Gill diseases in marine salmon aquaculture with an emphasis on amoebic gill disease

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Abstract

Gill diseases are a growing health challenge in salmon farming worldwide, but many gaps remain in our knowledge. Gill diseases are generally complex and multifactorial disorders, often with presumable spatial and temporal distribution patterns, but are highly difficult to effectively prevent and control. The term complex gill disease (CGD) includes a wide range of clinical disease presentations on the gills of farmed salmon; usually, CGD presents from the end of summer to early winter. The pathogens involved include *Neoparamoeba perurans*, *Tenacibaculum maritimum*, Candidatus *Piscichlamydia salmonis*, Candidatus *Branchiomonas cysticola*, *Desmozoon lepeophtherii* (syn. *Paranucleospora theridion*) and viruses, such as the Atlantic salmon paramyxovirus (ASPV) and salmon gill poxvirus (SGPV). Amoebic gill disease (AGD) is perhaps the most significant disease in terms of gill health and economic impact. AGD results in high mortality, reduced production performance and impaired fish welfare. This review summarizes and analyses CGD research, outbreaks and treatment, with a focus on AGD, as well as on knowledge gaps and avenues for future research.

Keywords: Complex gill diseases, Salmon farming

Review Methodology: We searched the PubMed and CAB Abstracts databases (keyword search terms used: gill, infectious, diseases, salmon). In addition, we performed a general search of all keywords using EndNote X9 software (Clarivate Analytics, Boston, MA, USA).

Introduction

The gill is a vital multifunctional organ that provides gas exchange and assists in osmotic and ionic regulation and the excretion of nitrogenous waste. Gill disorders represent a significant challenge to salmon producers worldwide and are a cause of high direct and indirect losses [1]. The gills are mainly predisposed to pollutants, environmental changes and parasitic, bacterial and viral infections because they are in direct contact with the environment. Gill diseases are generally complex and multifactorial disorders, often with presumable spatial and temporal distribution patterns, but they are very difficult to effectively prevent and control. Compromised gill function can lead to significant economic losses due to poor food conversion performance, direct mortalities and the cost of treatment.

The difficulty in establishing the cause of multifactorial gill disease has resulted in an inconsistent classification. Amoebic gill disease (AGD), caused by *Neoparamoeba perurans* (syn. *Paramoeba perurans*), is perhaps the most significant disease in terms of gill health and economic impact [2–6], but several other pathogens are also potentially associated with gill disease [7]. The term 'proliferative gill inflammation' (PGI) has been used to describe recurrent gill disease outbreaks that occur in autumn in salmon farms in Norway [8, 9]. Similar pathologies to PGI also have been described as occurring in Scotland and Ireland [10]. 'Proliferative gill disease' (PGD) is used as a nonspecific term derived from the appearance of gross lesions in the salmon gill. In Scotland, gill disease occurs during the same season as in Norway and has been referred to as PGD due to the proliferative histological features and uncertain aetiology. Gill disease

Table 1 Pathogens associated with gill disease in marine salmon aquaculture

Pathogen	Salmon species	Pathology/ syndrome	Geographical distribution	References
Neoparamoeba perurans	Atlantic salmon, rainbow trout and coho salmon	Amoebic gill disease (AGD)	Australia, Norway, Chile, Scotland, Ireland, USA and Canada	[21] [22] [23] [2] [4] [24, 25] [26] [27]
Candidatus *Branchiomonas cysticola*	Atlantic salmon	Epitheliocystis	Norway and Ireland	[28] [29]
Candidatus *Piscichlamydia salmonis*				[30]
Desmozoon lepeophtherii	Atlantic salmon	Complex gill disease (CGD)	Norway, Scotland and Ireland	[31] [32] [33]
Atlantic salmon paramyxovirus (ASPV)				[34, 35]
Salmon gill poxvirus (SGPV)				
Tenacibaculum maritimum	Atlantic salmon, rainbow trout, coho salmon and chinook salmon	Tenacibaculosis	Australia, Norway, Chile, Scotland, Ireland, USA and Canada	[12] [13] [14] [15]

presentation in Scotland is considered to be virtually synonymous with PGI in Norway, but with a less pronounced inflammatory response and inconsistent vascular changes. Similar gill diseases have been reported in Ireland, Chile and Canada [11].

Complex gill disease (CGD) is the term currently used to refer to this varied syndrome of probable multifactorial aetiology and variable histopathology, and the term encompasses the syndromes referred to as PGI or PGD in previously published articles [11]. CGD includes a wide range of clinical disease presentations on the gills of farmed salmon, and these diseases usually appear from the end of summer to early winter, and environmental insults from phytoplankton and/or zooplankton are frequently involved.

Pathogens Involved in CGD

Table 1 summarizes the main pathogens associated with gill diseases in marine salmon aquaculture. Bacteria such as *Tenacibaculum maritimum* are the causative agents of tenacibaculosis, gill rot and gliding bacterial diseases [12–16], and associated gill lesions were first described in chinook salmon *Oncorhynchus tshawytscha* [15]. Gill infections due to tenacibaculosis tend to present in lethargic fish with an increased respiratory rate and increased mucus on the gills, along with pale patches of necrosis [12–15]. Preliminary diagnoses of symptomatic fish are carried out via a microscopic examination of affected tissue that shows motile filamentous bacteria [12–15]. Further definitive confirmation should be performed through the isolation of bacterial colonies or using molecular diagnostics [16–18]. Transmission of the bacterium can be through seawater or directly from host to host [16]; however, jellyfish (e.g. *Phialella quadrata* and *Pelagia noctiluca*) may act as a vector for this pathogen [19, 20]. Atlantic salmon have been found to be particularly susceptible to tenacibaculosis, with fish age (juvenile) and temperature (above 15 °C) being identified as risk factors [16].

Epithelial cysts in the gills of seawater-farmed Atlantic salmon are a common finding during gill disease outbreaks [30] and have been associated with various putative pathogens, including the bacteria Candidatus *Branchiomonas cysticola* and Candidatus *Piscichlamydia salmonis* [28, 36]. Epitheliocystis is characterized by the development of inclusions/cysts in the branchial epithelium in addition to chloride cells [28, 29, 37] and has also been documented as occurring in skin epithelial cells [38]. The pathology in gills associated with epitheliocystis includes hyperplasia, lamellar fusion and focal necrosis of epithelial cells [30].

