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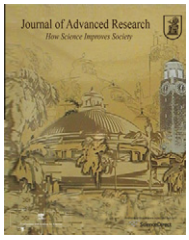

REVIEW ARTICLE

Bacterial coldwater disease of fishes caused by *Flavobacterium psychrophilum*

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Abstract Coldwater disease (CWD) is a bacterial disease that affects a broad host-species range of fishes that inhabit cold, fresh waters. This disease occurs predominately at water temperatures of 16 °C and below, and is most prevalent and severe at 10 °C and below. Coldwater disease occurs in cultured and free-ranging populations, with hatchery-reared young trout and salmon species especially vulnerable to infections. *Flavobacterium psychrophilum* is the etiological agent of CWD. This Gram-negative bacterium may be recovered from affected host tissues and characterized using standard biochemical techniques, providing that reduced nutrient media are used. There are numerous reports that describe sensitive and specific serologic and genomic diagnostic techniques for CWD. The entire genome of a virulent isolate of *F. psychrophilum* has been sequenced and described. Rainbow trout (*Oncorhynchus mykiss*) fry syndrome is also caused by *F. psychrophilum* with mortalities >50% possible among affected fish lots. Evidence suggests that pathogen transmission occurs both horizontally and vertically. Analogous to many diseases to other animals, prevention and control are essential to avoid losses to CWD, particularly since there is currently no commercially available vaccine and a limited number of antimicrobials have been approved for treating food fish worldwide. This review provides current host and geographic ranges of the pathogen, and covers epizootiology, transmission, pathogenicity, diagnostics, and prevention and treatment.

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Flavobacterial diseases of freshwater fishes

There are three *Flavobacterium* spp. that are primary pathogens to freshwater hatchery-reared and wild fish populations: *Flavobacterium columnare*, the cause of columnaris disease, *Flavobacterium branchiophilum*, the cause of bacterial gill disease, and *Flavobacterium psychrophilum* the cause of bacterial coldwater disease. Combined, the diseases and mortality caused by these pathogens constitutes one of the broadest host- and geographic ranges of any of the bacterial pathogens to fishes. Fish pathogenic *Flavobacterium* spp. are presumed

ubiquitous in temperate freshwater aquatic environments and occur in water temperatures ranging from just above freezing (*F. psychrophilum*) to 30 °C and above (*F. columnare*). Most, if not all cultured freshwater fish species may be affected by at least one of these pathogens. Other members of the Family Flavobacteriacea have been associated with diseases of fishes. For example, *Chryseobacterium piscicola* is an emerging pathogen of Flavobacteriaceae having been reported from Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) [1,2].

Columnaris disease, affects many cool- and warmwater fish species, typically in warm waters at 20–25 °C and above; however, it is not unusual to diagnose columnaris disease in fish, including trout species, in water as cool as 12–14 °C. Many cultured and free-ranging fish species are considered at risk for infection and possible disease. Columnaris disease affects aquaculture species, particularly the catfish species, as well as many aquarium species. *F. columnare* can be cultured from external sites on fish, including lesions, skin/mucus, and gills, and internal tissues, primarily the kidneys of fish with systemic infections. Primary cultures can be made on Anacker and Ordal [3] Cytophaga agar or the selective medium of Hawke and Thune [4]. The resulting colonies on primary plates are very characteristic: pale yellow, rhizoid and adhere tightly (i.e., sticky) to the medium surface. Colonies may be subcultured and confirmed using a few relatively simple diagnostic tests [5].

Bacterial gill disease, caused by *F. branchiophilum* [6–8], is primarily a disease to young hatchery-reared salmonids; it is not recognized as a problem in wild fish populations [9–13]. In endemic areas, bacterial gill disease outbreaks in aquaculture occur regularly and often in conjunction with increased host stressors. Although bacterial gill disease has been experimentally induced in healthy fish of various ages [14], many workers have noted that this disease typically occurs in association with certain predisposing factors such as overcrowding, reduced dissolved oxygen, increased ammonia, and particulate matter in the water [9,10,13]. Consequently, alleviating these host stressors has often been shown to reduce severity of active outbreaks and prevent further outbreaks. Mortality can rise quickly and be high if the culture conditions are not improved or a treatment is not promptly administered. Bacterial gill disease is common in spring, which coincides with production cycles at fish hatcheries when they have their greatest numbers of small fish after spawning and prior to stocking. A diagnosis of bacterial gill disease can often be accurately made by experienced workers simply by knowing the previous bacterial gill disease history of the hatchery and observing characteristic signs displayed by affected fish. Infected fish are typically lethargic, will be high in the water column and gasping for air at the surface and align near and into the incoming water, all of which are obvious signs of respiration difficulty. A Gram-stained gill smear will show numerous Gram-negative, long-thin rods. Combined, these criteria generally constitute a confirmed diagnosis. Bacterial primary isolation of *F. branchiophilum* is typically not attempted because this bacterium is particularly difficult to culture.

Bacterial coldwater disease

The etiological agent of bacterial coldwater disease (CWD) is *F. psychrophilum*, formerly known as *Cytophaga psychrophila* and *Flexibacter psychrophilus* [15]. This bacterial pathogen has been recovered from a broad geographic range and from a number of free-ranging and cultured salmonid fish species and a variety of non-salmonid fish hosts (Table 1). Coldwater disease results in significant disease and mortality to coldwater fish species, particularly to certain trout and salmon populations. Disease typically occurs at water temperatures below 16 °C, and is most prevalent and serious at 10 °C and below [16]. Although all ages of fish are affected, small fish (fry and fingerling size) are particularly vulnerable to infections [16,17]. Coldwater disease presents as different manifestations with the 'classic' or most prevalent form of disease producing characteristic open lesions on the external body surfaces of fish. These lesions may be initially observed as areas of rough-appearing skin or fin tip fraying. As the infection continues, necrosis develops at the sites of bacterial colonization, often noted as dorsal and adipose fin pathology. Lesion development has a predilection for the caudal peduncle and caudal fin regions. Along with the external pathology, systemic bacterial infections and extensive internal pathology will also be present among many specimens. As the disease form is more acute, the external lesions will be less prevalent and systemic infections and internal pathology will predominate.

F. psychrophilum was initially described and recovered in 1948 from a die-off in coho salmon *Oncorhynchus kisutch* from the Pacific Northwest United States [18]. This disease affected the adipose-caudal fin region and in some specimens with late-stage infections and prior to death, the vertebral column could be fully exposed. While usually fatal to fish with late-stage disease signs, the prevalence and mortality in affected fish populations were low. Davis [19] observed slender, Gram-negative rods 3–5 μm long and noted that overcrowding seemed to be a host predisposing factor in 'peduncle disease' outbreaks in rainbow trout in 1941 and 1945 at a hatchery in the Eastern United States (West Virginia). To control peduncle disease, Davis [19] suggested culling out those fish with obvious clinical signs in an effort to minimize the continuous shedding of pathogenic cells into the water column that served to infect other fish. It was also suggested to properly sterilize contaminated rearing troughs or ponds and all equipment, such as boots and nets, which were used to handle infected fish or water.

The pathologies and clinical disease signs associated with CWD are varied and extensive [20–24]. Listlessness, loss of appetite, and eroded fin tips are initial signs of CWD. Bacterial colonization may appear as faint, white areas on the fins, with some fish showing separation of the fin rays. Other disease signs may include exophthalmia, abdominal distension with increased volumes of ascites, and pale gills. In advanced cases of coldwater disease, necrosis of the caudal region may be severe and progress until caudle vertebra are exposed (Fig. 1). Lesions can also be noted on the lateral sides, snout-jaw region, and musculature often between the dorsal fin and back of the head. Histological examinations show extensive pathology in host tissues, including: focal necrosis in spleen, liver, and kidneys; increased vacuolar degeneration; increased eosinophilia and haemosiderin in the kidney; necrosis, pyknosis and lymphocyte infiltration in the dermis and underlying lateral musculature of skin lesions.

Rainbow trout fry syndrome [25–30] and a relatively more chronic form [31,32] are other disease manifestations caused by *F. psychrophilum*. Rainbow trout fry syndrome, as the name implies, affects the early life-stage fish, or the sac fry to

Table 1 Host and geographic records of *Flavobacterium psychrophilum*.

