors. Even though the '*Ca. P. salmonis*' specific FISH probes worked on the cloned bacterial inserts *in vitro*, there may be some secondary or tertiary structure blocking probe binding once the bacterium is *in situ* within the gill tissues. Alternatively, the bacterial concentration of rRNA may have been too low for detection by FISH. Ribosomal content of bacterial cells in dormancy or with low physiologic activity can be below the threshold for detection by this method. Finally, even though unlikely, it is feasible that there were only small numbers of cysts containing '*Ca. P. salmonis*' alongside the more numerous '*Ca. B. cysticola*' cysts and these were not included in the sub-sampled test tissues.

The question inevitably arises as to what the significance of '*Ca. P. salmonis*' is in terms of epitheliocystis and gill disease in general. Steinum *et al.* (2010) suggested there is a moderately positive association between this bacterium and the severity of PGI. While this may be true, the role of '*Ca. P. salmonis*' as an epitheliocystis-causing agent must remain ambiguous. Chlamydia-like bacteria have been detected and sequenced in association with epitheliocyst infections in a number of fish species (Draghi *et al.* 2004; 2007; 2010; Meijer *et al.* 2006; Karlsen *et al.* 2008; Polkinghome *et al.* 2010, Mitchell *et al.* 2010). While *in situ* evidence based on ribo-probing has been presented in

some cases (Draghi *et al.* 2004, 2007; 2010; Karlsen *et al.*, 2008) the link between retrieved DNA sequences and the bacterial cells within the respective epitheliocysts has not always been conclusively confirmed (Meijer *et al.*, 2006; Polkinghome *et al.*, 2010; Mitchell *et al.*, 2010). The possibility exists therefore that some of the chlamydial sequences detected by PCR and associated with epitheliocyst infections may in fact represent organisms external to the epitheliocyst and possibly even external to the fish gill.

More convincing *in situ* hybridization experiments, perhaps with alternative fluorescent probes would assist in defining the micro-ecological position of '*Ca. P. salmonis*' in gill tissues, and help elucidate its role in disease.

Culture of '*Ca. B. cysticola*' is likely to prove challenging, as it may be an obligate intracellular pathogen and previous efforts to culture '*Ca. P. salmonis*' (Terje Steinum, Elena Tönshoff, personal communications) would more than likely have resulted in incidental culture of '*Ca. B. cysticola*' if it were a straightforward process. This is unfortunate as challenge experiments would be extremely useful to characterise disease caused by this bacterium. Nevertheless, dose-response trials utilising serial dilutions of infected gill material may be possible. Results from such trials would provide much information on the pathogenicity of '*Ca. B. cysticola*', particularly in light of the speculated quantitative effects.

Retrospective analysis of archived material from previous studies on epitheliocystis in Atlantic salmon may enhance our knowledge of the prevalence of and connection between '*Ca. B. cysticola*' and gill disease. In addition, surveys of other fish species could be undertaken to establish if this bacterium is exclusively associated with marine Atlantic salmon. The location of '*Ca. B. cysticola*' in the environment while not infecting Atlantic salmon is another area requiring investigation. Molecular screening of different species of zoo or phyto-plankton or protist parasites, particularly those affecting the gills of fish, may help identify if this bacterium has an intermediate host. Finally, giving the potential impact of '*Ca. B. cysticola*' infection to gill health, field trials investigating whether antibiotic treatment is effective may prove valuable.

In summary we have shown the recently identified novel cyst-forming bacterium '*Ca. B. cysticola*' is widely distributed within farmed Atlantic salmon in both Norway and Ireland and the bacterial load significantly correlates with the severity of pathological changes observed in the fish. '*Ca. B. cysticola*' appears to comprise the dominating agent of epitheliocystis, despite detection of '*Ca. P. salmonis*' in a large number of the study fish, the agent previously exclusively associated with

the condition.

5 Challenge trials with '*Candidatus Clavochlamydia salmonicola*'

Statement of Contribution: All work in this chapter was carried out by the author of this thesis.

5.1 Introduction

The transmission and mode of infection by the agents of epitheliocystis identified remain undetermined to date. The lack of successful *in vitro* culture techniques has made it difficult to assess the real significance of infections caused by these organisms, as Koch's postulates cannot be tested. Furthermore, the absence of an experimental challenge model has hampered our understanding of the transmission and pathophysiology of the disease (Nowak and LaPatra, 2006).

The Chlamydiae are exclusively intracellular organisms, making it likely there is at least one, or perhaps even several, different environmental reservoirs or vectors for each species. However, such reservoirs of the epitheliocystis-causing Chlamydiae in fish remain elusive. Amoebae are ubiquitous protista that frequently contain bacterial endosymbionts, including many different species of Chlamydiae. Estimates suggest approximately 5% of all endosymbiont isolates from members of the genus *Acanthamoeba* are novel Chlamydia-like bacteria (Fritsche *et al.*, 2000). Free-living amoebae such as these abundant *Acanthamoeba* species have also been shown to act as environmental reservoirs for many of the pathogenic Chlamydial species, indicating they may serve as reservoirs for transmission and vehicles of dispersal of many important diseases (Horn, 2008).

Co-cultivation of amoebae has been used successfully to recover novel Chlamydiae from environmental samples, such as activated sludge (Collingro *et al.*, 2005). However, despite several attempts, isolation of either of the two agents of epitheliocystis from Atlantic salmon, '*Ca. P. salmonis*' or '*Ca. C. salmonicola*,' using co-cultivation techniques has been unsuccessful. Attempts to culture either of these bacteria using different fish and insect cell lines have also failed to produce any results (unpublished data, Terje Steinum, Veterinary Institute, Oslo and Elena Toshoff, Department of Microbial Ecology, University of Vienna).

Challenge protocols in fish are varied and can involve bath exposure, intra-peritoneal and intramuscular injection techniques or co-habitation. These different methods are extensively described in the literature for individual challenge models. Ideally, a challenge method should closely mimic natural exposure to a pathogen and ensure that the immune mechanisms located in the appropriate body surfaces for the disease play their role (Nordmo, 1997). Bath and co-habitation challenge methods probably reflect natural infections more closely than the other techniques. However, these methods are more difficult to control and standardise than injection methods (Nordmo, 1997). Animal models of human Chlamydial infections, such as those used in trachoma, frequently use purified elementary bodies, the infectious stage of the chlamydial life cycle, for challenge studies (Taylor, 1985). For this epitheliocystis trial, an alternative strategy had to be established due to the

lack of availability of a pure culture of the bacterium.