A number of bacterial agents are associated with epitheliocystis in salmonids [39]; however, Candidatus *B. cysticola* has recently been identified as a potential agent of epitheliocystis in marine-cultured Atlantic salmon [28]. A molecular study found Candidatus *B. cysticola* at a far greater density in fish with large numbers of epithelial cysts; in addition, *in situ* hybridization (ISH) allowed identification of the agent within cysts, which indicated a potential role for the agent in gill disease [30]. Some studies have indicated that epitheliocystis is merely coincidental, whereas others have observed it during PGI outbreaks with associated mortality [8, 9]. Candidatus *P. salmonis* has

previously been associated with gill disease. However, the role of Candidatus *P. salmonis* is still relatively unclear, particularly with respect to whether it is a primary or secondary pathogen [36].

A microsporidian parasite, *Desmozoon lepeophtherii* (syn. *Paranucleospora theridion*), has recently been described [31, 40]. *D. lepeophtherii* is believed to have a complex life cycle involving both *Lepeophtheirus salmonis* and Atlantic salmon [31], although salmon have been found to be infected with the microsporidian in the absence of lice [41]. The true significance of this parasite as a gill pathogen is still unclear as it is frequently the most prevalent agent detected in gill samples, even in gills with no reported pathologies [7, 9]. However, it was present in fish with PGI at up to 30 times the levels observed in unaffected fish in one study [9], with a four-fold increase in another [7].

In a case from Scotland, *D. lepeophtherii* was the apparent causative agent of the gill disease outbreak recorded which was associated with a distinct proliferative and necrotic pathology [10]. *D. lepeophtherii* may encourage immune suppression, thereby increasing the susceptibility of the host as well as facilitating the proliferation of pathogens already present in the fish [7, 31]. The densities of the microsporidian appear to be influenced by environmental conditions, with higher densities being recorded during periods with the highest temperatures [7, 41]. Further work is required to fully understand the relationship between the marine environment and potential gill disease pathogens [10]. The role of other parasites during CGD, such as *Parvicapsula pseudobranchiola*, *Ichthyobodo* spp. and *Trichodina* spp., may accentuate gill disease, but these parasites appear to be secondary pathogens [11].

Atlantic salmon paramyxovirus (ASPV) and salmonid gill poxvirus (SGPV) have been identified as having some association with gill disease in Atlantic salmon, but their effect remains relatively unclear. In 1995, a previously undescribed virus belonging to the paramyxoviridae genus was isolated from the gills of Atlantic salmon suffering from PGI and was named ASPV [32]. However, subsequent infection trials failed to determine the pathology or mortalities in disease-free salmon, but the virus was associated with two cases of mortality in salmon farms in Norway [32]. Further studies examining the multifactorial aetiology of PGI found no evidence for the involvement of ASPV [31].

During a number of outbreaks of PGI in Norway, a DNA virus, SGPV, was first observed to infect epithelial cells, causing hypertrophy and the degeneration of the nucleus, in addition to causing 20 and 80% mortality in freshwater and marine sites, respectively [32, 42]. During the outbreak in the marine site, *Neoparamoeba* sp. was also present, which may have contributed to the mortality [31, 39, 42].

The effect of SGPV appears to be greatest when recorded during freshwater production, and when it coincides with smoltification, significantly increased levels of mortality have been recorded as the infection particularly affects the gills and chloride cells [34, 35]. With advances in molecular techniques, SGPV has been shown to be far more widely distributed than previously believed and is often found in addition to a number of other pathogenic agents [35], which further highlights the multifactorial nature of gill disease.

In Ireland, Downes *et al*. [43] observed that between 12 and 16 weeks after seawater transfer, colonization of the gills by *D. lepeophtherii*, Candidatus *B. cysticola*, *T. maritimum*, SGPV and *N. perurans* commenced, and by week 16 of marine production, each of the pathogens was detected. *D. lepeophtherii* and Candidatus *B. cysticola* were by far the most prevalent of the potential pathogens detected. *T. maritimum* was found to be significantly correlated with temperature, thereby showing distinct seasonality. SGPV was found to be highly sporadic and was detected in the first sampling point, suggesting a carryover from the freshwater stage of production. The results of model indicated no clear effect between any of the pathogens, but the models showed that the only variable that had a consistent effect on the histology score was *N. perurans*.

However, noninfectious disorders due to harmful algae blooms (HABs) and other challenges also play roles in the mortalities attributable to CGD [43]. Several species of marine phytoplankton have been recorded to be associated with fish mortalities, including *Karenia mikimotoi*, which has been implicated in Atlantic salmon mortalities in Ireland, Scotland and Norway [44–47]. A HAB of *Pseudochattonella* cf. *verruculosa* during the 2016 austral summer (January–March) killed nearly 12% of Chilean salmon produced, causing the worst mass mortality of fish and shellfish ever recorded in the coastal waters of western Patagonia [48]. The algae bloom coincided with a strong El Niño event and the positive phase of the Southern Annular Mode that altered the atmospheric circulation in southern South America and the adjacent Pacific Ocean. These events led to very dry conditions and allowed higher than normal solar radiation to reach the surface. Using a time series of atmospheric, hydrologic and oceanographic data, León-Muñoz *et al*. [48] showed that an increase in the surface water temperature and reduced freshwater input resulted in a weakening of the vertical stratification in the fjords and sounds of this region, which allowed the advection of more saline and nutrient-rich waters, ultimately resulting in an active harmful algal bloom in coastal southern Chile.

Although the worldwide occurrence of severe HABs in recent decades suggests a connection with anthropogenic climate change [49], the causal link needs to be established at the regional scale [50]. Temperature has been identified as the most important environmental factor shaping the structure of ocean plankton communities [51]. Although the occurrence of HABs is controlled by multiple processes, temperature is a central organizing factor that determines the potential for HABs to occur [52]. The continuance of ocean warming through the twenty-first century will promote the intensification and redistribution of HABs around the world [53].