Geographic origin	Hosts	References
Australia	Rainbow trout *Oncorhynchus mykiss*, Atlantic salmon *Salmo salar*	[59,69]
Canada	Rainbow trout, brook trout *Salvelinus fontinalis*, Atlantic salmon, Arctic char *Salvelinus alpinus*, coho salmon *O. kisutch*, sea lamprey *Petromyzon marinus* L.	[71,84,113–115]
Chile	Rainbow trout, Atlantic salmon	[94,106,116,117]
Denmark	Rainbow trout	[27,38,40]
Estonia	Grayling *Thymallus thymallus*	[118]
Finland	Rainbow trout, brown trout *S. trutta* morpha *lacustris*, sea trout *S. trutta* morpha *trutta*, brook trout, Arctic char, whitefish *Coregonus muksun*, perch *Perca fluviatilis* L., roach *Rutilus rutilus*	[28,38,73,86,118,119]
France	Rainbow trout, common carp *Cyprinus carpio*, eel *Anguilla anguilla*	[25,28,45,57]
Germany	Rainbow trout, eel *A. anguilla*, common carp, crucian carp *Carassius carassius*, tench *Tinca tinca*	[120,121]
Japan	Rainbow trout, coho salmon, chum salmon *O. keta*, amago salmon *O. rhodurus*, common carp, yamame salmon *O. masou*, iwana salmon *S. leucomaenis pluvius*, eel *A. japonica*, Japanese dace (ugui) *Tribolodon hakonensis*, ayu *Plecoglossus altivelis*, pale chub (oikawa minnow) *Zacco platypus*, Japanese crucian carp (ginbuna) *C. auratus langsdorfii*, and two species of goby *Chaenogobius urotaenia* and *Rhinogobius brunneus*	[48,77,85,122,123]
Korea	Ayu	[124]
Northern Ireland	Rainbow trout	[28]
Norway	Brown trout *S. trutta* morpha *lacustris*	[28]
Peru	Rainbow trout	[71]
Scotland	Rainbow trout	[125]
Spain	Rainbow trout, eel *A. anguilla*	[30,126]
Sweden	Rainbow trout, sea trout, Baltic (Atlantic) salmon *S. salar*	[52,118]
Switzerland	Rainbow trout	[28]
Turkey	Rainbow trout	[127]
United Kingdom	Rainbow trout, Atlantic salmon	[26,29,59,116]
United States	Rainbow trout, brook trout, brown trout *S. trutta* morpha *lacustris*, lake trout *S. namaycush*, steelhead trout *O. mykiss* (migrating), Atlantic salmon, coho salmon, Chinook salmon *O. tshawytscha*, white sturgeon *Acipenser transmontanus*, chum salmon, goldfish *Carassius auratus*, cutthroat trout *O. clarkii*	[16,18,19,32,51,58,71,109,128,129]

early-feeding developmental stage. This disease form is acute and may result in high percentages of deaths among fish lots, perhaps 50% or greater total mortality. A bacteremia develops in conjunction with extensive internal pathology, including anemic and pale kidneys and livers. Lethargy, exophthalmia (often bilateral), dark skin pigmentation and pale gills are additional characteristic disease signs of rainbow trout fry syndrome. Lorenzen et al. [28] showed that *F. psychrophilum* isolates recovered from fish with rainbow trout fry syndrome were phenotypically homogeneous with isolates recovered from larger fish with classical CWD. Daskalov et al. [33] noted that the effects of high oxidized lipids in fish showed similarities in signs of rainbow trout fry syndrome. Some of the same histologic characteristics of rainbow trout fry syndrome were also noted in nutritional diseases caused by feeding diets high oxidized lipids [33]. Rainbow trout fed a diet with high levels of oxidized lipids had a greater mortality, relative to controls, by *F. psychrophilum* after exposure to the pathogen by scarifying and immersion or IP challenges.

With the chronic form of CWD, affected fish may show spiral or erratic swimming behavior, blackened caudal (tail) regions and/or spinal column deformities [31,32]. The reported disease signs and behavior appeared similar to those associated with whirling disease in fish caused by *Myxobolus cerebralis* [31]. However, with subsequent diagnostic evaluation, a whirling disease etiology can be eliminated and a correct diagnosis of CWD can be made based upon a case history along with primary culture and characterization of *F. psychrophilum* from affected tissues, including brain, spleen, kidney, liver, and lesion-skin. Kent et al. [32] showed the ataxic, spiral swimming behavior was associated with *F. psychrophilum* infections and chronic inflammation of the cranium and vertebrae in coho salmon. Fish showing this behavior did not recover and died. Based on epizootiological analyses, Kent et al. [32] concluded that *F. psychrophilum* was the cause of this disease presentation because it was only observed in populations that had recovered from acute CWD. Histologic evaluations showed periostitis, osteitis, meningitis, and periosteal proliferation of vertebrae at the junction of the vertebral column and cranium. This chronic CWD manifestation has occurred in fish that have recovered from a previous outbreak of acute clinical CWD [32] or it was diagnosed in fish lots with no recent history of CWD [31]. The bacterium may be cultured from the brain, kidney, liver, spleen and heart, but not necessarily from all tissues from each specimen or from all apparently infected specimens [31,32].

Concurrent infections in fish of *F. psychrophilum* with other fish pathogens are not uncommon. Dalsgaard and Madsen [34]

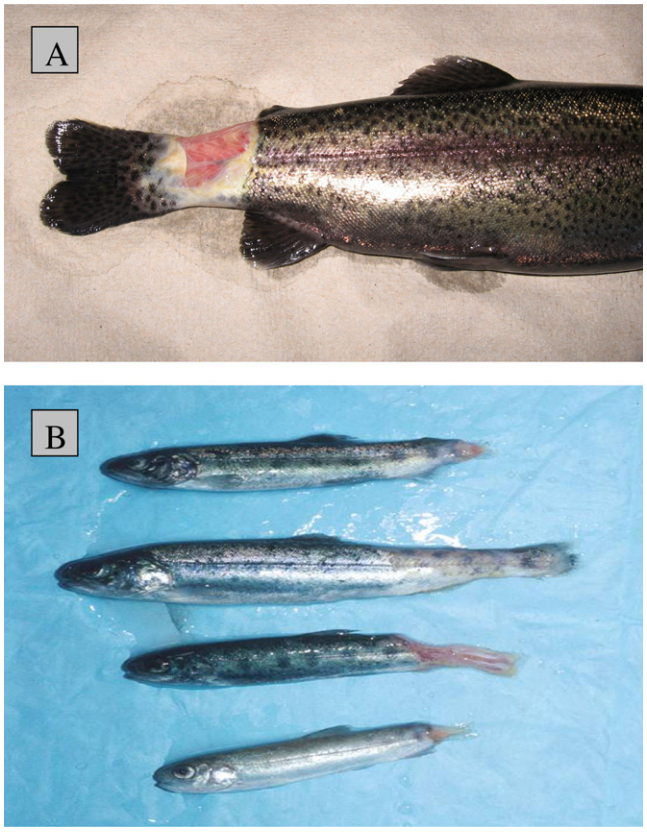

Fig. 1 Typical coldwater disease caudal lesions in rainbow trout *Oncorhynchus mykiss* (Panel A) and coho salmon *O. kisutch* (Panel B) caused by *Flavobacterium psychrophilum*. Photographs courtesy of Vermont Fish and Wildlife Department, Waterbury, VT and Wisconsin Department of Natural Resources, Madison, WI.

reported a concurrent infection in rainbow trout with the Gram-negative bacterium *Yersinia ruckeri*, the causative agent of enteric redmouth disease. There are other co-infections of *F. psychrophilum* with viruses, namely, infectious pancreatic necrosis virus, infectious hematopoietic necrosis virus, and erythrocytic inclusion body syndrome [35–37]. *F. psychrophilum* does not cause diseases in other animals or humans. The impact of fish losses at hatcheries reduces the numbers of fish available for raising or for stocking for sport fishing purposes and can impact restoration or population augmentation successes of certain endangered fish species.

Epizootiology and transmission

Since *F. psychrophilum* is horizontally transmitted, the water column is the medium in which viable cells move. The reservoir(s) of *F. psychrophilum* include pathogen-carrier fish, bacteria-shedding diseased and dead fish, and water supplies. *F. psychrophilum* has a demonstrated ability to survive for long periods outside fish hosts and to occur in non-fish hosts. Madetoja et al. [38] showed that rainbow trout that died from an infection with *F. psychrophilum* shed very high numbers of bacteria. Cell shedding rates depended on water temperatures, and cells were shed for at least 80 days. Madsen et al. [39] isolated *F. psychrophilum* from water samples that were collected near farmed rainbow trout or eggs. The results from laboratory waterborne challenges, the equivalent to natural horizontal transmission, with *F. psychrophilum* are equivocal [16,40] and an abrasion artificially created on the body surface, such as with a pre-challenge bath exposure to 0.005% formalin, facilitates disease [40]. Aoki et al. [41] noted success in *F. psychrophilum* laboratory challenges in 1.3 or 5.6 g rainbow trout depended on the growth stage of the bacterial challenge culture used to expose the fish. It was important to use log-phase cultures for experimental bath infections to produce typical clinical disease signs and mortality. Aoki et al. [41] showed that 18 and 24 h *F. psychrophilum* cultures with challenge doses of 2.00×10^7 and 8.50×10^7 cfu/mL, respectively, resulted in significantly greater mortalities than was obtained with a 48 h culture, even though the 48 h culture had a greater number of cells (3.40×10^8 cfu/mL).

Injection challenge methods are often used to expose experimental groups of fish to *F. psychrophilum* [36,42–44]. Decostere et al. [42] noted that only 10-week old rainbow trout developed clinical signs and mortality following IP injections with 1.00×10^6 cfu, while fish 5 or 15 months old did not. Also, spleen phagocytes from the 10-week old fish contained viable *F. psychrophilum* cells, and these cell numbers increased with exposure time. This contrasted with the two groups of older fish in which no *F. psychrophilum* cells were detected in spleen phagocytes.