We describe a challenge trial with '*Candidatus* *Clavochlamydia salmonicola*' using fresh gill material with relatively high levels of the bacterium and a direct novel per-gill infection technique adapted from Miyazaki *et al.* (2008). This technique was deemed to be the most accurate means of simulating natural infection in the gills, as opposed to using parenteral injection techniques or bath / cohabitation methods.

Though only very occasionally associated with gill pathology (Karlsen *et al.*, 2008; Mitchell *et al.*, 2010), '*Ca. C. salmonicola*' was selected for the epitheliocystis challenge model for a number of reasons. Firstly, freshwater salmon pre-smolts are readily available and easy to maintain in small tanks. Secondly, a high prevalence and intensity of infection had been recorded in a number of sites in Ireland during a previous study (Mitchell *et al.*, 2010), therefore it was envisaged that there would be a ready supply of suitable infectious material for the challenge.

The aims of the experiment were to establish a disease model for '*Ca. C. salmonicola*' in order to investigate infection transmission dynamics, such as whether direct infection from fish to fish was possible or if an intermediate host may be required. It was also anticipated that the model might give some insight into the pathophysiology of epitheliocystis infections in general.

5.2 Materials and methods

Selection of suitable fish populations for the challenge trial

In early February 2011, fish were sampled from six freshwater hatcheries in Ireland in an attempt to identify naïve fish for the challenge trial, with neither histological nor molecular evidence of '*Candidatus* *Clavochlamydia salmonicola*' infection. However, all sites had fish that tested positive for '*Ca. C. salmonicola*' on RT PCR. In light of the lack of a site totally free from infection, a freshwater site in Donegal with extremely low levels of infection by RT PCR, and no evidence of infection histologically, was selected to provide the challenge fish. Very high threshold cycle (Ct) values were recorded by real-time PCR indicating low quantities of bacteria were present on the gills of these fish. A comparative analysis using quantitative real-time PCR (Mitchell *et al.*, 2010) would then be used to ascertain if there was an increase in infection levels in fish from the Donegal site post challenge. A site in the west of Ireland in Galway with readily visible '*Ca. C. salmonicola*' cysts

and low Ct values on molecular analysis was selected to provide the infectious fish.

Challenge fish

60 fish were randomly selected using a hand-net from one tank at a freshwater site in Donegal in Ireland, randomly assigned to four groups of 20 and transported in oxygenated water to the Vet Aqua Laboratories in Galway on the 21st February 2011. On arrival, three fish from each group were removed and euthanised. The 2nd gill arch on the left hand side was removed for histopathology and a portion of the 2nd gill arch on the right hand side was suspended in RNAlater for real-time PCR. These fish were sampled pre-trial to assess the background levels of infection in the challenge fish at the start of the experiment. The remaining fish were transferred in their previously assigned groups to four separate 80 liter aquarium tanks with appropriate filtration and aeration systems. Water in the tanks was sourced from the mains supply, and had been allowed to de-chlorinate naturally by passive diffusion over 48 hours prior to the experiment. Fish were allowed to acclimatise and then were observed twice daily for signs of unusual behaviour or ill health. Oxygen and temperature readings were taken once daily and recorded. Ammonia, nitrite and nitrate levels were measured every 3-4 days, or more frequently if a decline in water quality was suspected. The experiment ran for 30 days in total. Disturbance of the fish was kept to a minimum in the first 2 days which allowed them to acclimatise to their new environment prior to the challenge. At this point, two challenge and two control tanks were assigned at random.

Infectious Fish and production of infected gill homogenate

15 fish from a site in Galway with moderate levels of '*Ca. C. salmonicola*' infection were collected and transported to the Vet Aqua Laboratory. These fish were immediately euthanized, the 2nd gill arch on the left hand side removed for histopathology and the 2nd gill arch on the right hand side excised and cut into two sections of approximately 1cm² each. One of these was collected in RNAlater for molecular analysis while the other was used to create the gill homogenate. For each fish, the gill tissue cells were gently scraped from the cartilage using a sterile scalpel blade, the tissue suspended in a 2ml eppendorph of sterile saline and homogenized by suction and expulsion for 30 seconds using a 23 gauge needle and 2.5 ml syringe. The homogenate from all the fish was pooled and refrigerated at 5°C immediately prior to the challenge.

Infectious challenge

Challenge fish were anaesthetised in a bath with 30mg / L of the anaesthetic methane trisulphate (MS 222) until an adequate plane of anaesthesia was reached. This was the point where the fish had totally lost their righting reflex and had a significantly diminished response to external stimuli. The fish were extracted from the anaesthetic bath and placed in dorsal recumbancy. The per-gill infection technique was modified from Miyazaki *et al.*, (2008). In their KHV challenge experiment, they inoculated a pure culture of the virus directly onto the gills. In this case a gill cell homogenate containing infected cells was used. The operculae were gently opened and 0.75 ml of infectious gill homogenate sprayed on both left and right sets of gill arches using a syringe and 23 gauge needle, ensuring all the gills were covered in homogenate. Post inoculation, Miyazaki *et al.* (2008) left the fish for 5 minutes wrapped in paper to allow the KHV to attach to the gill cells. The fish in this case were maintained in dorsal recumbancy for only 30 seconds post inoculation due to the higher oxygen requirement of Atlantic salmon. Each fish was then returned directly to the appropriate tank and monitored until it recovered from anesthesia, namely until it regained its righting response and was swimming steadily in the water column. Control fish underwent an identical procedure but the gills were sprayed with sterile saline in place of the infectious gill homogenate.

Sampling post challenge

Approximately five fish were sampled from each of the four tanks on 7, 14 and 21 days post-challenge (details in Table 1). At each sampling, gills were removed for both histopathology and molecular analysis as described.