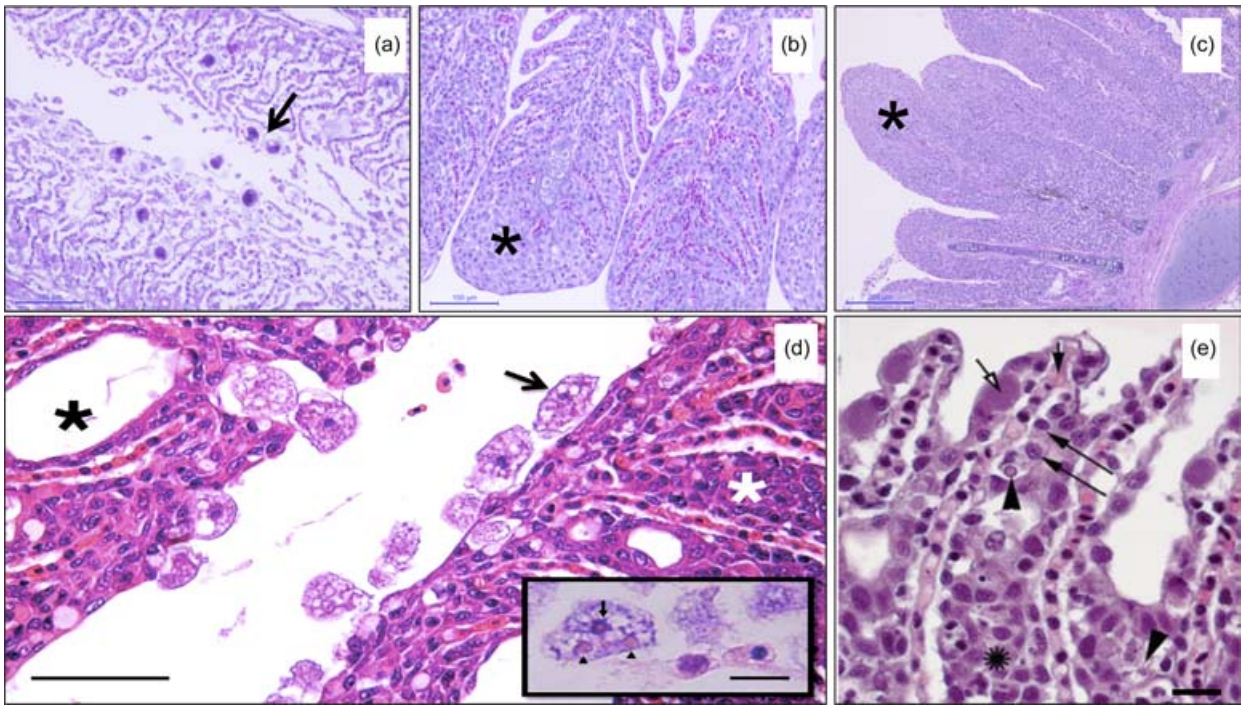

Figure 1. CGDs in Atlantic salmon. (a) Note the presence of microalgae organisms (arrow) retained in mucin and cellular detritus in the gill interlamellar space (H&E, 100 µm). (b, c) The pathology in the gills associated with PGD includes hyperplasia, lamellar fusion (black asterisk), focal necrosis of epithelial cells and mucous cells hyperplasia (H&E, 100×). (d) Gill lesion from an AGD-affected fish. Epithelial gill hyperplasia with an attached amoeba (black arrow), secondary lamellae fusion (white asterisk) and presence of interlamellar vesicles (black asterisk) (H&E, 80 µm, Rozas *et al*. [55]). Magnification showing the details of the amoeba nucleus, which has an amphiphilic core surrounded by an irregular basophilic ring (black arrow), and parasomes appeared within the eosinophilic cytoplasm (black head arrow) (H&E, 20 µm). (e) Gill pathology associated with PGI included presence of fibrin and/or dead cells in the lamellar blood vessels (short filled arrow), hyperplasia of epithelial cells (black asterisk), death of cells (filled arrowhead), and inflammatory cells (long filled arrow) in the epithelium. Many epitheliocysts is present (short unfilled arrow) (H&E, 20 µm [9]).

Rozas *et al*. [54] officially monitored gill diseases under commission by the National Fisheries and Aquaculture Service (Sernapesca) during the summer algae bloom of 2016. A histological study was conducted on 182 surviving fish sampled from 60 seawater farms during and after the algal bloom (February–April). The histological lesions observed were grouped into cellular changes (degeneration and necrosis) (36.26%; 66/182 fish), growth disorders (epithelial hyperplasia, mucosal cell hyperplasia and eosinophilic granular hyperplasia) (67.58%; 123/182 fish) and circulatory disorders (telangiectasia, thrombosis and haemorrhage) (53.29%; 97/182 fish).

Initially (February–March), most of the fish presented acute circulatory and cellular changes and the presence of microalgae (76.05%; 108/142 fish) (Figure 1), probably associated with the algae bloom, whereas in the last samples (April), lesions were observed in the process of recovery; these lesions included chronic progressive lesions associated with biological agents, and specifically, an increase in the frequency of *N. perurans* (77.5%; 31/40 fish) was observed [53].

Finally, Bloecher *et al*. [56] recently confirmed the negative impact that the hydroid nematocyst *Ectopleura larynx* can have on salmon gill health and highlighted the potential risks that net cleaning poses to fish welfare. However, *in situ* measurements of gill health before and after net cleaning conducted in the field are necessary to validate these findings. Both laboratory and field research should examine situations in which salmon are subject to repeated exposure to cnidarian cleaning waste at realistic intervals.

Amoebic Gill Disease

AGD is caused by the parasitic amoeba *N. perurans* and affects Atlantic salmon gills [21]. The disease was first identified in the mid-1980s, when it infected salmonids farmed in Washington State, USA and Tasmania, Australia [55]. AGD has also been reported in Chile, Canada, Norway, Scotland, Faroe Islands and Ireland [2, 4, 21–27, 55] (Table 2). After 2010, the occurrence of AGD in farmed Atlantic salmon has significantly increased in the Northeast Atlantic, initially in Ireland and Scotland in 2011–2012 and later northwards to the Orkney Islands in Shetland, Norway and the Faroe Islands in 2012–2013 [6]. In Norway, AGD was observed for the first time in association with health problems in farmed Atlantic salmon

Table 2 Salmonid species in which *N. perurans* infections have been verified, either by ISH or by PCR (conventional or real-time PCR). All verified *N. perurans* infections represent farmed fish diagnosed with AGD

Host/Country	Agent detection	References
Atlantic salmon		
Australia (Tasmania)	ISH, PCR	[21, 23]
United States of America	ISH, PCR	[23, 57]
Chile	ISH, PCR	[4, 24, 25]
Ireland	ISH	[22, 23]
Scotland	ISH	[23, 58]
Norway	PCR	[2]
Canada	PCR	[27]
Faroe Islands	PCR	A. K. Olsen (personal communication) in [6]
Rainbow trout		
Australia (Tasmania)	ISH	[23]
Chile	PCR	[24, 25]
Norway	PCR	[26]
Chinook salmon		
New Zealand	ISH	[23]
Coho salmon		
Chile	PCR	[24, 25]

at four sites in the autumn of 2006 [2]. Bustos *et al.* [4] described AGD in Atlantic salmon farmed in Chile and confirmed that *N. perurans* was the causal agent.