F. psychrophilum has a demonstrated ability to adapt to a variety of environments, and not only survive, but also maintain pathogenicity. This bacterium has been recovered from broad host and geographic ranges, it resists lysozyme up to 2 mg/mL, and a small percentage of cells survived 100 ppm povidone–iodine for 30 min, a compound frequently used as an egg surface disinfectant. *F. psychrophilum* can survive in stream water for months and adopts a different morphology apparently to withstand the conditions of starvation [45]. Madetoja et al. [46] showed that *F. psychrophilum* cells in freshwater at 15 °C remained culturable through 300 days. Attachment to n-hexadecane and unfertilized eggs was significantly greater by *F. psychrophilum* cells maintained in either stream water or cytophaga broth for 1 month, in contrast to cells from 3-day-old cultures in cytophaga broth [45]. Adaptability of *F. psychrophilum* was further demonstrated by Brown et al. [17] when they recovered the bacterium from the brain of a newt Pleurodelinae, a non-fish host. Additionally, using PCR *F. psychrophilum* was detected from benthic diatoms [47] and from algae [48]. These studies suggest that perhaps any number of non-fish hosts could serve as a reservoir for *F. psychrophilum*. Although the contribution of aquatic non-fish hosts to the biology of CWD is not known, the capability of *F. psychrophilum* to survive in aquatic environments is illustrated.

Evidence suggests that *F. psychrophilum* is also vertically transmitted. For example, this bacterium has been recovered from ovarian fluids, intraovum, egg surfaces, milt, mucus samples and kidneys from sexually mature chum, coho and Chinook salmon, rainbow and steelhead trout, and Atlantic salmon [16,17,39,49–51]. Brown et al. [17] recovered *F. psychrophilum* from the insides of fertilized and eyed eggs. Ekman et al. [52] isolated *F. psychrophilum* from both male and female reproductive products from Baltic salmon (*S. salar*) returning from the Baltic Sea to spawn. Similar to other fish pathogens, *F. psychrophilum* can also contaminate the surface of pathogen-free fish eggs, which is a form of horizontal transmission

[17,53–55]. Kumagai et al. [54] exposed *F. psychrophilum* to groups of eggs before and after water hardening, as well as to eyed eggs. All of the groups were then disinfected with 50 mg/L povidone-iodine for 15 min. *F. psychrophilum* was subsequently recovered from only those eggs that were exposed to the pathogen prior to water hardening. Cipriano [49] recovered between 5.00×10^2 and 2.50×10^8 cfu *F. psychrophilum* per gram from Atlantic salmon eggs that were treated with 50–100 mg/L povidone–iodine at fertilization, post-water hardened and eyed egg stages. Further evidence that *F. psychrophilum* is internalized within eggs was reported by Kumagai et al. [53] who demonstrated that disinfection with 50 mg/L povidone-iodine for 15 min was not effective in eliminating the bacterium from either eyed- or fertilized eggs that had been pathogen-exposed prior to the water hardening process. Kumagai et al. [54] showed the importance of water hardening the eggs in pathogen-free water to prevent (egg) surface contamination.

Diagnosis and isolate characterization

A successful diagnosis of CWD considers all relevant information. Important factors include facility disease history, the rearing conditions for the fish, water temperature, host(s) involved and their ages, presence of characteristic clinical disease signs, the observation of characteristic bacterial cells in Gram-stained tissue preparations, and confirmation of *F. psychrophilum* as the causative agent from moribund or freshly dead specimens through primary culture and biochemical identifications, serological, or genotypic assays.

Microscopic examination of *F. psychrophilum* cells in infected tissues reveals long, thin, rod-shaped cells typically in a size range of 0.75–1.0 μm wide by 3–5 μm long (Fig. 2). Some cells may be attached end-to-end and consequently will appear longer.

F. psychrophilum can be recovered from a number of external and internal sites including skin/mucus, gills, brain, ascites, lesions, mucus, kidney and spleen and reproductive products of spawning adults. However, not all apparently affected fish could have sufficient number of viable cells in internal tissues for successful primary culture. Recovery of the pathogen from lesions is often more challenging than from internal sample sites due to the presence of environmental bacteria or oomycetes that will readily grow on primary isolation bacteriological media. Taking cultures from a greater number of fish or samples will enhance the chance to recover the bacterium. With some diagnostic cases, it may be possible to observe characteristic *F. psychrophilum* cells from infected tissues on histologic slides, yet be unsuccessful in culturing the bacterium from those same tissues, or vice versa, particularly from asymptomatic fish having reduced infection levels. The pathology to fish caused by *F. psychrophilum* can be extensive, for example, focal necrosis in various organs, and periostitis, osteitis, meningitis, ganglioneuritis and pyknotic nuclei are possible [26,32]. Particularly with chronic coldwater disease, masses of *F. psychrophilum* may be seen in the cranial area and anterior vertebra as well as inflammation and cartilage necrosis along the vertebral column.

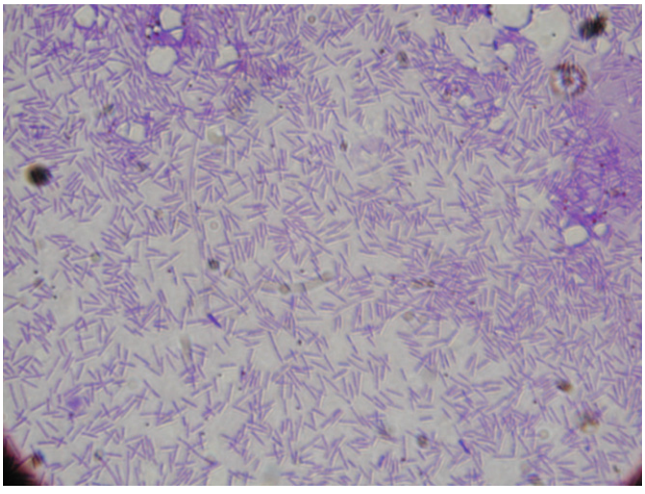

Fig. 2 Simple stain (crystal violet; 1000×) of *Flavobacterium psychrophilum* cells. External lesion material smear from a rainbow trout *Oncorhynchus mykiss* affected with coldwater disease. Photomicrograph courtesy of Vermont Fish and Wildlife Department, Waterbury, VT.

Homogenization of sample tissues prior to the inoculations may enhance recovery, especially from fish with low-level infections. Primary culture plates can be inoculated using one of several techniques, such as direct streak-plating or preparing a dilution series and drop-inoculating specific volumes on the medium surface to yield viable cell numbers (i.e., cfu/g). Several bacteriological media may be used for primary culture of *F. psychrophilum*. Cytophaga medium [3] is frequently employed in diagnostic laboratories; the recipe consists of 0.05% tryptone, 0.05% yeast extract, 0.02% sodium acetate, 0.02% beef extract, and pH 7.0–7.2. Agar may be added if desired. Cytophaga medium was developed to support the growth of bacteria that require a reduced nutrient load requirement. Holt et al. [21] described tryptone yeast extract salts (TYES) consisting of 0.4% tryptone, 0.04% yeast extract, 0.05% magnesium sulfate, 0.05% calcium chloride, and pH 7.2 as an excellent liquid medium, that diagnosticians routinely supplement with agar for use as a primary isolation medium for *F. psychrophilum*. Other reduced nutrient concentration media have also been used [16,56–59]. Some authors report improved growth of *F. psychrophilum* after supplementing the medium with serum, a component typically used for slow growing or fastidious bacteria that will grow on rich nutrient media. Lorenzen [60] and Brown et al. [17], for example, incorporated 5.0% and 0.5%, respectively, of new born calf serum. Obach and Baudin Laurencin [61] supplemented Cytophaga medium with 10% fetal calf serum for recovery of *F. psychrophilum* from rainbow trout. Daskalov et al. [62] utilized Cytophaga medium as a basal medium to which they added galactose, glucose, rhamnose and skimmed milk. Rangdale et al. [59] modified cytophaga medium by increasing the tryptone concentration ten-fold (to 0.5%) and the beef extract from 0.02% to 0.05%. Increased tryptone (to 0.5%) in Cytophaga medium has since been used by various researchers who reported excellent growth of laboratory cultures. Lorenzen [60] showed the importance of the brand of beef extract to culture *F. psychrophilum*, with optimal results using the semi-solid form. Kumagai et al. [63] suggested the incorporation of 5 μg/mL tobramycin to primary culture media to aid recovery of *F. psychrophilum* by retarding the growth of environmental bacterial contaminants.

The optimum incubation temperature for primary isolation and culture growth of *F. psychrophilum* is 15–16 °C. Colonies on Cytophaga agar are pale-yellow and about 2–3 mm in diameter after 2–3 days of incubation. Colonies form a characteristic fried egg appearance with a slightly raised center and mild spreading, irregular margin (Fig. 3). Colonies do not adhere to the medium surface in the similar manner that *F. columnare* colonies do. Suspect *F. psychrophilum* colonies can readily be subcultured onto fresh media, e.g., Cytophaga agar, for characterization and identification using standard biochemical and physiological methods [9,15,28,57–58,64–69]. Unless growth/no growth on select media is to be evaluated, the basal medium for biochemical testing must be reduced nutrient to support bacterial growth, even for negative test reactions. For example, the basal medium of Pacha [70], which consists of 0.2% peptone, 0.2% sodium chloride, 0.03% potassium phosphate, 0.00015% bromothymol blue, and 0.3% agar, pH 7.0–7.2, is an excellent choice as a basal medium to evaluate acid production from assimilation of sugars.