Table 1. Fish sampled at each time point during the challenge trial

Date	Description	Number of fish sampled	Challenge / Controls
March 1 st 2011	Pre-challenge Donegal site	12	N/A
March 1 st 2011	Infectious fish Galway site	15	N/A
March 8 th 2011	7 days post challenge	20	9/11
March 15 th 2011	14 days post challenge	18 [*]	8/10
March 22 nd 2011	21 days post challenge	14 ^{**}	4/10
	Total	82	N/A

^{*}Two fish from one of the challenge tanks died prior to sampling. ^{**} Three fish escaped and three fish died due to ammonia toxicity from each of the challenge tanks prior to sampling.

Histopathology

The second gill arch from the left hand side of each fish was chosen for histopathological examination. Samples were fixed in 10% neutral buffered formalin; 5µm sections from paraffin embedded tissue were cut and stained with hematoxylin and eosin. Samples were then scanned at X100 magnification and the number and morphology of bacterial cysts recorded (“cysts” are defined aggregates of basophilic, host membrane-bound, bacterial organisms, readily visible on histopathology).

Molecular sampling and Real-Time PCR

1cm² gill tissue samples were suspended in 2ml eppendorphs of RNeasy Lysis Buffer (Ambion Inc., Texas, U.S.) and stored at -20°C. DNA was extracted from gill tissues using the High Pure PCR Template Preparation Kit (Roche Ltd., Basel, Switzerland). The assay targeting a 178 bp sequence of the ‘*Ca. C. salmonicola*’ 16S rRNA gene (Mitchell *et al*, 2010) used 20 µl reactions with Taqman Universal PCR Mastermix (Roche molecular systems Inc. Branchburg, USA) according to the manufacturer’s recommendations. The reaction protocol, run on the Mx3005P™ real-time PCR system (Stratagene, Texas, USA), was as follows: 2 minutes at 50°C, 10 minutes at 95°C, followed by 50 cycles of 95°C for 30 seconds and 60°C for 1 min. Analysis of real-time PCR data was done with MxPro software

(Stratagene, Texas, USA). Negative and positive controls using DEPC-treated water (Invitrogen) and DNA from an Irish gill tissue sample, confirmed positive by sequencing, were included in all testing. All gill tissues sampled in RNAlater from the fish were tested by real time PCR.

5.3 Results

General Sampling and Husbandry

The temperature of the tanks was maintained at 17 – 18°C over the 4 week period. Oxygen levels were maintained above 6 mg / L in all tanks, ranged from 6.0 – 8.9 mg / L and averaged at 6.8 mg / L. Nitrate and nitrite levels remained within the normal range during the period. Two weeks after the start of the challenge the ammonia levels spiked in one of the challenge tanks (free ammonia was >0.08mg / L), most likely as a result of failure of the biofilter to operate efficiently in this case. The levels of ammonia observed are known to be toxic to salmonids and consequently there were a number of mortalities in this tank. In addition there were some escapees from this tank. This loss of fish from one of the challenge tanks meant it was not possible to sample fish from this tank at 21 days post challenge.

Histopathology

One gill arch from each fish in the trial was screened for histopathology.

Infectious fish: 15 fish were screened. All fish had evidence of '*Ca. C. salmonicola*' on histopathology and low (13 fish) to moderate (2 fish) levels of cysts were observed (low = < 10 cysts per field of view, Moderate 10 – 99 cysts per field of view, X 10 magnification (Mitchell *et al.*, 2010). Low levels of the sessile ciliophore *Capriniana piscium* were also observed on 6 / 15 fish. No histopathology of any significance was observed in the gill lamellar tissues.

Pre Trial / Control / Challenge Fish: There was no histopathological evidence of '*Ca. C. salmonicola*' cysts in any of the fish from these groups. Minor gill pathology was observed in fish sampled from one of the challenge groups on day 14 post challenge. This was in the form of epithelial cell hypertrophy and occasional single cell necrosis. This tank had experienced a spike in free ammonia levels which is likely to have been responsible for the pathology observed. Otherwise

no histopathology of any significance was observed in the gill lamellar tissues.

Real Time PCR

The prevalence and intensity of infection in the fish from each group is detailed in Table 2. The infector fish had an average Ct Value of 31.26 which was 6.5 Ct values lower than the levels of infection detected in the pre-trial screening of the challenge fish. This difference indicates there was nearly 20 times as many bacteria in the gills of infector fish when compared with the challenge fish (each reduction in Ct value is equivalent to a three-fold increase in bacterial number). With successive sampling over time the average Ct values increased substantially, at 7 days post challenge the average was 39.54 and at 14 days it was 41.88. No molecular evidence of '*Ca. C. salmonicola*' remained in any of the samples at 21 days post challenge.

Data were not subjected to statistical analysis due to very small sample sizes. The results are illustrated below in Figure 1.

Table 2. Prevalence of infection in each group sampled by RT PCR and Histology. Ct- values given in each case indicate the levels of bacteria present in each group of fish.

Fish Group	RT PCR Prevalence %	RT PCR – Infected and Challenge Fish Mean Ct. (± SD) (Range)	RT PCR – Pre-challenge and Control Fish Mean Ct. (± SD) (Range)	Histology Prevalence %
Infected Fish	100%	31.26 (±2.51) (28.33-32.26)	N/A	100%
Pre- challenge	100%	N/A	37.76 (±1.15) (35.22 – 39.58)	0%
7 days post- challenge	45%	40.30 (±1.09) (39.24-41.42)	39.16 (±1.89) (38.11-41.70)	0%
14 days post- challenge	11%	41.88 (±1.71) (40.17-43.58)	42.56 (±0) (42.56)	0%
21 days post challenge	0%	0	0	0%