During an AGD epizootic in Chile, rainfall lower than the 15-year average was recorded from May to November 2007, which was believed to be the most likely environmental factor for the timing of the outbreak [4, 24, 25]. The prevalence of AGD in Atlantic salmon farms was 55.7% (29/52 farms), and the epidemic curve was observed between May 2007 and June 2008, closely related to low rainfall and high salinity (>32‰) [4, 24, 25]. Fish weighing more than 300 g reared in the Los Lagos Region during summer and autumn showed a 3.7 ($P = 0.0004$), 4.2 ($P = 0.0178$) and 6.2 ($P = 0.0031$) times greater risk of being AGD positive, respectively [24]. The reduction in Atlantic salmon biomass reared in Chile was closely related to the infectious salmon anaemia outbreaks, which may have considerably increased the infection pressure of *N. perurans* on rainbow trout (63.2%, 12/19 farms) and coho salmon (90.9%, 10/11 farms) [25].

The occurrence of AGD in Ireland over the 2011/2012 period presented some unique challenges for the Irish salmon industry, in particular, a shortage of well boats for treating infected fish and permission for the use of water sources by local authorities. Farms in Tasmania, which are located at sites with a strong influence of fresh water due to high levels of rainfall or with a strong freshwater input, are less impacted by AGD [59]. However, AGD has also been observed in farms in Tasmania at temperatures of 10.6 °C and salinity of 7.2 ppt [60].

Outbreaks of AGD reported in Norway and Scotland were described as being associated with higher water temperatures [2, 26]. The temporal and spatial distribution of AGD cases in Chile may be closely related to the ubiquitous nature of *N. perurans*, low rainfall and high salinity [4, 24, 25]. The increased AGD outbreaks during summer may not be solely due to increased thermal stress in fish but also to increased amoebae attachment at 15 °C, which causes an increased gill pathology [60].

The agent

The distinguishing feature separating *Paramoeba*, *Neoparamoeba* and *Janickina* from other species of amoeba is the presence of the endosymbionts or parasomes from the family *Paramoebidae*; an exception is the *Paramoeba eilhardi*, which sometimes lacks parasomes [61, 62]. When in motion, trophozoites of the genus *Paramoeba* and *Neoparamoeba* usually possess several dactylopodia. A comparative study completed by Dyková *et al.* [63] acknowledged the importance of molecular characterization, as differentiation between amoebae at the morphological level is almost impossible.

Although recent molecular evidence suggests that the genera *Neoparamoeba* and *Paramoeba* are paraphyletic and could be synonymized [64]; however, doing so would be premature, as nuclear SSU rDNA is highly conserved and insufficient by itself to formalize such a change [65, 66]. Therefore, until more scaled amoebae are sequenced and genes other than SSU rDNA are investigated, evidence is insufficient to change the currently used nomenclature [67–69].

The phylogeny of the amoebae associated with the Chilean epizootic was examined using the 18S rRNA gene, and these findings were compared with 18S rRNA gene sequences from 46 isolates of *Neoparamoeba* and an outgroup [4]. Phylogenetic analysis of the Chilean gene (GQ407108) sequence found that it clustered with the Australian and Norwegian isolates (EU326494) with 98.4–99.2 and 99.6% similarity, respectively, which suggests that *N. perurans* has a universal distribution [4].

For many years, advancement of research into the aetiology of AGD was inhibited due to the inability to culture the causative agent [5, 6]. However, Crosbie *et al.* [70] completed the isolation and *in vitro* culture of *N. perurans*, and they were therefore able to fulfil Koch's postulates. The culture was maintained using malt yeast agar with seawater overlaid and subcultured every 3–4 days, from which a clonal culture was established. After 70 days in culture, a clone successfully infected Atlantic salmon, causing AGD, which was subsequently reisolated and confirmed by polymerase chain reaction (PCR) and ISH [70].

Risk factors

Amphizoic marine amoebae are believed to be ubiquitous in the environment, whereas the role of reservoir

populations, a possible mechanism of transmission to and among farmed fish for many disease-causing amoebae, has not been fully elucidated [57, 71]. Amoebae that can cause parasitic infections in farmed fish are known to be free-living in the environment and may alter their life strategies given the correct circumstances [72].

Such infections are generally poorly understood but may occur due to adverse impacts on hosts from environmental stress factors, in particular, elevated temperatures, salinity or the initial insult from zooplankton, which can leave the gills susceptible to infection [11]. *N. perurans* is transmitted from fish to fish through the water, but information regarding the dynamics of this spread is lacking.

Water, biofouling, sediments and salmon parasites have been tested for the presence of *N. perurans* to determine where the amoebae are located when they are not parasitic [69]. Concentrations of *N. perurans* were found to be low in seawater from salmon farms in Tasmania [73–75] and Norway [76], even during AGD outbreaks, with maximum concentrations of up to 62.3 amoebae/l [73], which is much lower than the concentration used in challenge trials in the laboratory [57, 77]. The only time that any difference was observed in the abundance of *N. perurans* was during an AGD outbreak in early autumn (2014), when *N. perurans* was more common in surface waters than at other depths [73]. The abundance of *N. perurans* in the water column was affected by changes in salinity following rainfall, where amoebae were present only at higher salinities in deeper waters and no obvious relationship existed between the depth distribution of *N. perurans* and Atlantic salmon [74].

There is no scientific evidence that sea lice have a significant role in spreading *N. perurans*. However, while *N. perurans* was recovered from sea lice in the USA [57], it was not recovered in Norway. During an AGD epizootic outbreak in Chile, exceptionally high levels of coinfection with *Caligus rogercresseyi* may have contributed to the observed mortality [4]. A heavy infestation of salmon lice may influence a case of AGD by increasing the burden on an already weakened fish [4].