Isolates typically do not grow, or grow poorly on high-nutrient concentration media routinely used in fish disease diagnostic laboratories, including brain heart infusion agar, tryptic soy agar, triple sugar iron agar and blood agar. Most *F. psychrophilum* isolates are reported to produce oxidase and catalase, hydrolyze gelatin and casein, produce flexirubin-like pigments (chromogenic shift from yellow to orange in 10% KOH), degrade tyrosine, and lyse killed *Escherichia coli* cells. Most isolates are negative for assimilation of a suite of sugars (production of acid indicated by a pH drop in a basal medium with a pH indicator), indole production, starch hydrolysis, and degradation of tributyrin and xanthine. Variable results are reported for elastin hydrolysis, nitrate reduction, and chondroitin sulfate AC lyase. Some of the variability reported in line-data for certain biochemical tests might be attributed to differences in isolate origins or the methods employed to determine the results. An example of this is the unique phenotype of some *F. psychrophilum* isolates from Australia, which produce brown pigment when grown on a medium containing tyrosine [69]. Lorenzen et al. [28] showed that the concentration of certain medium supplements, or biochemical test substrates, may affect the test results. If the concentration of a substrate in a medium is too low, this could result in a false-negative interpretation. Furthermore, they emphasized the need to use fresh growth cultures as the inoculum for biochemical characterization tests, and the use of sensitive test procedures for certain characters, such as the use of lead acetate to detect weak production of hydrogen sulfide.

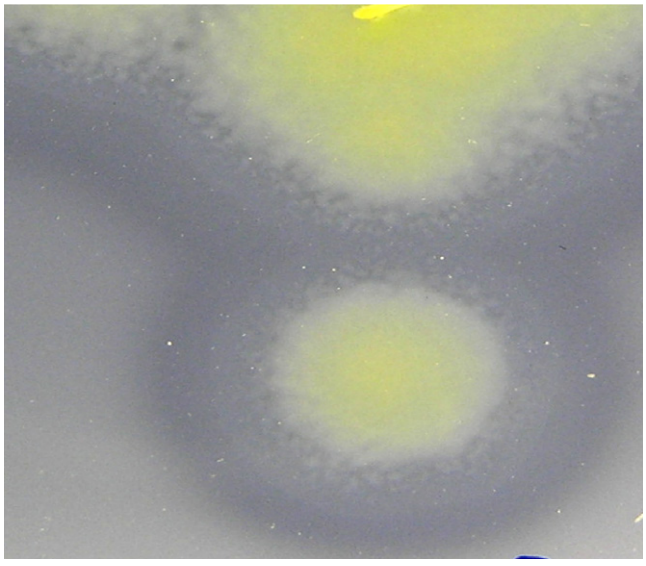

Fig. 3 *Flavobacterium psychrophilum* colonies on Cytophaga agar [3] supplemented with 0.2% gelatin. The bacterial colonies were gelatinase positive, as indicated by clear zones adjacent to and surrounding the colonies.

Other sensitive diagnostic techniques in addition to bacterial culture have been employed to detect *F. psychrophilum* in water, in fish, and fish sex products, or to diagnose or confirm standard culture diagnostics for coldwater disease. A number of clinicians have used antisera raised against *F. psychrophilum* in the immunofluorescence antibody technique [41,48,71–74] and for immunohistochemistry [35,38,75]. Enzyme-linked immunosorbent assays have been developed using antibodies *F. psychrophilum* cell surface components for detection of the pathogen in fish [71,76]. Misaka et al. [77] used nitrocellulose bacterial colony blotting off culture media plates and immunostaining to quantify viable *F. psychrophilum* from kidneys and ovarian fluids of chum salmon *Oncorhynchus keta*.

Fish disease diagnosticians are increasingly employing and relying on nucleic acid genotype based assays to detect fish pathogens, including *F. psychrophilum*, or to confirm the identifications made using other methods, such as standard phenotypic characterizations. A number of procedures using polymerase chain reaction assays (PCR), and particularly the more specific nested PCR assays, have been described [47,51,72–74,78–89]. Amita et al. [48] detected *F. psychrophilum* in a water sample and in algae using PCR. Izumi et al. [47] used a nested PCR to detect *F. psychrophilum* from benthic diatoms samples from surfaces of stones. Suzuki et al. [90] compared the sensitivities of various PCR primers for *F. psychrophilum* and found that the primer targeting the 16S rDNA was the more sensitive; however, this primer resulted in a level of false-positive reactions. Because of this, they concluded that PCR primers targeting the DNA gyrase subunit gene *gyr*B and the peptidyl-prolyl *cis–trans* isomerase C gene *ppi*C were the preferred primers for *F. psychrophilum*. A multiplex PCR was developed by del Cerro et al. [82] to detect three fish pathogens simultaneously, which included *F. psychrophilum*.

Pathogenicity and immunity

The genome of a virulent *F. psychrophilum* isolate has been delineated [91]. The circular chromosome consists of 2,861,988 base pairs, which is relatively small compared to other environmental bacteria within the family; the average genome size for the genus *Flavobacterium,* estimated by DNA reassociation assays, is 4.1 ± 1 Mb [92]. The G + C content of *F. psychrophilum* is 32.54% [64].

Potential gene products related to virulence for *F. psychrophilum* were described [91]. Proteases are considered to be essential virulence components, and potential secreted proteases were identified in the genome [93]. Genes coding

for cytolysins and haemolysin-like proteins are considered important virulence determinants, while fibronectin-type adhesins may have an essential role in the bacterium's attachment capability. Other enzymes act to negate host defense mechanisms. Avendaño-Herrera et al. [94] employed pulsed-field gel electrophoresis of *Sac* I restriction patterns of Chilean *F. psychrophilum* field isolates and demonstrated two distinct genetic groups that correlated with host of origin, rainbow trout and Atlantic salmon.

Innate immunity to *F. psychrophilum* in rainbow trout has been correlated with spleen size [95]. Hadidi et al. [95] screened 71 full-sibling crosses and found that the resistant or susceptible phenotypes were stable. The spleen-somatic indices of 103 fish created high, medium, and low spleen-index groups. Specimens having the larger spleen indices were significantly more resistant to *F. psychrophilum*. Acute serum amyloid A (A-SAA) is normally thought to be a major acute-phase reactant and effector of innate immunity in vertebrates. When challenged with whole cell *F. psychrophilum*, lipopolysaccharides (LPS), or CpG oligonucleotides, A-SAA was strongly induced in many immune-relevant rainbow trout tissues [96]. Unlike mammalian A-SAA, trout A-SAA does not increase in the plasma of diseased fish. Therefore, the role of this molecule in protection against *F. psychrophilum* is perhaps more important in localized defense mechanisms.

Numerous studies have been done that demonstrate protective immune responses in an effort to develop a vaccine for CWD. Passive immune protection to *F. psychrophilum* with serum from convalescent, and previously immunized rainbow trout was demonstrated (in rainbow trout) by LaFrentz et al. [97]. Protection to specific molecular mass *F. psychrophilum* cell fractions was shown by LaFrentz et al. [36], also to the P18 surface antigen [98], and to formalin- and heat-inactivated *F. psychrophilum* cells [99]. Additionally, protection against *F. psychrophilum* was shown by vaccination with an outer membrane fraction [100] and a 70–100 kD cell fraction [36] composed of O-polysaccharide components of LPS. Aoki et al. [101] showed that membrane vesicles were released in *F. psychrophilum* stationary phase growth cultures. Stationary phase *F. psychrophilum* cells or membrane vesicles alone provided no protection to rainbow trout; however, host survival to challenge was 94–100% when these two components were combined in experimental vaccines. Analysis of virulent and avirulent strains of *F. psychrophilum* by comparative immunoproteomic methods demonstrated eight proteins that were unique to the virulent strain [102]. Two highly immunogenic heat shock proteins (HSP 60, HSP 70) shared extensive homology with the heat shock proteins of other, related bacteria. LaFrentz et al. [103] developed an attenuated strain of *F. psychrophilum* through repeated passage on increasing concentrations of rifampicin. Intraperitoneal injection with the attenuated strain conferred significant protection in rainbow trout to challenge with the virulent parent strain. The protected fish showed elevated specific antibody titers. More importantly, LaFrentz et al. [103] showed that immersion exposure to the attenuated strain also elicited a protective immune response in fish. Álvarez et al. [104] also demonstrated protection in rainbow trout fry using an attenuated strain of *F. psychrophilum*; this strain was attenuated using transposon insertion mutagenesis. LaFrentz et al. [105] suggested that the glycocalyx of *F. psychrophilum* may be an antigen for the development of a vaccine for protection against CWD and rainbow trout fry syndrome. Johnson et al. [44] showed that the major histocompatibility gene region MH-IB was linked to survivability to CWD in rainbow trout that were IP injection challenged to *F. psychrophilum*.