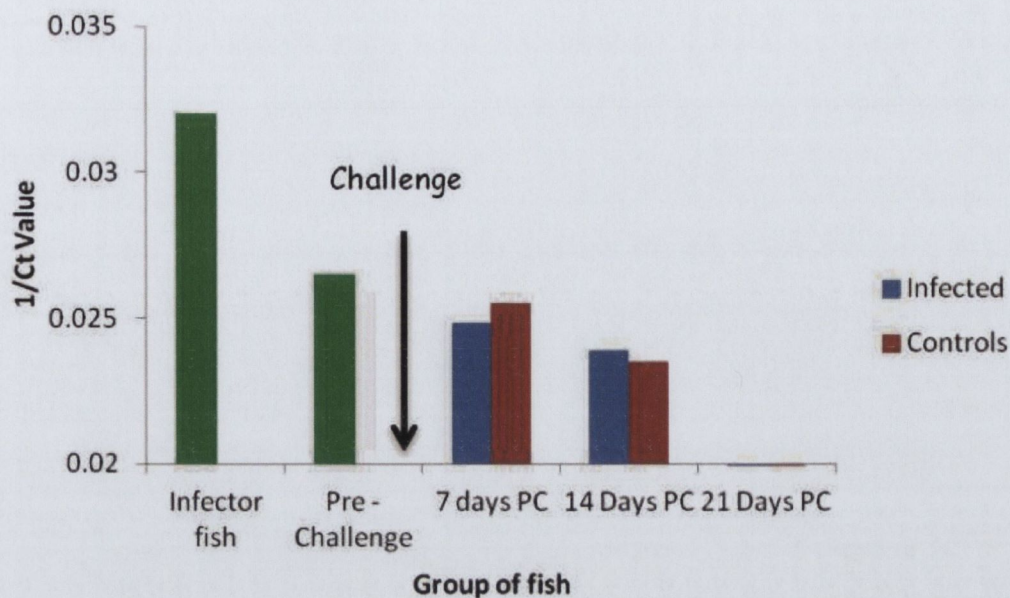


Figure 1. Levels of bacteria in each group of fish sampled during the challenge trial. PC = post challenge. The inverse Ct value is used to enable the trend in bacterial quantity to be visualised more clearly.

5.4 Discussion

The current experiment describes an attempt to create a challenge model using ‘*Ca. C. salmonicola*’. While the model may have failed to demonstrate any evidence of successful infection transmission, the results did provide some unusual findings that enable us to at least speculate about the life cycle and infection dynamics of this agent of epitheliocystis.

Attempting to design an effective challenge model using an unculturable bacterium such as ‘*Ca. C. salmonicola*’ has many limitations. The use of crude homogenates of infected tissues becomes a necessity in many cases, meaning that there is a distinct probability that other pathogens may also be present in the challenge material. This is especially true when the infective material is made of gill tissues, which have an abundant diverse natural bacterial flora (Steinum *et al* 2009). And possibly other microorganisms such as viruses or parasites, like the *Capriniana piscium* species, as observed in the infector fish in this study. The potential of any of these microorganisms to affect the outcome of a challenge trial is difficult to discern. The efficacy of the per-gill infection technique appears to be good in recreating Koi herpes virus infections (Miyazaki *et al*, 2008). However, spraying infected

gill tissue cells directly onto the gills of fish is not an accurate reflection of natural wild transmission, which may have been problematic during this experiment.

It is clear from the results that the challenge did not cause any increase in infection levels in the exposed fish. There may be many reasons for this. A fundamental failure of infection may have occurred, possibly due to the non-viability of the infectious particles within the cysts. The infectious particles of Chlamydiae are known as elementary bodies. If the cysts were made up of bacteria in the alternative stages of development (reticulate and / or intermediate bodies) it may be feasible that they were incapable of becoming endocytosed into the lamellar epithelial cells on contact. However, transmission electron microscopy of '*Ca. C. salmonicola*' infected tissue generally shows a mixture of particle types co-exist within the cysts (Schmidt-Posthaus *et al.*, 2011; Karlsen *et al.*, 2008).

Another possibility why the challenge failed is that the suction homogenization was insufficient to rupture the cysts containing the bacteria and the gills of the challenge fish may not have been exposed to sufficient quantities of infectious organisms. In retrospect, addition of tissue lysis buffer may have been useful to ensure the rupture of gill epithelial cells and release of the infectious particles. The effects of tissue lysis buffer on '*Ca. C. salmonicola*' cells would however need to be established.

Another potential reason for failure is there were simply inadequate levels of bacteria in the gill tissue homogenate to create an infectious dose. Fresh frozen gill material from fish that were sampled in 2010 with very high levels of '*Ca. C. salmonicola*' was available (Ct value = 23.90), However, the viability of Chlamydiae may be reduced significantly by freezing (Corsaro & Greub, 2006) so the fresh material with lower levels of bacteria was preferentially selected. The fact that the mechanism of entry into and survival of Chlamydiae inside host cells remains poorly understood does not help when attempting to design a trial, or speculating as to why infection failed. Whether entry is facilitated via endocytosis or phagocytosis remains controversial. It has been suggested that Chlamydiae may even be capable of both depending on circumstances and the host (Bavoli *et al.*, 2000).

Assuming that fundamental mechanical failure of infection was not the case however, and that the organisms were viable and infectious, other possibilities why the challenge failed must be considered. It would seem reasonable to speculate that the previous exposure of the challenge fish to '*Ca. C. salmonicola*' may have allowed for the development of some specific immune response to the bacterium. However, previous findings during the longitudinal freshwater study would not

support this hypothesis (Chapter 1, Mitchell *et al.*, 2010). During the longitudinal study, the same cohort of fish was sampled first in November 2009, then in February 2010. Despite the fact the fish had clearly been exposed to '*Ca. C. salmonicola*' and infected for a number of months, the Ct values dropped dramatically between November (27.62) and February (23.90) indicating a 12-fold increase in the quantity of bacteria per fish. There was certainly no evidence in this case of an effective immune response over time. In human Chlamydial infections such as ocular trachoma protective immunity does not occur post exposure to the bacteria, and repeated infections are actually necessary for the development of the characteristic disease which includes chronic effects such as trachomatous follicles in the tarsal conjunctiva and conjunctival scarring and corneal pannus. It is thought that with many chlamydial infections a paradoxical effect exists – although the immune response confers partial protection, it also appears to be responsible for much of the pathology that characterizes the disease (Taylor, 1985). In addition, many species of Chlamydiae frequently show an ability to resist the mechanism of action of macrophages and can effectively evade the host immune response (Corsaro & Greub, 2006). In fact this may be the reason why there is so little pathology or inflammation observed adjacent to the cysts in '*Ca. C. salmonicola*' infections.