Paramoeba spp. have been detected in the gills of wild couta, *Thyrsites atun*, caught in the vicinity of Atlantic salmon farms [76]. As greater emphasis is placed throughout the industry on the reduction of medicinal treatments for sea lice, a renewed interest in these cleaner fish as biological controls has emerged [77, 78]. The identification of *N. perurans* on the gills of cleaner fish species such as lumpsuckers (*Cyclopterus lumpus*) and wrasse (*Labrus bergylta*) is a major concern to the industry, as these cleaner fish may act as potential reservoirs or asymptomatic carriers and can infect to naive Atlantic salmon [76, 79, 80].

The pathogenesis of reinfection in the post-treatment period has been found to be identical to that of the initial infection, although the source of the reinfection was not identified [81]. Potentially, the treated salmon themselves may be the main source of reinfection, as some amoebae remain on the gills following treatment [82]. Additionally, the potential impacts on AGD of alternative strategies against sea lice, such as 'snorkel cages' and the use of lights [83, 84], which encourage fish to spend more time in deeper waters, should be investigated. Recently, Wright *et al*. [74] showed that snorkel technology has a place in the toolkit of commercial salmon sea-cage farmers comanaging salmon lice and AGD outbreaks.

When amoebae are isolated from a natural environment, they invariably bring bacteria to the culture [85], even when they are surface disinfected. Although many *Neoparamoeba* isolates do not appear to be bacterivorous, if they are subcultured on a regular basis, bacteria are always present, based on transmission electron microscopy [63], and in some isolates taken from sediment, bacteria are present and multiply inside the amoebae.

Although paramoebae feed on bacteria, their relationship with bacteria may be more complex than just as a source of nutrition. Similar to other phagotrophic eukaryotes, *Neoparamoeba* and *Paramoeba* are exposed to foreign DNA, which provides opportunities for horizontal gene transfer [86]. Currently, no evidence exists to support the hypothesis that bacterial infection or the presence of nonpathogenic bacteria contributes to the severity of AGD [69]. This lack of evidence does not, however, mean that bacteria are not somehow involved. Egan and Gardiner [87] recently suggested that many diseases in the marine environment are the result of a microbiome disturbance or microbial dysbiosis. The possibility that some or all paramoebiases are caused by imbalances in the microbiome of the afflicted animal should therefore not be ignored. While fish microbiome research is progressing, the focus has been on the gut microbiome, with the 16S rRNA gene being used to explore bacterial diversity [88]; little is known about the microbiome of the gill. AGD can clearly be caused by exposure to *N. perurans*; however, neither the potential for coinfection nor the possibility that microbial dysbiosis contributes to disease susceptibility can be dismissed [87].

Environmental factors, such as temperature, and long-term trends, such as climate change, in particular, may also play a role in the increased effects of AGD on the salmon industry. In most geographical locations, this disease was first reported when temperature was above average [6]. Climate change has been suggested to serve as a trigger for microbial dysbiosis in the marine environment [87]. Increasing temperatures or other stressful environmental conditions will serve to stress marine hosts, thereby impairing the immune responses of the hosts, which can indirectly affect the composition of the host microbiome and trigger an imbalance that leads to a disease outbreak [87]. Microbial dysbiosis can also occur when environmental factors directly affect an animal microbiome.

Chalmers *et al*. [89] suggested that ploidy does not affect the manifestation or severity of AGD pathology or the serum innate immune response. Additionally, the serum immune response of diploid and triploid Atlantic salmon may not be significantly affected by AGD.

Pathology

AGD clinically manifests as lethargy, anorexia, congregation at the water surface and an increased ventilation rate [59, 60]. Preliminary diagnosis of the infection is often done through scoring of the white mucoid patches present on the gills of infected fish [58, 75, 90], and these scores have been shown to be good indicators of AGD when the checks are performed by an experienced examiner [24, 60, 75, 81, 91].

The pathology of AGD has been well defined and is characterized by localized host tissue responses, including epithelial oedema, hyperplasia of the epithelial cells as well as mucous cells, fusion of lamellae and the development of interlamellar vesicles [43] (Figure 1). Amoebae may also attach in the histological examination, and these amoebae should contain at least one *Perkinsiella amoebae*-like organism [2, 4, 21, 23–25, 60, 81, 91] (Figure 1), which is considered to be case defining [23–25, 60].

By transmission electron microscopy, Wiik-Nielsen *et al.* [92] observed enlarged swellings in affected gill filaments with fusion among adjacent lamellae, in addition to spherical amoebae, which appeared to be embedded within the epithelium and that subsequently left indentations with visible fenestrations. These fenestrated structures appeared to correspond with the presence of pseudopodia, which were observed to penetrate the epithelium.

Concentrations of amoeba from 10 to 500 amoeba/l have been documented as causing AGD in naïve Atlantic salmon, with the pathology observed in both gross and histological examinations appearing to be proportional to the concentration of amoeba initially used [93]. Differences in virulence between the amoebae extracted from AGD-infected fish and amoebae cultured *in vitro* have been recorded throughout research into AGD, and ideally, studies should be conducted using well-characterized strains of *N. perurans*. Some evidence exists to show that cultures maintained in a lab for extended periods of time have displayed differences in virulence based on gill score [94]. Furthermore, the clonal strain of *N. perurans* originally used to fulfil Koch's postulates [70] was found to have lost virulence after 3 years in culture [95].

Diagnosis

The most financially viable nondestructive means for diagnosing AGD at the commercial scale is through gross pathological assessment [24, 59, 60, 81] using various gill-scoring methods developed by Taylor *et al.* [3]. Tools such as gill scoring can be used to determine the severity of the AGD infection and the frequency of treatment [3, 5, 60, 81, 91]. However, this approach is a presumptive means by which to confirm the presence of AGD and is open to misinterpretation. The detection of lesions and patches only indicates an altered gill condition but lacks the ability to identify the causative agent [60, 81]. Lesions and patches on the gills do not always coincide with AGD in salmon and are less reliable in the early stages of an infection [23, 24].

Histology has been one of the primary methods for identifying and diagnosing the causal agent, and it has also been utilized in the investigation of the host response [60, 81]. Mitchell *et al.* [96] developed a histopathological gill scoring method that assigned a score of 0–3 for each parameter associated with changes in gill health, including lamellar oedema, lamellar hyperplasia, lamellar fusion and circular anomalies (necrosis and sloughing).

Rozas *et al.* [24] showed a moderate concordance level ($k = 0.5319$) between gross pathology and histology. The sensitivity and specificity of the gross pathology was 77.91 and 71.05%, respectively. Although gross and histological screenings have provided valuable tools to the industry for the regulation of AGD, they are still limited in their capacity to identify the infectious agent [60, 81, 91, 96].