Prevention, control, and treatment

As with all fish diseases, including CWD, management strategies that minimize the risks of pathogen introductions or transmission, and reduce the severity of overt disease outbreaks are desired alternatives to chemical or antimicrobial treatment therapies. Prevention of diseases is the most prudent form of disease control and treatment; this especially pertains to cultured fish populations, and ultimately to wild fish populations restored or augmented with fishes reared at hatcheries. Proper fish husbandry will alleviate host stressors that are often involved or suspected in the disease processes, such as factors that compromise the integrity of the mucus covering the fin tips [106,107]. Disease preventative techniques include rearing small (i.e., most susceptible) fish in pathogen-free water, maintaining safe carrying capacities for the water supply and flow, the use and proper storage of quality fish food, cleanliness of the fish holding tanks, minimizing organic material and nitrite [108], and effective sanitization of equipment used in fish production [109]. High numbers of *F. psychrophilum* cells are shed into the water column by fish that died from CWD. It was shown to be very important to quickly remove dead fish from the population thereby reducing re-infection [38]. Periodic health and pathogen inspections on statistically significant numbers of specimens from each fish lot to detect a pathogen prior to the expression of clinical disease are an essential part of a disease prevention strategy. If a pathogen is detected early, the affected fish and therefore, the pathogen can be confined (i.e., quarantined) within a designated area of a facility and a containment and treatment strategy begun. Caution should always be exercised when moving fish between culture facilities, especially if fish are suspected to be diseased or if the source facility has a disease history.

Povidone–iodine is commonly used as a fish egg surface disinfectant to fertilized and eyed eggs [107]. Although this treatment is not 100% effective to inactivate *F. psychrophilum* in all situations, it reduces egg-associated pathogen transmission. Brown et al. [17] showed that 2% of *F. psychrophilum* cells survived an exposure to 100 ppm povidone–iodine for 30 min. Kumagai et al. [53] treated fertilized rainbow trout, coho and masu salmon eggs with 50 ppm povidone–iodine for 15 min and subsequently recovered *F. psychrophilum* from 60% to 80% of the treated eggs; additionally, they treated eyed coho salmon eggs with up to 1000 ppm povidone-iodine for 15 min or 200 ppm for up to 120 min and both resulting data sets for treated eggs were comparable to infected, but untreated controls. At the 1000 ppm concentration, for example, 8.0×10^4 cfu/g egg were recovered. Results clearly show that standard egg treatment protocols may not be relied upon to effectively disinfect salmonid eggs and control the spread of *F. psychrophilum* [17,53,110].

In the United States, antimicrobial agents or other drugs to be used in fish destined for human consumption must be approved by the U.S. Food and Drug Administration and used

in accordance with product label information. Certain factors should be considered when using a therapeutic agent, such as tissue clearance time, toxicity to fishes in different water chemistries, and the organic load in the water. If it is unclear whether a drug will result in adverse effects to fish in a certain water chemistry profile, it may be advisable to initially try the treatment in a pilot study on a small number of individuals to identify a potential problem, rather than simply treating large numbers of fish and discovering toxicity with no means to quickly stop the treatment.

For fish bacterial diseases treated with oral delivery of medicated food, early intervention is paramount to achieve a successful treatment for CWD. This is especially true since one of the earliest disease signs is the fish's loss of appetite, which will directly affect the efficacy of treatment. A successful antimicrobial treatment is dependant on an early and accurate diagnosis of *F. psychrophilum* as the causal agent of disease. However, prophylactic or indiscriminate antimicrobial therapy should be avoided because of the risk to develop antimicrobial-resistant bacterial strains [59,111,112]. Prior to the use of an antimicrobial agent, it is desirable to recover the causative bacterium of the disease, confirm the identification, and perform in vitro sensitivity testing to ensure that the particular bacterial isolate is susceptible to the drug to be used. If the isolate is resistant to the antimicrobial agent, then therapy will be ineffective and perpetuate the resistant isolate at the facility, and will result in a financial loss for the medicated food.

Two drugs are approved for treatment of CWD in captive-reared fish in the United States (www.fda.gov/cvm). Both antimicrobials are delivered to affected fish orally via medicated feed. Florfenicol (Aquaflor®) may be used for freshwater-reared salmonids and must be prescribed by a licensed veterinarian. Dosage is 10 mg florfenicol per kilogram of fish per day for 10 consecutive days. The withdrawal time is 15 days. Oxytetracycline dihydrate (Terramycin®) is similarly permitted for freshwater-reared salmonids, at 3.75 g per 45.4 kg of fish per day for 10 consecutive days, and with a 21-day withdrawal time. Either treatment should be used in conjunction with improved environmental parameters that may reduce stressors to fish. It is important to maintain clean holding tanks and to promptly remove dead fish to minimize *F. psychrophilum* cells in the water column.

Currently, there are no vaccines commercially available to protect fish against bacterial CWD. A problem unique to vaccination of fish is the need for the vaccine delivery method to be easily and effectively given to large numbers (e.g., thousands) of fish held in hatchery systems. This is particularly so for rainbow trout fry syndrome, in that fish will be just beyond sac fry stage when vaccinated. Ideally, the delivery method will be an immersion or waterborne exposure, which is not only efficient for the fish culturist, but will also be minimally stressful (e.g., handling) for the fish.

Recent research on vaccine development for *F. psychrophilum* has been related to specific proteins produced by the bacterium. Plant et al. [43] demonstrated high antibody responses in rainbow trout to heat shock proteins 60 and 70, singularly or in combination, which were administered (IP) with Freunds complete adjuvant. Eight weeks post-immunization, the fish were exposed to 5.0×10^6 or 1.25×10^7 cfu *F. psychrophilum* by subcutaneous injections. Mean mortality in the heat shock protein treatment groups was 74% or greater and significant protection compared to control groups was not afforded to the fish. Plant et al. [43] concluded that these proteins did not seem to be useful for further vaccine development. LaFrentz et al. [130] identified and analyzed specific proteins of *F. psychrophilum* cultures grown *in vivo* and *in vitro* in an iron-limited medium. Through evaluations using 2-D polyacrylamide gel electrophoresis, numerous proteins from the cultures showed increased intensities, while others showed lesser intensities. The expressed (upregulated) proteins may be important in the course of CWD in fish (LaFrentz et al. [130] and perhaps warrant utilization in the development of a fish vaccine.

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References

[1] Ilardi P, Fernández J, Avendaño Herrera R. *Chryseobacterium piscicola* sp. nov., isolated from diseased salmonid fish. Int J Syst Evol Microbiol 2009;59(12):3001–5.
[2] Ilardi P, Abad J, Rintamäki P, Bernardet JF, Avendaño Herrera R. Phenotypic, serological and molecular evidence of *Chryseobacterium piscicola* in farmed Atlantic salmon, *Salmo salar* L., in Finland. J Fish Dis 2010;33(2):179–81.
[3] Anacker RL, Ordal EJ. Studies on the myxobacterium *Chondrococcus columnaris* 1,2 I. Serological typing. J Bacteriol 1959;78(1):25–32.
[4] Hawke JP, Thune RL. Systemic isolation and antimicrobial susceptibility of *Cytophaga columnaris* from commercially reared channel catfish. J Aquat Anim Health 1992;4:109–13.
[5] Griffin BR. A simple procedure for identification of *Cytophaga columnaris*. J Aquat Anim Health 1992;4:63–6.
[6] Kimura N, Wakabayashi H, Kudo S. Studies on bacterial gill disease in salmonids. 1. Selection of bacterium transmitting gill disease. Fish Pathol 1992;12:233–42.
[7] Von Graevenitz A. Revised nomenclature of *Campylobacter laridis*, *Enterobacter intermedium* and "*Flavobacterium branchiophila*". Int J Syst Bacteriol 1990;40(2):211.
[8] Wakabayashi H, Huh GJ, Kimura N. *Flavobacterium branchiophila* sp. nov., a causative agent of bacterial gill disease of freshwater fishes. Int J Syst Bacteriol 1989;39(3):213–6.
[9] Bullock GL. Studies on selected myxobacteria pathogenic for fishes and on bacterial gill disease in hatchery-reared salmonids 1972. Technical paper 60. Washington, DC, U.S.: Fish and Wildlife Service; 1972.
[10] Bullock GL. Bacterial gill disease of freshwater fishes. Fish disease leaflet 84. Washington, DC, U.S.: Fish and Wildlife Service; 1990.
[11] Daoust PY, Ferguson HW. Gill diseases of cultured salmonids in Ontario. Can J Comp Med 1983;47(3):358–62.
[12] Farkas J. Filamentous *Flavobacterium* sp. isolated from fish with gill diseases in cold water. Aquaculture 1985;44(1):1–10.
[13] Schachte JH. Bacterial gill disease. In: Meyer FP, Warren JW, Carey TG, editors. A Guide to integrated fish health management in the great lakes basin (Special publication). Ann Arbor, Mich: Great Lakes Fishery Commission; 1983. p. 181–4.
[14] Ferguson HW, Ostland VE, Byrne P, Lumsden JS. Experimental production of bacterial gill disease in trout by horizontal transmission and by bath challenge. J Aquat Anim Health 1991;3:118–23.