The fact that other environmental or fish factors that may have had an effect on the efficacy of the challenge, such as temperature, oxygen levels or life stage of the fish, cannot be ruled out. Some species of Chlamydiae that exist within amoebae have been shown to be endosymbiotic at one temperature and lytic at another which may affect transmission dynamics and release of infectious stages into the environment (Corsaro & Greub, 2006).

The failure of the direct transmission challenge to work raises the possibility that '*Ca. C. salmonicola*' requires an intermediate host to facilitate infections. As the fish were maintained in mains-treated water, there would be no living organisms in the water such as amoebae that could act as potential vectors. It is feasible to consider that '*Ca. C. salmonicola*' may require a developmental stage within an intermediate host to become infectious. The lack of such hosts in the trial due to the stringent water conditions may be the reason why over the three-week period all indications of infections disappeared from the fish. The natural infection cycle present in the fish at the start of the experiment may have run its course. Once the bacteria were expelled from the gill tissues, with no intermediate hosts or vectors being available, transmission and infection could not be propagated.

The only documentation in the literature to date of challenge trials with epitheliocystis were reported anecdotally by Wolf *et al.* (1988) over 20 years ago. He describes an experiment where a

crude emulsion of gill material from bass (*Morone* spp.) was added to a tank of goldfish (*Carassius auratus*) and several of the goldfish went on to develop epitheliocystis within 3-4 weeks. He also describes an experiment where G.L. Hoffman infected healthy bluegills (*Lepomis macrochirus*) by placing them in a tank with infected gill arches. The challenge fish developed epitheliocystis within 25 days and unexposed control fish apparently remained healthy. It is likely that several other attempts have been made over the years to establish some form of infectious challenge model for epitheliocystis but unfortunately, due to a lack of a successful outcome or a robust scientific experimental design, these results may have remained unpublished.

In terms of future work, attempts could be made to develop a challenge model with an alternative agent of epitheliocystis. Sampling of infected gills during a serious outbreak of disease would be the best means of sourcing good challenge material, even if it meant having to risk reducing the viability of the bacteria through freezing so a challenge experiment could be set-up. Although more challenging logistically it would be useful to attempt another trial using seawater Atlantic salmon where the more severe proliferative pathology is frequently observed.

The challenge trial has in particular highlighted the question as to the location of '*Ca. C. salmonicola*' in the environment when not infecting piscine hosts. Knowledge of this would be useful in gaining a better understanding of the cycle of transmission and infection dynamics. Collection and molecular screening of amoebae, especially those of the genus *Acanthamoeba*, from rivers or lakes connected to fish farms where there is a high prevalence of infection may be a means to identifying an intermediate host and consequently increase the chance of developing a successful challenge model in the future.

6 General Discussion

It is clear from reviewing the literature that there are many different infectious and non-infectious agents which currently pose a challenge to the gill health of marine-farmed salmonids. Synergisms between infectious agents are not well understood, nor are potential interactions between these agents and environmental parameters such as phytoplankton blooms and zooplankton swarms. The study of gill disorders must involve exploring these multifactorial interactions in order to attain a clear understanding of the disease dynamics during outbreaks. Achieving this effectively will prove extremely challenging, as multifactorial disease models would be complex both to create and reproduce.

One of the most frequently pondered questions about epitheliocystis is the extent of its significance in terms of gill disease. The presence of the condition is frequently unaccompanied by pathology. However, in some cases, epitheliocystis is observed associated with extreme proliferative disease. The cause of this intermittent pathological response is unknown, and many mechanisms have been suggested. It is possible that there may be a quantitative effect, a tipping point, that triggers a pathological response in the host, or there may be some relationship to the developmental stage of the pathogen. Alternatively there may be some link to host factors such as species, strain, age or immune status. Potentially, a synergism with another as yet unidentified pathogen, such as a virus, may be required to trigger the pathological changes. Finally it is possible that management or environmental factors may play a role in causing the disease. These questions could be elucidated with a better understanding of what the patho-physiological mechanisms are that actually trigger the proliferative response in salmonid gills. In addition, isolation of the bacteria that cause epitheliocystis would undoubtedly provide us with a better understanding of the disease mechanisms involved, in addition to potentially enabling a challenge model and vaccine development in the future.

The discovery of the first non-chlamydial agent of epitheliocystis, '*Candidatus* Branchiomonas cysticola' may assist in the explanation of the observed intermittent proliferative response. The presence of this bacterium within epitheliocysts was clearly demonstrated by fluorescence *in situ* hybridization in gills with extensive proliferative

pathology. Although '*Ca. B. cysticola*' is present in many fish without gill disease, there is a significant association between the levels of the bacterium and the severity of the pathology observed in the gills of diseased fish. Draghi *et al.* (2004) observed two very different disease pictures when examining fish from different outbreaks of epitheliocystis, one with proliferative pathology, the other non-proliferative. On electron microscopy, marked ultrastructural differences were observed between the developmental stages of the bacteria in the two different cases. While the authors speculated that two different chlamydia-like developmental cycles may exist in farmed Atlantic salmon, one associated with proliferative lesions and the other with non-proliferative infections, they also considered the possibility that the two types of infection may have been caused by different species of *Chlamydia*. Perhaps this was in fact the case, but in light of the current findings there is a possibility that one of these infections was caused by '*Ca. B. cysticola*.' Until very recently, it had been assumed that the only agent of epitheliocystis in seawater Atlantic salmon was '*Ca. P. salmonis*' (Draghi *et al.*, 2004). In retrospect it is plausible that '*Ca. B. cysticola*' may be the more significant pathogen, or at least the one responsible for the proliferative pathology that is intermittently associated with cases of epitheliocystis. Retrospective screening of previous cases would help elucidate if this were the case.

No significant correlation with the levels of '*Ca. P. salmonis*' and the numbers of cysts observed on histopathology was uncovered by Steinum *et al.* (2010) or in the present work (Chapter 3). This fact, in conjunction with the lack of convincing detection of this bacterium within the epitheliocysts, using either FISH or other means of *in situ* hybridization, means there is now ambiguity about the significance of '*Ca. P. salmonis*' as an agent of epitheliocystis. Nevertheless, '*Ca. P. salmonis*' is frequently detected in Atlantic salmon gills, and may be of pathological importance. Steinum *et al.* (2010) showed an association between the levels of this bacterium and the severity of PGI.