A number of laboratory techniques have been developed to confirm AGD in presumptively diagnosed fish [97–99]. Following the identification of *N. perurans*, Young *et al.* [97] developed a PCR assay that amplified a 636-bp region of the 18S rRNA gene. Further investigation allowed for the development of ISH using oligonucleotides that bind to the 18S rRNA gene, and this technique was utilized to confirm that *N. perurans* was the predominant aetiological agent of AGD in Tasmania, despite other amoebae species being previously associated with the disease [97]. The 18S rRNA gene is generally chosen due to its high copy number, which allows for high sensitivity, and the 18S rRNA gene is an established marker for microbial identification, with a database of species-specific sequences [75]. This assay was found to be specific and highly sensitive for the detection of *N. perurans* in gill samples and isolates of noncultured gill-derived amoebae.

Bridle *et al.* [75] developed and validated a real-time PCR assay using SYBR® Green chemistry and an iQ5 Real-Time PCR detection system (Bio-Rad NSW, Australia). The primers used in this assay amplified a 146-bp portion of the 18S rRNA gene from base 677 to 822 of *N. perurans*. The correlation between the PCR results of gill swabs taken from infected salmon and gross gill scores showed potential for the development of a nondestructive sampling regime for the detection of AGD [75].

Rozas *et al.* [24] developed a PCR assay to amplify the *N. perurans* 18S rRNA gene from gill clinical samples of AGD-affected fish. The oligonucleotides designed and used for the detection of *N. perurans* showed amplicons of the expected size (462 bp) from all analysed fish. This PCR was able to detect *N. perurans* genetic material in the gills of fish with gross pathology and histological lesions characteristic of AGD. High concordance ($k = 0.95$) between the PCR results and the histological examination was observed.

A quantitative duplex real-time TaqMan®-based PCR was developed for the detection of *N. perurans* in Atlantic salmon and rainbow trout, with a set of primers and probes being used to amplify a 139-bp fragment specific to the *N. perurans* 18S rRNA gene [100]. Although the differences

observed by Fringuelli *et al.* [100] between the parasite load and AGD score were not statistically significant, the gill histopathology that was microscopically observed was not always associated with the presence of amoebae. This result, together with the lack of agreement between the results obtained by PCR and the gill histopathology examination, is consistent with the histopathology of some of the samples having been caused by other pathogens, waterborne irritants or a combination of these factors [100].

Treatments are triggered when moribund fish or fish with advanced clinical signs of disease are sampled [99]. A diagnostic method that allows for the early identification of the aetiological agent is essential, particularly as the cost of treatment is highly demanding [96]. Fish with no obvious pathology, either gross or histological, have previously tested positive via PCR when sampled using a gill swab, suggesting that once correctly optimized, a PCR assay could potentially be more sensitive than traditional diagnostic methods [97].

Immune response

Early studies on the transcriptional responses to AGD have shown no differences in the gill tissue expression of tumour necrosis factor (TNF)-α1, TNF-α2, interleukin (IL)-1β, inducible nitric oxide synthase and interferon (IFN)-γ mRNAs compared with that of tissue from healthy fish during the early onset of the disease in Atlantic salmon [101]. With the progression of the disease, the IL-1β mRNA level was found to be upregulated and lesion-restricted [102]. In AGD-affected tissue, significant downregulation of the major histocompatibility complex (MHC) class I (MHC-I) pathway-related genes occurred during the later stages of infection and appeared to be mediated by the downregulation of IFN regulatory factor (IRF)-1, independent of type I IFN, IFN-γ and IRF-2 expression [103]. Within this condition, suppression of the MHC-I and possibly the MHC-II pathways may inhibit the development of acquired immunity and could explain the unusually high susceptibility of Atlantic salmon to AGD.

However, anterior gradient-2 (AG-2), which is involved in inhibiting the tumour suppressor protein p53 (p53) and required for mucin (MUC) 2 post-transcriptional synthesis and secretion, was upregulated in AGD-affected gill tissue, whereas p53 tumour suppressor protein mRNA was concurrently downregulated in AGD lesions, suggesting a role for AG-2 and p53 in AGD pathogenesis [104]. MHC class II+ cells, considered to be antigen-presenting cells, were found within gill lesions by immunohistochemistry, and these cells exhibited variable levels of expression [104].

A recent study has shown that the mRNA expression levels of proinflammatory cytokines (IL-1β, TNF-α), cellular markers of cell-mediated immunity (T cell receptor (TCR)-α chain, cluster of differentiation (CD) 4, CD8, MHC-I, MHC-IIα), and antibody-mediated immunity (IgM, IgT) are correlated with a classical inflammatory response in the gills of AGD-affected Atlantic salmon at 10 days postinfection [105]. Contrary to previous studies, Pennachi *et al.* [105] suggested that *N. perurans* elicits a classical inflammatory response in the gills of AGD-affected fish and indicates that the mRNA expression of immune genes within gill lesions misrepresents the cellular immune response in the gills during AGD. The results obtained during this study, most notably the increased expression of TCR mRNA, which was strongly correlated with CD8 mRNA, suggest the infiltration of T-cells and highlight the importance of CD8+ T-cells and the possible involvement of gill intraepithelial lymphoid tissue (ILT) (Table 3).

Fish affected by AGD show an increased length in the ILT 28 days postexposure in the dorsal area of the gill arch, with a peak in lymphocyte density 7 days postexposure [108]. Benedicenti *et al.* [106] examined the immune response involvement in AGD using Atlantic salmon post-smolts sampled 3 weeks after exposure to either 500 or 5000 cells/l *N. perurans*. Gene expression analysis was performed on the first gill arch including the ILT. The Th1, Th17 and Treg pathways were found to be significantly downregulated, mainly in samples from fish given the higher dose. By contrast, the Th2 pathway was found to be significantly upregulated by both infection doses. These results seen during late stage AGD suggest that either an immune evasion strategy, similar to the responses driven by helminthic parasites to avoid cell-mediated killing mechanisms, or an allergic reaction caused by the parasite is occurring (Table 4).