[15] Bernardet JF, Segers P, Vancanneyt M, Berthe F, Kersters K, Vandamme P. Cutting a gordian knot: Emended classification and description of the genus Flavobacterium, emended description of the family Flavobacteriaceae and proposal of *Flavobacterium hydatis* norn. nov. (Basonym, *Cytophaga aquatilis* Strohl and Tait 1978). Int J Syst Bacteriol 1996;46:128–48.

[16] Holt RA. *Cytophaga psychrophila*, the causative agent of bacterial cold-water disease in salmonid fish. Ph.D. Thesis. Corvallis, OR: Oregon State University; 1987.

[17] Brown LL, Cox WT, Levine RP. Evidence that the causal agent of bacterial coldwater disease *Flavobacterium psychrophilum* is transmitted within salmonid eggs. Dis Aquat Organ 1997;29:213–8.

[18] Borg AF. Studies on myxobacteria associated with disease in salmonid fishes. Wildlife Dis 1960;8:85.

[19] Davis HS. Care and diseases of trout. 1947. Rep.no. 12. Fish and Wildlife Service, United States Department of the Interior; 1947.

[20] Cipriano RC, Holt RA. Fish disease leaflet *Flavobacterium psychrophilum*, cause of bacterial cold-water disease and rainbow trout fry syndrome. Rep.no. 86. United States, Kearneysville, WV: Department of the Interior, U.S. Geological survey; 2005.

[21] Hotl RA, Rohovec JS, Fryer JL. Bacterial cold-water disease. In: Inglis V, Roberts RJ, Bromage NR, editors. Bacterial disease of fish. New York: Wiley-Blackwell; 1993. p. 3–22.

[22] Nematollahi A, Decostere A, Pasmans F, Haesebrouck F. *Flavobacterium psychrophilum* infections in salmonid fish. J Fish Dis 2003;26(10):563–74.

[23] Shotts Jr EB, Starliper CB. Flavobacterial diseases: Columnaris disease, cold-water disease and bacterial gill disease. In: Woo PTK, Bruno DW, editors. Fish diseases and disorders: Volume 3: Viral, bacterial and fungal infections. Wallingford, UK: CAB Publishing; 1999. p. 559–76.

[24] Wood JW. Diseases of pacific salmon: Their prevention and treatment. 2nd ed. Washington State Dept Fisheries, Hatchery Division; 1974.

[25] Bernardet JF, Baudin Laurancin F, Tiserant G. First identification of *'Cytophaga psychrophila*' in France. Bull Eur Assoc Fish Pathol 1988;8:104–5.

[26] Bruno DW. *Cytoghaga psychrophila* (='*Flexibacter psychrophilus*')(Borg), histopathology associated with mortalities among farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum) in the UK. Bull Eur Assoc Fish Pathol 1992;12:215–6.

[27] Lorenzen E, Dalsgaard I, From J, Hansen EM, Horlyck V, Korsholm H, et al. Preliminary investigations of fry mortality syndrome in rainbow trout. Bull Eur Assoc Fish Pathol 1991;11:77–9.

[28] Lorenzen E, Dalsgaard I, Bernardet JF. Characterization of isolates of *Flavobacterium psychrophilum* associated with coldwater disease or rainbow trout fry syndrome I Phenotypic and genomic studies. Dis Aquat Organ 1997;3(197):208.

[29] Santos Y, Huntly PJ, Turnbull A, Hastings TS. Isolation of *Cytophaga psychrophila* (*Flexibacter psychrophilus*) in association with rainbow trout mortality in the United Kingdom. Bull Eur Assoc Fish Pathol 1992;12:209–10.

[30] Toranzo AE, Barja JL. Fry Mortality Syndrome (FMS) in Spain. Isolation of the causative bacterium *Flexibacter psychrophilus*. Bull Eur Assoc Fish Pathol 1993;13:30–2.

[31] Blazer V, Stark K, Starliper C. Unusual histologic manifestations of *Flexibacter psychrophila* in hatchery salmonids, vol. 10. Cipriano RC, editor. 21st Annual Eastern Fish Health Workshop. Virginia: Gloucester Point; 1996.

[32] Kent ML, Groff JM, Morrison JK, Yasutake WT, Holt RA. Spiral swimming behavior due to cranial and vertebral lesions associated with *Cytophaga psychrophila* infection in salmonid fishes. Dis Aquat Organ 1989;6:11–6.

[33] Daskalov H, Robertson PAW, Austin B. Influence of oxidized lipids in diets on the development of rainbow trout fry syndrome. J Fish Dis 2000;23(1):7–14.

[34] Dalsgaard I, Madsen L. Bacterial pathogens in rainbow trout, *Oncorhynchus mykiss* (Walbaum), reared at Danish freshwater farms. J Fish Dis 2000;23(3):199–209.

[35] Evensen O, Lorenzen E. Simultaneous demonstration of infectious pancreatic necrosis virus (IPNV) and *Flavobacterium psychrophilum* in paraffin-embedded specimens of rainbow trout Oncorhynchus mykiss fry by use of paired immunohistochemistry. Dis Aquat Organ 1997;29(3):227–32.

[36] LaFrentz BR, LaPatra SE, Jones GR, Cain KD. Protective immunity in rainbow trout *Oncorhynchus mykiss* following immunization with distinct molecular mass fractions isolated from *Flavobacterium psychrophilum*. Dis Aquat Organ 2004;59(1):17–26.

[37] Piacentini SC, Rohovec JS, Fryer JL. Epizootiology of erythrocytic inclusion body syndrome. J Aquat Anim Health 1989;1:173–9.

[38] Madetoja J, Nyman P, Wiklund T. *Flavobacterium psychrophilum*, invasion into and shedding by rainbow trout *Oncorhynchus mykiss*. Dis Aquat Organ 2000;43(1):27–38.

[39] Madsen L, Moller JD, Dalsgaard I. *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Walbaum), hatcheries: Studies on broodstock, eggs, fry and environment. J Fish Dis 2005;28(1):39–47.

[40] Madsen L, Dalsgaard I. Reproducible methods for experimental infection with *Flavobacterium psychrophilum* in rainbow trout *Oncorhynchus mykiss*. Dis Aquat Organ 1999;36(3):169–76.

[41] Aoki M, Kondo M, Kawai K, Oshima S. Experimental bath infection with *Flavobacterium psychrophilum*, inducing typical signs of rainbow trout *Oncorhynchus mykiss* fry syndrome. Dis Aquat Organ 2005;67(1–2):73–9.

[42] Decostere A, D'Haese E, Lammens M, Nelis H, Haesebrouck F. *In vivo* study of phagocytosis, intracellular survival and multiplication of *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Walbaum), spleen phagocytes. J Fish Dis 2001;24(8):481–7.

[43] Plant KP, LaPatra SE, Cain KD. Vaccination of rainbow trout, *Oncorhynchus mykiss* (Walbaum), with recombinant and DNA vaccines produced to *Flavobacterium psychrophilum* heat shock proteins 60 and 70. J Fish Dis 2009;32(6):521–34.

[44] Johnson NA, Vallejo RL, Silverstein JT, Welch TJ, Wiens GD, Hallerman EM, et al. Suggestive association of major histocompatibility IB genetic markers with resistance to bacterial cold water disease in rainbow trout (*Oncorhynchus mykiss*). Mar Biotechnol 2008;10(4):429–37.

[45] Vatsos IN, Thompson KD, Adams A. Adhesion of the fish pathogen *Flavobacterium psychrophilum* to unfertilized eggs of rainbow trout (*Oncorhynchus mykiss*) and n-hexadecane. Lett Appl Microbiol 2001;33(3):178–82.

[46] Madetoja J, Nystedt S, Wiklund T. Survival and virulence of *Flavobacterium psychrophilum* in water microcosms. FEMS Microbiol Ecol 2003;43(2):217–23.

[47] Izumi S, Fujii H, Aranishi F. Detection and identification of Flavobacterium psychrophilum from gill washings and benthic diatoms by PCR-based sequencing analysis. J Fish Dis 2005;28(9):559–64.

[48] Amita K, Hoshino M, Honma T, Wakabayashi H. An investigation on the distribution of *Flavobacterium psychrophilum* in the Umikawa river. Fish Pathol 2000;35(4):193–7.