The discovery and investigation of '*Ca. B. cysticola*', the first non-chlamydial agent of epitheliocystis, has some significance in terms of potential impacts on the Atlantic salmon farming industry. Since it appears to be an important pathogen capable of causing severe proliferative gill pathology, more focus in the future should be placed on this novel bacterium. Investigations into the prevalence of '*Ca. B. cysticola*' in further outbreaks of PGI

and other gill diseases using molecular methods should be prioritized. Culture should be attempted using either cell lines or axenic amoebal techniques and antibiotic therapy tested in the face of outbreaks.

'*Ca. B. cysticola*' forms a completely novel lineage within the Betaproteobacteria. Bacteria of the genus *Burkholderia* are also members of the Betaproteobacteria. This genus contains many pathogenic members such as *Burkholderia cepacia*, an important pathogen causing pulmonary infections in people with cystic fibrosis (Mahenthiralingam *et al.*, 2008) and also a contributor to community-acquired pneumonia (Burns *et al.*, 1996). The most closely related Betaproteobacteria to '*Ca. B. cysticola*' are bacteria from the genus *Pandoracea*, some of which are also involved in respiratory pathogenesis in humans and can cause infections in human cystic fibrosis patients (Caraher *et al.*, 2008). '*Ca. B. cysticola*' may employ similar methods of invading epithelial cells to *B. cepacia* and *Pandoraea* spp.. Invasion of epithelial cells is a good strategy in terms of avoidance of many host defence processes. It is plausible that '*Ca. B. cysticola*' may also upregulate inflammatory mediators in a similar pattern to that seen with both these human pathogens, resulting in the proliferative pathology we frequently see with high levels of this bacterium.

Demonstration of a causal link between many of the newly discovered Chlamydia-like bacteria and disease has frequently been challenging to achieve, not just in fish but in humans and animals also. This applies to many other species of bacteria other than the Chlamydiae, especially those that cannot be cultured *in vitro* and are only detectable by molecular methods. In their reconsideration of Koch's postulates, Fredericks and Relman (1996) provide some good guidelines as to how we can define causality with these problematic bacteria. The criteria are as follows: the putative pathogen should be present in most cases and be found in areas known to be diseased; the pathogen should be present at the cellular level and absent or less abundant in those cases without disease; as disease resolves the pathogen should decrease; the abundance of the pathogen should correlate with the severity of the disease and finally, as evidence of a causal relationship should be reproducible. With the possible exception of the last, '*Candidatus Branchiomonas cysticola*' fits these criteria, demonstrating a causal relationship between it as an agent of epitheliocystis and proliferative disease. '*Ca. P. salmonis*' does not meet all the above

criteria. It is possible that '*Ca. P. salmonis*' may act as an opportunistic bacterium rather than as a primary pathogen, and only manifest in a disease state in compromised hosts.

The routes of transmission and the identity of the environmental reservoirs / hosts of all these different gill pathogens require elucidation. Although not established fully, the gills are considered to be an important port of entry for systemic pathogens such as infectious salmon anemia virus (ISAv) and salmonid alpha virus (SAV) (Rodger *et al.*, 2011). Compromised or diseased gills may allow an easier route of entry into the host. There is very little evidence as to how gill-specific pathogens are presented to and infect gill epithelia, nor where they reside when not infecting their hosts. Amoebae have been considered a likely reservoir for the agents of epitheliocystis, particularly those of Chlamydial aetiology. Screening of different species of amoebae may provide us with some indication of the environmental reservoirs of these cyst-forming bacteria. Non cyst-forming bacteria such as *Tenacibaculum* spp. have been detected by molecular techniques in jellyfish (Ferguson *et al.*, 2010). These and other zooplankton may be important reservoirs of infection for agents of epitheliocystis or other pathogens such as viruses and parasites, and they warrant further investigation. Perhaps this is one of the reasons why we frequently see significant gill pathology involving infectious agents after an environmental insult, as suggested by Rodger (2007).

The advent and more frequent use of molecular techniques in recent years has lead to widespread application of a suite of diagnostics based on PCR and sequencing analysis in all areas of fish health and disease. Being quick and easy to perform, these techniques have frequently replaced the more classical methods such as culture as a means of pathogen detection, in particular for the more difficult organisms. Until the early 2000's, the literature on epitheliocystis was largely based on clinical observations, gross and histopathology and electron microscopy, after which it was replaced by molecular detection and phylogenetic studies. Molecular techniques to detect pathogens in the gills were applied during a longitudinal study investigating the aetiology of PGI (Steinum *et al.*, 2010), to detect the microsporidean *Desmozoon lepeophtherii* (Freeman & Sommerville, 2009) and '*Ca. P. salmonis*'. Although the microsporidean was present in approximately 80% of fish without PGI, a higher load of the pathogen was found in fish with PGI, and a role for the parasite in

development of the disease was suggested.

This quantitative effect was also observed with '*Ca. P. salmonis*', the bacterial load increasing mirroring the severity of the PGI. The possibility that diseased and compromised tissues allow these microorganisms to reproduce rather than act as primary pathogens was not addressed, but should be considered. Regrettably the demonstration of causality will always be difficult without robust disease models to test the pathogenic potential of different organisms. In addition to these two microorganisms, '*Ca. B. cysticola*' was also detected in healthy fish, at lower levels than in diseased fish. The mere presence of potentially pathogenic organisms must be always interpreted with caution.