Valdenegro *et al.* [107] identified 186 and 322 non-redundant proteins in gill and skin mucus, respectively, based on stringent filtration criteria, and statistical analysis demonstrated that 52 gill and 42 skin mucus proteins were differentially expressed in mucus samples from AGD-affected fish. By generating protein–protein interaction networks, some of these proteins formed part of the cell-to-cell signalling and inflammation pathways, such as C-reactive protein, apolipoprotein 1, granulin, cathepsin and angiogenin-1. In addition, Marcos-López *et al.* [110] showed the upregulation of prohibitin, cyclophilin A, apolipoprotein A1, ictacalcin, RhoGDP dissociation inhibitor α, components of the heat shock proteins 70 family and histones H3a and H4 and downregulation of peroxiredoxin-5 and cofilin. Among the protein functions identified were cell cycle regulation, cytoskeletal regulation, oxidative metabolism and immunity.

After four successive AGD challenges, no significant differences in the plasma or skin mucus levels of IgM were observed between AGD-naïve and challenged fish, but IgM gene expression in gill lesions of AGD-affected fish increased by up to 31 days after infection [109], which is possibly explained by weak correlations between the protein and mRNA abundances in cells and tissues. After a single infection, Valdenegro *et al.* [109] showed that the levels of serum or skin mucus IgM antibodies were not affected, and no changes in the IgM or IgT transcription were induced.

Table 3 Immune gene expression in the gills of Atlantic salmon during an AGD experimental challenge

Infection stage	Results	Interpretation	Ref.
Early stage	MUC-2, AG-2 upregulation p53 downregulation	AG-2 and p53 could have important roles in AGD pathogenesis	[104]
Early stage	TNF*α*, IL-1β, TCR*α*, CD4, CD8, MHC-I, MHC-II*α*, IgM, IgT upregulation	*N. perurans* elicits a classical inflammatory response in the gills of AGD-affected fish	[105]
Early stage	TNF*α*, IL-1β, COX-2 upregulation	*N. perurans* induces an early proinflammatory response in the gills of AGD-affected fish	[101]
Early stage	Th1, Th17, Treg downregulation Th2 upregulation	*N. perurans* promotes an allergic reaction and an immune-evasion strategy to avoid cell-mediated killing mechanisms	[106]
Late stage	IgM upregulation	*N. perurans* induces a humoral response in the gills of AGD-affected fish	[107]
Late stage	MHC-I, MHC-II, IRF-1, IRF-2 downregulation IL-1β, IL-8, COX-2 upregulation	This condition promotes the proinflammatory response, but the suppression of the MHC I and MHC II pathways could inhibit the development of acquired immunity	[103, 104]
Late stage	IL-1β upregulation	The proinflammatory response is maintained in late stages of the AGD infection	[102]

Table 4 Activity of proteins related to the immune response and oxidative stress in Atlantic salmon affected with AGD under experimental and field conditions

Day post-infection	Challenge	Results	Interpretation	Ref.
Serum/gill-skin mucus	Experimental	C-reactive protein, apolipoprotein 1, granulin, cathepsin, angiogenin-1 upregulation. Levels of serum or mucus IgM antibody were not affected.	*N. perurans* modifies the cell cycle regulation, cytoskeletal regulation, oxidative metabolism and immunity.	[107, 109]
Gills	Experimental	Prohibitin, cyclophilin A, apolipoprotein A1, ictacalcin, RhoGDP dissociation inhibitor *α*, HSP70, H3a, H4 upregulation Peroxiredoxin-5 and cofilin downregulation.	*N. perurans* modifies biological processes such as cell signalling and inflammation pathways.	[110]
Gill mucus	Field	Decreased activity for peroxidase, lysozyme, esterase, protease and low IgM levels.	Results obtained highlight the capacity of gills to elicit a local response to the infection, indicate an impaired immune response at the later stages of the disease.	[111]
Gills	Field	HAA and CAT activity decreased, whereas GR activity increased.	The oxidative stress and impaired antioxidant defences could contribute to the pathogenesis of late-stage AGD.	[112]

In the gill mucus, the IgM levels and activities of peroxidase, lysozyme, esterase and protease decreased in fish that had a greater time of exposure to infection by *N. perurans* and greater severity of the disease as well as a sequential increase after treatment [111]. These results demonstrate the ability of the gills to elicit a local response to the infection and indicate an impaired immune response at the later stages of the disease, followed by a partial reestablishment of the host immune status after treatment with fresh water.

Hydrophilic antioxidant activity (HAA) and antioxidant enzyme activity, including superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR), were determined in the gills obtained from a natural AGD-infection at gill scores of (GS) 0 and GS 2 and after a post-freshwater treatment, and separate samples comprising gill areas without and with lesions were taken [112]. HAA analysis revealed a significant depletion of the antioxidant capacity in infected gills and a recovery to previous antioxidant levels after freshwater treatment. SOD activity did not differ between the lesion and lesion-free areas, and CAT activity was diminished in the lesion areas of both infected and treated fish, whereas the GR activity at GS 2 was increased in lesion areas relative to lesion-free areas [112]. Oxidative stress and impaired antioxidant defences could contribute to the pathogenesis

of late-stage AGD, although knowledge gaps still exist, and this area requires further investigation.

Treatment and control

Freshwater bathing has been the standard method of treating the disease in Tasmania but is limited by access to freshwater [6]. In cooler production areas, hydrogen peroxide is an effective treatment, but the treatment is recognized as having a narrow safety margin at higher temperatures [70] or where fish are compromised by advanced AGD [113]. Affected animals are treated with freshwater bathing, a practice that costs the Australian salmon industry A$41 million/year and adds A$1.12 per kg to production costs [114]. Some estimates have put the cost of AGD-related mortality between $12.55 million in Norway and $81 million in Scotland [114].

Although significant research has been conducted on treatments since AGD was first recorded, freshwater bathing remains one of the most effective and essential methods for the removal of the majority of amoebae that cause AGD [6, 71, 82, 115]. Current treatment strategies in Australia involve monitoring of gross gill lesions and prophylactic freshwater baths [3]. Reinfection of the gills can occur relatively quickly, varying from 1 to 2 weeks post-freshwater bath, and increase in severity within 4 weeks [60, 81, 82]. Since the initial outbreaks in Australia in the 1980s, farms have seen a requirement for an increase in the frequency of treatments, with some fish being treated up to 15 times a year [81, 82, 115, 116].

The mechanism by which freshwater bathing treats AGD is by the osmotic effect, removing excess mucus and associated amoebae, thereby promoting healing of the gills [81, 82]. Findlay *et al.* [116] considered a number of factors, such as interactions among immune responses, health of the fish and the gills, number of amoebae remaining following treatment, and environmental variables to be important relative to reinfection with AGD.