[49] Cipriano RC. Intraovum infection caused by *Flavobacterium psychrophilum* among eggs from captive Atlantic salmon broodfish. J Aquat Anim Health 2005;17(3):275–83.
[50] Rangdale RE, Richards RE, Alderman DJ. Isolation of *Cytophaga psychrophila*, causal agent of Rainbow Trout Fry Syndrome (RTFS) from reproductive fluids and egg surfaces of rainbow trout (*Oncorhynchus mykiss*). Bull Eur Assoc Fish Pathol 1996;16(2):63–7.
[51] Taylor PW. Detection of *Flavobacterium psychrophilum* in eggs and sexual fluids of pacific salmonids by a polymerase chain reaction assay: Implications for vertical transmission of bacterial coldwater disease. J Aquat Anim Health 2004;16:104–8.
[52] Ekman E, Borjeson H, Johansson N. *Flavobacterium psychrophilum* in Baltic salmon *Salmo salar* brood fish and their offspring. Dis Aquat Organ 1999;37(3):159–63.
[53] Kumagai A, Takahashi K, Yamaoka S, Wakabayashi H. Ineffectiveness of iodophore treatment in disinfecting salmonid eggs carrying *Cytophaga psychrophila*. Fish Pathol 1998;33(3):123–8.
[54] Kumagai A, Yamaoka S, Takahashi K, Fukuda H, Wakabayashi H. Waterborne transmission of *Flavobacterium psychrophilum* in coho salmon eggs. Fish Pathol 2000;35(1):25–8.
[55] Ranfdale RE, Richards RH, Alderman DJ. Colonisation of eyed rainbow trout ova with *Flavobacterium psychrophilum* leads to rainbow trout fry syndrome in fry. Bull Eur Assoc Fish Pathol 1997;17:108–11.
[56] Anderson JI, Conroy DA. The pathogenic myxobacteria with special reference to fish diseases. J Appl Bacteriol 1969;32(1):30–9.
[57] Bernardet JF, Kerouault B. Phenotypic and genomic studies of ''*Cytophaga psychrophila*'' isolated from diseased rainbow trout (*Oncorhynchus mykiss*) in France. Appl Environ Microbiol 1989;55(7):1796–800.
[58] Cipriano RC, Schill WB, Teska JD, Ford LA. Epizootiological study of bacterial cold-water disease in pacific salmon and further characterization of the etiologic agent, *Flexibacter psychrophila*. J Aquat Anim Health 1996;8:28–36.
[59] Rangdale RE, Richards RH, Alderman DJ. Minimum inhibitory concentrations of selected antimicrobial compounds against *Flavobacterium psychrophilum* the causal agent of Rainbow Trout Fry Syndrome (RTFS). Aquaculture 1997;158(3–4):193–201.
[60] Lorenzen E. The importance of the brand of the beef extract in relation to the growth of *Flexibacter psychrophilus* in Anacker and Ordals medium. Bull Eur Assoc Fish Pathol 1993;13(64):65.
[61] Obach A, Baudin Laurencin F. Vaccination of rainbow trout *Oncorhynchus mykiss* against the visceral form of coldwater disease. Dis Aquat Organ 1991;12(13):15.
[62] Daskalov H, Austin DA, Austin B. An improved growth medium for *Flavobacterium psychrophilum*. Lett Appl Microbiol 1999;28(4):297–9.
[63] Kumagai A, Nakayasu C, Oseko N. Effect of tobramycin supplementation to medium on isolation of *Flavobacterium psychrophilum* from *Ayu Plecoglossus altivelis*. Fish Pathol 2004;39:75–8.
[64] Bernardet JF, Grimont PAD. Deoxyribonucleic acid relatedness and phenotypic characterization of *Flexibacter columnaris* sp. nov., nom. rev., *Flexibacter psychrophilus* sp. nov., nom. rev., and *Flexibacter maritimus* Wakabayashi, Hikida and Masumura 1986. Int J Syst Bacteriol 1989;39(3):346–54.
[65] Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. Group 15 nonphotosynthetic, nonfruiting gliding bacteria. Bergey's manual of determinative bacteriology. 9th ed. Baltimore, MD: Williams & Wilkins; 1994. p. 483–514.
[66] Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn Jr WC. Color atlas and textbook of diagnostic microbiology. 4th ed. Philadelphia, PA: Lippincott Company; 1992.
[67] MacFaddin JF. Biochemical tests for identification of medical bacteria. 3rd ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2000.
[68] Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH. Manual of clinical microbiology. 7th ed. Washington, DC: American Society Microbiology; 1999.
[69] Schmidtke LM, Carson J. Characteristics of *Flexibacter psychrophilus* isolated from Atlantic salmon in Australia. Dis Aquat Organ 1995;21(2):157–61.
[70] Pacha RE. Characteristics of *Cytophaga psychrophila* (Borg) isolated during outbreaks of bacterial cold-water disease. Appl Microbiol 1968;16(1):97–101.
[71] Lindstrom NM, Call DR, House ML, Moffitt CM, Cain KD. A quantitative enzyme-linked immunosorbent assay and filtration-based fluorescent antibody test as potential tools to screen broodstock for infection with *Flavobacterium psychrophilum*. J Aquat Anim health 2009;21(1):43–56.
[72] Madetoja J, Wiklund T. Detection of the fish pathogen *Flavobacterium psychrophilum* in water from fish farms. Syst Appl Microbiol 2002;25(2):259–66.
[73] Madetoja J, Dalsgaard I, Wiklund T. Occurrence of *Flavobacterium psychrophilum* in fish-farming environments. Dis Aquat Organ 2002;52(2):109–18.
[74] Vatsos IN, Thompson KD, Adams A. Colonization of rainbow trout, *Oncorhynchus mykiss* (Walbaum), eggs by *Flavobacterium psychrophilum*, the causative agent of rainbow trout fry syndrome. J Fish Dis 2006;29(7):441–4.
[75] Lorenzen E, Karas N. Detection of *Flexibacter psychrophilus* by immunofluorescence in fish suffering from fry mortality syndrome: A rapid diagnostic method. Dis Aquat Org 1992;13:231–4.
[76] Crump EM, Perry MB, Gale S, Crawford E, Kay WW. Lipopolysaccharide O-antigen antibody-based detection of the fish pathogen *Flavobacterium psychrophilum*. J Mol Microbiol Biotechnol 2003;6(3–4):182–90.
[77] Misaka N, Nishizawa T, Yoshimizu M. Quantitative detection of viable *Flavobacterium psychrophilum* in chum salmon *Oncorhynchus keta* by colony blotting and immunostaining. Fish Pathol 2008;43(3):117–23.
[78] Baliarda A, Faure D, Urdaci MC. Development and application of a nested PCR to monitor brood stock salmonid ovarian fluid and spleen for detection of the fish pathogen *Flavobacterium psychrophilum*. J Appl Microbiol 2002;92(3):510–6.
[79] Cepeda C, Santos Y. Rapid and low-level toxic PCR-based method for routine identification of *Flavobacterium psychrophilum*. Int Microbiol 2000;3(4):235–8.
[80] Chen YC, Davis MA, LaPatra SE, Cain KD, Snekvik KR, Call DR. Genetic diversity of *Flavobacterium psychrophilum* recovered from commercially raised rainbow trout, *Oncorhynchus mykiss* (Walbaum), and spawning coho salmon, *O. kisutch* (Walbaum). J Fish Dis 2008;31(10):765–73.
[81] Crumlish M, Diab AM, George S, Ferguson HW. Detection of the bacterium *Flavobacterium psychrophilum* from a natural infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum), using formalin-fixed, wax-embedded fish tissues. J Fish Dis 2007;30(1):37–41.
[82] Del Cerro A, Marquez I, Guijarro JA. Simultaneous detection of *Aeromonas salmonicida*, *Flavobacterium psychrophilum*, and *Yersinia ruckeri*, three major fish pathogens, by multiplex PCR. Appl Environ Microbiol 2002;68(10):5177–80.
[83] Del Cerro A, Mendoza MC, Guijarro JA. Usefulness of a TaqMan-based polymerase chain reaction assay for the