The diagnosis of infectious disease should be based not on the detection of microorganisms alone, but should result from a combination of diagnosis and clinical presentation, in conjunction with gross and histopathological observations. Fish diseases, in particular gill disorders, may be of multifactorial aetiology and for this reason over-reliance on the results of specific molecular testing can be misleading. A similar example, where the link between pathogen and disease is not clear, is the case of the recently discovered Piscine Reo-virus (PRV), thought to be responsible for causing the economically important disease of Atlantic salmon in Norway, heart and skeletal muscle inflammation (HSMI). PRV has been found in farmed fish that do not show clinical signs of HSMI. Moreover, PRV has been also detected in wild Atlantic salmon. Nonetheless, based on the tissue distribution and load of PRV in naturally and experimentally infected salmon, the authors claim a significant correlation with disease (Palacios *et al.*, 2010). Although not considered by the authors, again it is feasible that viral replication may be merely facilitated by compromised diseased tissues. Techniques such as pyrosequencing which are now being more frequently applied, are likely to increase our discovery of new "pathogens" in the future, however, their significance should be interpreted with caution until causality can be established.

The impact of environmental parameters such as phyto- and zooplankton is substantial both in terms of being directly harmful to the gills but also in predisposing the gills to secondary infectious agents. The pathophysiology and pathogenesis when gills are exposed to harmful zooplankton and phytoplankton have not been extensively explored. Baxter *et al.* (2011) recently described the effects of a challenge trial with *Aurelia aurata* on

Atlantic salmon. Significant pathology in the form of epithelial sloughing, necrosis and haemorrhage were observed, mediated by nematocyst damage. This was mirrored by a recently reported clinical event in Donegal, Ireland (Mitchell *et al.*, 2011). Prior to these findings the adult stages were *Aurelia aurata* were assumed to be harmless to fish, with the only reports of mortality being caused by suffocation by massive numbers of the tiny ephyra stages of this jellyfish. Challenge trials to identify which species of zooplankton are noxious would be highly beneficial, in addition to establishing what densities of the different species are required to cause significant pathology.

The gill health longitudinal study (described in Chapter 3) was performed to explore if a relationship between different environmental and infectious agents, in particular epitheliocystis, emerged over time. Unfortunately, levels of epitheliocystis remained low during the study period, only fluctuated slightly and did not appear to be associated with any pathology. However, a zooplankton swarm did have a significant effect on the gill pathology, and caused high levels of mortality in the fish on the site. When describing the epidemiology from the study, there was no evidence of infectious spread of the gill disease through the population. Such a phenomenon had been described by Rodger *et al.* (2011). They observed gill disease clearly spreading from pen to pen in an epidemic pattern characteristic of an infectious disease. This infectious spread of disease was not observed during any outbreaks studied by this author (fish sampled from Irish farms 1 and 2, Chapter 3). On these sites, all pens appeared to be affected simultaneously, even though an infectious agent was involved on both farms (both sites had many fish with high levels of epitheliocystis in conjunction with proliferative gill disease). The reasons for this remain to be explained. The case described by Rodger *et al.* (2011) involved a heavy burden of parasites which may have resulted in different transmission dynamics. No evidence for infectious spread of PGI has been described to date. More in depth descriptive epidemiology of gill disease in aquaculture would be beneficial.

In recent years greater interest in water-borne irritants such as plankton blooms and zooplankton swarms has led to the establishment of research projects that aim to increase scientific knowledge and be of benefit to science and industry. Examples of such initiatives include the GILPAT project in Ireland and others such as ECOHAB, a USA national research

plan, GEOHAB, an international project concerned with Global Ecology and Oceanography of Harmful Algal Blooms, EUROHAB a European Commission funded initiative, and EUROGEL (European Gelatinous zooplankton: mechanisms behind jellyfish blooms and their ecological and socio-economic effects).

Many of the potential mitigation strategies against these water-borne irritants in aquaculture are in the early stages of development and are insufficiently advanced to ensure their safe use, although examples such as the underwater sensor developed by a team at NOAA's National Centres for Coastal Ocean Science and the Monterey Bay Aquarium Research Institute (Scholin *et al.* 2009) and the physical-biological model developed by a team at the Woods Hole Oceanographic Institution (McGillicuddy *et al.* 2003) hold much promise. Development of early warning systems and the employment of oceanographic tracking models would be advantageous to predict deleterious coastal blooms. Such advance warning would allow for a wider choice of protective strategies and for greater time to put the chosen strategy in place. Devices such as LOKI (Lightframe Onsite Key species Investigation) have been developed with the aim of enabling the study of zooplankton distribution in direct relation to environmental parameters (Mengedocht *et al.* 2007).

Methods for the rapid identification of harmful zooplankton at farm level, especially the small species and hydroids, will be an essential part of increasing the general awareness of the impact of these organisms. Mitigation strategies need further investigation. Although cessation of feeding and oxygenation are two common strategies employed by aquaculture operations in the face of a phytoplankton bloom / zooplankton swarm, the effects of these strategies have never been properly quantified and require further research. Finally, investigation of the synergies between pathogens and these non-infectious agents in the progressive development of gill disease would be very beneficial in increasing our comprehension of gill disorders as multifactorial entities.

Scoring and grading is widely employed in human and veterinary histopathology. It is a useful tool in terms of quantification of disease, enabling management / treatment decisions and for comparative analysis during investigative studies and research. A novel histopathological gill scoring system was successfully developed and applied during this

work. Histopathology will always remain a subjective science and opinions frequently differ. Standardisation of different scoring methods would therefore be useful and attempts should be made to use published methods where they exist, as long as they are adequate for the task, to enable meaningful comparisons between different studies and cases.

The term proliferative gill inflammation (PGI) was first introduced by Kvellestad *et al.* (2005) to describe a gill disease syndrome frequently observed in seawater-farmed Norwegian Atlantic salmon. Diagnosis is based on four co-localized histopathological changes; inflammation, epithelial hyperplasia, cell death and circulatory disturbances. The Norwegians view PGI as a defined disease but the criteria define more of a syndrome than a disease. The case definition is currently obscure. Case definitions frequently include clinical, epidemiological and laboratory criteria but can be any combination of the above. Some clinical syndromes do not have confirmative laboratory tests and are diagnosed based on clinical and epidemiological criteria, whereas some require laboratory confirmation for diagnosis regardless of the clinical symptoms. The clinical signs of PGI have not been properly described in the literature, nor has the case definition included any laboratory diagnostics for the different pathogens that are potentially involved. Diagnosis is based partly on epidemiology, but mostly on the four pathological criteria described above. The issue with using these as a means of diagnosing PGI is the fact that the gill frequently responds to injury in the same way regardless of the insult, and these criteria are not really unique enough to define a particular disease. A better clinical description would help to clarify the case definition. For these reasons, there has been some reluctance to diagnose PGI in similar cases of proliferative disease in Ireland (personal communication, Hamish Rodger).