Treatments are generally triggered when farms observe 30–40% of fish with gill scores of 2 or above [58]. Although freshwater bathing is effective at significantly reducing the amoeba gill load, with an 86 ± 9.1% reduction in the number of live amoebae observed, the remaining amoebae could potentially cause a reinfection within one week [82]. Water hardness has a noticeable effect on the efficacy of freshwater bathing, with soft freshwater (19.3–37.4 mg/l $CaCO_3$) proving to be more effective at reducing the numbers of viable amoebae (73.9–40.9% of total count) [117].

The physiological effects on salmon of freshwater bathing have also been investigated, and as a treatment, freshwater bathing poses very little risk of side effects [118]. However, any form of bathing treatment can be problematic as it requires the fish to be confined by tarpaulin or cage skirt or be transferred to a well boat, which imposes a handling effect, causing acute stress to the fish [119].

Recently, Wright *et al.* [120] have shown that targeting cell detachment rather than cell death with repeated freshwater treatments of shorter duration than typical baths could be used in AGD management. Thus, AGD-affected Atlantic salmon subjected daily to 30 min (sublethal) and 120 min (lethal) freshwater treatments for 6 days consistently reduced the *N. perurans* cell numbers on gills compared with daily 3 min freshwater or seawater treatments for 6 days.

During the emergence of AGD in Australia, research focused on establishing an alternative chemotherapeutic agent; however, much of this research was relatively unsuccessful [6]. A commonly used treatment in the aquaculture industry is hydrogen peroxide, which is utilized in the treatment of many external parasites and gill infections as well as fungal, bacterial and protozoan infections, including sea louse infestations [121–123]. Farms in Ireland, Scotland and Chile have good experience with using hydrogen peroxide for the treatment of sea lice and had some success in treating cases of AGD in 2011 and 2012 at dosage levels between 1000 and 1400 mg/l for 18–22 min.

However, a major disadvantage of hydrogen peroxide for the treatment of AGD is that a narrow safety margin exists, and at temperatures >13.5 °C, its use becomes hazardous [58, 122]. Moreover, hydrogen peroxide is routinely used as a treatment for AGD and sea lice in Atlantic salmon aquaculture, but gills showing decreased antioxidant capacity may be more susceptible to hydrogen peroxide damage or toxicity [112]. A mortality of 6.5–7.1% was recorded during *in vivo* trials with a concentration of 1250 mg/l at 12 and 18 °C, which would be considered commercially unacceptable [71]. The effects of hydrogen peroxide on Atlantic salmon gills were investigated in relation to sea louse treatments, and it was determined that exposure to 2.58 g/l for 20 min causes complete mortality [124].

Fallowing of sites and cage rotation have been identified as having an effect on AGD, with fewer freshwater baths being required and increased growth rates observed where management practices were adjusted [125]. The current experience suggests that the development of a vaccine against this disease remains a significant challenge for the near future. Despite evidence for immunosuppression in the later stages of infection, experimental attempts to boost the immune response have not been successful [5]. To date, immunostimulants or experimental vaccines have had little effect on the survival of AGD affected fish [5]. However, an injection of CpG oligonucleotides and two experimental diets containing immunostimulants have produced some encouraging results. Possibly, the dose and timing of the applications tested were not optimal, and optimization could lead to the development of an effective immunostimulant [5]. Recently, Cano *et al.* [126] have shown that *in vitro* models can prove to be a promising tool to study host responses to amoebae and may therefore reduce the requirement for *in vivo* studies when evaluating alternative therapeutants to AGD control.

Breeding for disease resistance can contribute to the prevention and control of AGD, providing long-term cumulative benefits in selected stocks [125]. Robledo *et al*. [127] showed that resistance to AGD is a suitable trait for genomic selection, and the addition of this trait to Atlantic salmon breeding programmes can lead to more resistant stocks. Gill damage and the amoebic load are heritable ($h^2 \sim 0.25$–0.30) and show high positive correlations, indicating they may be good measurements of host resistance to AGD. Although the genetic architecture of resistance appears to be largely polygenic in nature, two regions on chromosome 18 are suggestive of an association with both AGD-resistance traits.

Conclusions and Future Research

Increasingly, CGD is a health challenge in salmon farming worldwide, but many gaps remain in our knowledge about them. Coinfections are not uncommon on farms and may have synergistic effects on the host or reduce the effects of immunomodulation or treatment. However, a lack of understanding exists regarding the interrelationships among all the pathogens involved in gill pathologies. These interactions should be explored using biological models for concomitant infections, and studies on the cohabitation of single pathogen-infected fish with naïve fish are required.

To assess the risk of future outbreaks, we must develop a better understanding of the biology of the pathogens involved, e.g. the relationship between *N. perurans* loads in seawater and environmental variables, such as salinity, temperature, bacterial load, turbidity and dissolved inorganic nutrients. Studies on the interaction of the different pathogens involved with gill diseases, with the particular oceanographic and hydrological variables of each geographical area of salmon farming as indicators that can be used as inputs for dispersion modelling, are highly desirable. The question remains: why are some amoeba species virulent and others not?

The available information on the ecology of *N. perurans* in nature outside fish farm cages is very limited. Evidence suggests the presence of an environmental reservoir, where amoebae reside in spring and summer. The reservoir could be constituted of biota, such as wild fish and invertebrates, or by water and sediments. The application of molecular methods on environmental samples is the simplest way to seek natural reservoirs.

Whereas, in the past, CGD and AGD affected only a few geographical areas, the disease is now being reported worldwide. Gill diseases represent a substantial risk to fish health in seawater fish farms. Global expansion and intensification of aquaculture and climate change will most likely increase these risks. Therefore, does climate change contribute to an increase in outbreaks of gill diseases in salmon aquaculture? Furthermore, investigating the pathogen and disease is essential to reduce the costs of AGD and ensure the sustainability of the salmon industry.

Further investigations into the potential for sea lice as a vector for AGD would be important to fully assess if and how great a risk factor a heavy sea louse infestation would be. Due to the complexity of the disease agents that potentially present on salmon farms at any time, an examination of hydroids is important in the context of multifactorial diseases and as potential reservoirs and subsequent carriers of disease agents. At the same time, further studying the relationship between bacteria and AGD, as well as the importance of microbial dysbiosis, is of interest [69].

To design effective control strategies for gill disease in salmon aquaculture, the promotion of public–private cooperation between all of the entities involved in the salmon farming industry at national and international levels is fundamental, with the purpose of sharing information and knowledge to ensure sustainability in the world salmon industry.

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