detection of the fish pathogen *Flavobacterium psychrophilum*. J Appl Microbiol 2002;93(1):149–56.
[84] Elsayed EE, Eissa AE, Faisal M. Isolation of *Flavobacterium psychrophilum* from sea lamprey, *Petromyzon marinus L*. with skin lesions in Lake Ontario. J Fish Dis 2006;29(10):629–32.
[85] Izumi S, Aranishi F, Wakabayashi H. Genotyping of *Flavobacterium psychrophilum* using PCR–RFLP analysis. Dis Aquat Organ 2003;56(3):207–14.
[86] Lönnström LG, Hoffrén ML, Wiklund T. *Flavobacterium psychrophilum* associated with mortality of farmed perch, *Perca fluviatilis* L. J Fish Dis 2008;31(10):793–7.
[87] Taylor PW, Winton JR. Optimization of nested polymerase chain reaction assays for identification of *Aeromonas salmonicida*, *Yersinia ruckeri*, and *Flavobacterium psychrophilum*. J Aqua Anim Health 2002;14(3):216–24.
[88] Urdaci MC, Chakroun C, Faure D, Bernardet JF. Development of a polymerase chain reaction assay for identification and detection of the fish pathogen *Flavobacterium psychrophilum*. Res Microbiol 1998;149(7): 519–30.
[89] Wiklund T, Madsen L, Bruun MS, Dalsgaard I. Detection of *Flavobacterium psychrophilum* from fish tissue and water samples by PCR amplification. J Appl Microbiol 2000;88(2): 299–307.
[90] Suzuki K, Arai H, Kuge T, Katagiri T, Izumi S. Reliability of PCR methods for the detection of *Flavobacterium psychrophilum*. Fish Pathol 2008;43(3):124–7.
[91] Duchaud E, Boussaha M, Loux V, Bernardet JF, Michel C, Kerouault B, et al. Complete genome sequence of the fish pathogen *Flavobacterium psychrophilum*. Nat Biotechnol 2007;25(7):763–9.
[92] Fogel GB, Collins CR, Li J, Brunk CF. Prokaryotic genome size and SSU rDNA copy number: Estimation of microbial relative abundance from a mixed population. Microb Ecol 1999;38(2):93–113.
[93] Bertolini JM, Wakabayashi H, Watral VG, Whipple MJ, Rohovec JS. Electrophoretic detection of proteases from selected strains of *Flexibacter psychrophilus* and assessment of their variability. J Aquat Anim Health 1994;6(3):224–33.
[94] Avendaño-Herrera R, Araya P, Fernández J. Molecular analysis of *Flavobacterium psychrophilum* isolates from salmonid farms in Chile. Bull Eur Assoc Fish Pathol 2009;29(6):184–92.
[95] Hadidi S, Glenney GW, Welch TJ, Silverstein JT, Wiens GD. Spleen size predicts resistance of rainbow trout to *Flavobacterium psychrophilum* challenge. J Immunol 2008;180(6):4156–65.
[96] Villarroel F, Casado A, Vásquez J, Matamala E, Araneda B, Amthauer R, et al. Serum amyloid A: A typical acute-phase reactant in rainbow trout. Dev Comp Immunol 2008;32(10):1160–9.
[97] LaFrentz BR, LaPatra SE, Jones GR, Cain KD. Passive immunization of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Flavobacterium psychrophilum*, the causative agent of bacterial coldwater disease and rainbow trout fry syndrome. J Fish Dis 2003;26(7):377–84.
[98] Dumetz F, Duchaud E, LaPatra SE, Le Marrec C, Claverol S, Urdaci MC, et al. A protective immune response is generated in rainbow trout by an OmpH-like surface antigen (P18) of *Flavobacterium psychrophilum*. Appl Environ Microbiol 2006;72(7):4845–52.
[99] Madetoja J, Lönnström LG, Björkblom C, Uluköy G, Bylund G, Syvertsen C, et al. Efficacy of injection vaccines against *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis 2006;29(1):9–20.
[100] Rahman MH, Kuroda A, Dijkstra JM, Kiryu I, Nakanishi T, Ototake M. The outer membrane fraction of *Flavobacterium psychrophilum* induces protective immunity in rainbow trout and ayu. Fish Shellfish Immunol 2002;12(2):169–79.
[101] Aoki M, Kondo M, Nakatsuka Y, Kawai K, Oshima SI. Stationary phase culture supernatant containing membrane vesicles induced immunity to rainbow trout *Oncorhynchus mykiss* fry syndrome. Vaccine 2007;25(3):561–9.
[102] Sudheesh PS, LaFrentz BR, Call DR, Siems WF, LaPatra SE, Wiens GD, et al. Identification of potential vaccine target antigens by immunoproteomic analysis of a virulent and a non-virulent strain of the fish pathogen *Flavobacterium psychrophilum*. Dis Aquat Organ 2007;74(1):37–47.
[103] LaFrentza BR, LaPatrab SE, Callc DR, Cain KD. Isolation of rifampicin resistant *Flavobacterium psychrophilum* strains and their potential as live attenuated vaccine candidates. Vaccine 2008;26(44):5582–9.
[104] Álvarez B, Álvarez J, Menéndez A, Guijarro JA. A mutant in one of two exbD loci of a TonB system in *Flavobacterium psychrophilum* shows attenuated virulence and confers protection against cold water disease. Microbiology 2008;154(4):1144–51.
[105] LaFrentz BR, Lindstrom NM, LaPatra SE, Call DR, Cain KD. Electrophoretic and Western blot analyses of the lipopolysaccharide and glycocalyx of *Flavobacterium psychrophilum*. Fish Shellfish Immunol 2007;23(4):770–80.
[106] Martínez JL, Casado A, Enríquez R. Experimental infection of *Flavobacterium psychrophilum* in fins of Atlantic salmon *Salmo salar* revealed by scanning electron microscopy. Dis Aquat Organ 2004;59(1):79–84.
[107] Wedemeyer GA. Fish hatchery management. 2nd ed. Bethesda, MD: American Fisheries Society; 2001.
[108] Nematollahi A, Decostere A, Pasmans F, Ducatelle R, Haesebrouck F. Adhesion of high and low virulence *Flavobacterium psychrophilum* strains to isolated gill arches of rainbow trout *Oncorhynchus mykiss*. Dis Aquat Organ 2003;55(2):101–7.
[109] Bebak JA, Welch TJ, Starliper CE, Baya AM, Garner MM. Improved husbandry to control an outbreak of rainbow trout fry syndrome caused by infection with *Flavobacterium psychrophilum*. J Am Vet Med Assoc 2007;231(1):114–6.
[110] Cipriano RC, Novak BM, Flint DE, Cutting DC. Reappraisal of the federal fish health recommendation for disinfecting eggs of Atlantic salmon in iodophor. J Aquat Anim Health 2001;13(4):320–7.
[111] Bruun MS, Schmidt AS, Madsen L, Dalsgaard I. Antimicrobial resistance patterns in Danish isolates of *Flavobacterium psychrophilum*. Aquaculture 2000;187(3–4):201–12.
[112] Schmidt AS, Bruun MS, Dalsgaard I, Pedersen K, Larsen JL. Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms. Appl Environ Microbiol 2000;66(11):4908–15.
[113] Hesami S, Allen KJ, Metcalf D, Ostland VE, MacInnes JI, Lumsden JS. Phenotypic and genotypic analysis of *Flavobacterium psychrophilum* isolates from Ontario salmonids with bacterial coldwater disease. Can J Microbiol 2008;54(8):619–29.
[114] Lumsden JS, Ostland VE, Ferguson HW. Necrotic myositis in cage cultured rainbow trout, *Oncorhynchus mykiss* (Walbaum), caused by *Flexibacter psychrophilus*. J Fish Dis 1996;19(2):113–9.
[115] Ostland VE, Byrne PJ, Lumsden JS, MacPhee DD, Derksen JA, Haulena M, et al. Atypical bacterial gill disease: A new form of bacterial gill disease affecting intensively reared salmonids. J Fish Dis 1999;22(5):351–8.
[116] Bustos PA, Calbuyahue J, Montana J, Opazo B, Entrala P, Solervicens R. First isolation of *Flexibacter psychrophilus*, as causative agent of Rainbow Trout Fry Syndrome (RTFS),

producing rainbow trout mortality in Chile. Bull Eur Assoc Fish Pathol 1995;15:162–4.
[117] Valdebenito S, Avendaño-Herrera R. Phenotypic, serological and genetic characterization of *Flavobacterium psychrophilum* strains isolated from salmonids in Chile. J Fish Dis 2009;32(4):321–33.
[118] Madetoja J, Hänninen ML, Hirvelä-Koski V, Dalsgaard I, Wiklund T. Phenotypic and genotypic characterization of *Flavobacterium psychrophilum* from Finnish fish farms. J Fish Dis 2001;24(8):469–79.
[119] Wiklund T, Kaas K, Lonnstrom L, Dalsgaard I. Isolation of *Cytophaga psychrophila* (*Flexibacter psychrophilus*) from wild and farmed rainbow trout. Bull. Eur Assoc Fish Pathol 1994;14(2):44–6.
[120] Lehmann J, Mock D, Sturenberg FJ, Bernardet JB. First isolation of *Cytophaga psychrophila* from a systemic disease in eel and cyprinids. Dis Aquat Organ 1991;10:217–20.
[121] Weis J. A cold water disease in rainbow trout (Ueber das Vorkommen einer Kaltwasserkrankheit bei Regenbogenforellen Salmo gairdneri). Tieraerztl Umsch 1987;42:575–8.
[122] Iida Y, Mizokami A. Outbreaks of coldwater disease in wild ayu and pale chub. Fish Pathol 1996;31(3):157–64.
[123] Wakabayashi H, Toyama T, Iida T. A study on serotyping of *Cytophaga psychrophila* isolated from fishes in Japan. Fish Pathol 1994;29(2):101–4.
[124] Lee KB, Heo GJ. First isolation and identification of *Cytophaga psychrophila* from cultured Ayu in Korea. Fish Pathol 1998;33(1):37–8.
[125] Ferguson HW, Girons A, Rizgalla G, LaPatra S, Branson EJ, MacKenzie K, et al. Strawberry disease in rainbow trout in Scotland: Pathology and association with *Flavobacterium psychrophilum*. Vet Rec 2006;158(18):630–1.
[126] Cepeda C. García-Márquez S, Santos Y. Improved growth of *Flavobacterium psychrophilum* using a new culture medium. Aquaculture 2004;238(1–4):75–82.
[127] Kum C, Kirkan S, Sekkin S, Akar F, Boyacioglu M. Comparison of *in vitro* antimicrobial susceptibility in *Flavobacterium psychrophilum* isolated from rainbow trout fry. J Aquat Anim Health 2008;20(4):245–51.
[128] Cipriano RC, Ford LA, Teska JD. Association of *Cytophaga psychrophila* with mortality among eyed eggs of Atlantic salmon (*Salmo salar*). J Wildlife Dis 1995;31(2):166–71.
[129] Rucker RR, Earp BJ, Ordal EJ. Infectious diseases of Pacific salmon. Trans Am Fish Soc 1953;83:297–312.
[130] LaFrentz BR, LaPatra SE, Call DR, Wiens GD, Cain KD. Proteomic analysis of *Flavobacterium psychrophilum* cultured *in vivo* and in iron-limited media. Dis Aquat Organ 2009;87(3):171–82.