Perhaps it would be more useful to consider PGI as a syndrome akin to pneumonia in humans and animals, merely a term to describe inflammation of the respiratory tissues, manifested clinically as increased respiratory rate, flaring of the operculae, gasping at the water surface and any other clinical signs that represent serious gill compromise. Pneumonia in humans and animals is a good analogy for proliferative gill disease in fish. It can be caused by viruses, bacteria, parasites, fungi or physical and chemical agents. It can be acute or chronic and frequently has a multifactorial aetiology with a number of different

agents involved. There are risk factors that contribute to the susceptibility of the animal, namely the health status of the host itself, the environment and the presence of one of the aforementioned pathogens. For intensively reared livestock for instance, stress is one of the main precipitators of disease (Radostits *et al.*, 2009). The same principles and aetiologies can also be applied to fish. Radostits *et al.* (2009) states that in terrestrial animals viruses often precede bacteria in pneumonic disease. Although Steinum *et al.* (2010) did not find any concrete indications of a viral aetiology of PGI, further investigations into whether other novel undiscovered viruses in fish may predispose to proliferative gill diseases would be interesting.

From the freshwater longitudinal studies, it seems that epitheliocystis caused by '*Candidatus* *Clavochlamydia salmonicola*' does not appear to be associated with any pathology. None of the fish displayed any histopathology of significance adjacent to the cysts, nor was there any evidence of pathology post-seawater transfer while the cysts persisted for up to six weeks. It is unlikely that this bacterium is of pathological significance in Atlantic salmon. However, the possibility remains that there may be some synergistic effects if it occurs in combination with other infectious agents or certain environmental conditions. Given that epithelial turnover in the gills is estimated at 7 – 8 days (Zenker *et al.*, 1987) it was surprising that the infections persisted for up to 6 weeks post seawater transfer before entirely disappearing, indicating that the *Chlamydiae* involved may have some mechanism of arresting cell turnover or interfering with apoptosis. Chlamydial infections in humans with both the important pathogens, *Chlamydia trachomatis* and *Chlamydia pneumoniae*, protect the host cell against apoptotic stimuli by degradation of proapoptotic BH-3 only proteins (Fischer *et al.*, 2004). This is likely to be an important mechanism for the ability of *Chlamydia* to reproduce within cells, and a similar means may also be employed by '*Ca. C. salmonicola*' to prolong the life of the infected cell. This phenomenon was again observed during the freshwater challenge trial, although on this occasion all evidence of infection had disappeared by three weeks post transfer of the salmon into mains freshwater. These findings strongly suggest that an intermediate host or some other factor, only found in the natural lake / river system housing the fish, may be essential in propagation of infections.

Creation of a challenge model for epitheliocystis was attempted, without success, during the study. Challenge models for different diseases are crucial to enable us to establish causality and understand mechanisms of infection, for examining interactions of different factors or variables, for exploring treatment regimens and their efficacy, and potentially helping us to develop treatments or even vaccines for disease prevention. The establishment of a challenge model that is an accurate reflection of a particular disease is laborious, and frequently the models developed fail to deliver a comparable picture to that created during natural infections. However their value cannot be contested in furthering our understanding of the dynamics of infectious diseases, and further efforts should be made in the future to create a model for epitheliocystis, perhaps using a different cyst-forming agent and alternative challenge technique.

Gill disease is a broad and complex topic. An extensive review of both published literature and unpublished data supplied by fish veterinarians, fish health professionals and biologists revealed that methods of disease prevention and effective treatment and mitigation strategies are currently very limited. Both infectious and environmental causes of gill disorders need to be better elucidated. There is extensive scope for further research in these areas. A very important fact that emerged during this work is that many types of gill disease are potentially of multifactorial aetiology. Synergisms between infectious agents are not well understood, nor are potential interactions between these agents and environmental parameters such as phytoplankton blooms and zooplankton swarms. Comprehending these interactions needs to be prioritized in order to gain a meaningful understanding of gill disease, and to formulate effective preventative, therapeutic and mitigative strategies to reduce the impact on the salmonid farming sector.

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8 Appendix – Publications arising from this thesis

1. Mitchell, S.O., Steinum, T., Rodger, H., Holland, C., Falk, K. and Colquhoun, D. J. (2010). Epitheliocystis in Atlantic salmon, *Salmo salar* L., farmed in fresh water in Ireland is associated with “*Candidatus* Clavochlamydia salmonicola” infection. *Journal of Fish Diseases*, **33**, 665 – 673.
2. Mitchell, S.O. and Rodger, H.D. (2011). A review of infectious gill disease in marine salmonid fish. *Journal of Fish Diseases*, **34**, 411-432.
3. Mitchell, S.O., Baxter, E.J. and Rodger, H.D. (2011) Gill pathology in farmed salmon associated with the jellyfish *Aurelia aurata*. *Veterinary Record*, **169 (23)**, 609.
4. Mitchell, S.O., Baxter, E.J., Holland, C. and Rodger, H.D. (2012) A novel histopathological gill scoring protocol for assessment of gill health during a longitudinal study in marine-farmed Atlantic salmon (*Salmo salar*). *Aquaculture International*.
5. Rodger, H.D., Henry, L. and Mitchell, S.O. (2011). Non-infectious gill disorders of marine salmonid fish. *Reviews in Fish Biology and Fisheries*, **21(3)**, 423-440.
6. Rodger, H. D., Murphy, K., Mitchell, S. O. and Henry, L. (2011). Gill disease in marine farmed Atlantic salmon at four farms in Ireland. *Veterinary Record*, **168 (25)**, 668.
7. Toenshoff E. R., Kvellestad A., Mitchell S. O., Steinum T., Falk K., Colquhoun, D. J. and Horn, M. (2012). A Novel Betaproteobacterial Agent of Gill Epitheliocystis in Seawater Farmed Atlantic Salmon (*Salmo salar*). *PLoS ONE* 7(3):e32696.doi:10.1371/journal.pone.0032696.