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Emergence of cold water strawberry disease of rainbow trout *Oncorynchus mykiss* in England and Wales: outbreak investigations and transmission studies

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ABSTRACT: Cold water strawberry disease (CWSD), or red mark syndrome (RMS), is a severe dermatitis affecting the rainbow trout *Oncorynchus mykiss*. The condition, which presents as multifocal, raised lesions on the flanks of affected fish, was first diagnosed in Scotland in 2003 and has since spread to England and Wales. Results of field investigations indicated the condition had an infectious aetiology, with outbreaks in England linked to movements of live fish from affected sites in Scotland. Transmission trials confirmed these results, with 11 of 149 and 106 of 159 naïve rainbow trout displaying CWSD-characteristic lesions 104 to 106 d after being cohabited with CWSD-affected fish from 2 farms (Farm B from England and Farm C from Wales, respectively). The condition apparently has a long latency, with the first characteristic lesions in the previously naïve fish not definitively observed until 65 d (650 day-degrees) post-contact with affected fish. Affected fish from both outbreak investigations and the infection trial were examined for the presence of viruses, oomycetes, parasites and bacteria using a combination of techniques and methodologies (including culture-independent cloning of PCR-amplified bacterial 16S rRNA genes from lesions), with no potentially causative infectious agent consistently identified. The majority of the cloned phylotypes from both lesion and negative control skin samples were assigned to *Acidovorax*-like β-Proteobacteria and *Methylobacterium*-like α-Proteobacteria.

KEY WORDS: RFLP · 16S rRNA · Clone library · *Flavobacterium psychrophilum* · Red mark syndrome

INTRODUCTION

Red mark syndrome (RMS), otherwise known as UK cold water strawberry disease (CWSD), is a disease of the rainbow trout *Oncorhynchus mykiss* (Walbaum), characterised by the appearance of single to multiple ulcerated skin swellings, of varying intensity, on the flanks of affected fish (Ferguson et al. 2006, Verner-Jeffreys et al. 2006).

First noted in Scotland in 2003, it shares some similarities with a warmer water form of strawberry disease (SD), as previously described in the UK and Europe (Fleury et al. 1985, St-Hilaire & Jeffery 2004). It has recently been proposed that the warmer water form of the disease be renamed warm water strawberry disease (WWSD) (Ferguson et al. 2006). Histologically, CWSD and WWSD are distinct only during the early phase of infection (before the epidermis is breached), and both conditions apparently respond to treatment with antibiotics, such as oxytetracycline (OTC). However, in the UK, differences between the 2 diseases, in terms of both their epidemiology (particularly the temperatures at which they are seen) and pathological effects, are sufficient to regard CWSD as a separate condition (Ferguson et al. 2006).

In early 2005, CWSD was diagnosed for the first time in fish farmed in England at a temperature below 14°C. Farmers in both Scotland and England

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report that the disease is prevalent at low water temperatures (<15°C), in comparison to the UK experience of WWSD, which generally occurs only when water temperatures exceed 14°C (a summer rather than winter condition). Early signs of CWSD can include severe scale loss prior to the emergence of the characteristic external lesions (Ferguson et al. 2006), and there are no signs of systemic infection (i.e. no affect on appetite, growth or mortality). However, the condition causes losses to farmers both in treatment costs and in downgrading of affected fish at harvest.

The objective of the present study was to determine if the disease had an infectious aetiology by conducting a disease investigation on farms affected by the condition. Laboratory trials evaluating whether the condition could be transmitted from affected to naïve fish were also conducted. As a recent report implicated *Flavobacterium psychrophilum* as potentially being linked to the condition (Ferguson et al. 2006), particular effort was made to identify whether this, or a closely related organism, was associated with diseased fish.

MATERIALS AND METHODS

Outbreak investigations

Outbreaks of CWSD at 3 farms in England and Wales were investigated between January 2005 and January 2006. Structured interviews with the farmers asked about the chronology of the disease outbreak, the sources and timing of live fish introductions and the spread of the disease in time and space within the affected farm. Using a diagram of the layout of the farm, these data were used to map the spread of disease among units (e.g. raceways) on the farm over time in relation to the timing of fish introductions and units where they were introduced. This descriptive analysis was used to identify potential routes of the spread of disease. Farms A and B were part of the same South of England business, producing ca. 600 tonnes and 250 tonnes of rainbow trout per year, respectively. Both farms received fingerlings (5 to 40 g) from external hatcheries in late August to September for subsequent ongrowing, predominantly for the table market. Both farms used concrete raceway systems, drawing and discharging their water to and from the same chalk stream river, with Farm B upstream of Farm A. Neither farm disinfected this water supply to remove pathogens. Both production sites maintained dissolved oxygen levels at 12 ppm at the intake to 7 ppm at the outflow by oxygenation. Water was reused up to 4 times on both sites.

Farm C, located in Wales, produced ca. 100 tonnes of trout, rearing its own fry from eggs. The farm drew and discharged water from the point of a neighbouring river that was fed with water discharging from neighbouring sandstone hill valleys; pH levels were typically 7.2 to 7.4 (sometimes higher if lime applied to pasture was washed off into the river). This water supply was not disinfected before entering the culture units. The river contained largely native brown trout and was not accessible to migratory salmonids. Each batch of hatched fry was moved sequentially through the production system until the fry were introduced into a single large maze-shaped raceway at ca. 100 g. Batch separation in the maze was achieved using screens. Stocking densities were between 40 and 110 kg m^3 on all 3 sites. At each visit, 10 fish with CWSD were examined and samples taken for diagnostic testing (bacteriology, mycology, virology, histopathology and parasitology).

Transmission experiments

Fish. Two of the farms visited, Farms B and C, provided affected fish for the transmission studies. CWSD-affected rainbow trout (ca. 450 g) were taken to Cefas Weymouth Laboratory (CWL). Between 5 and 10 of the affected fish from each farm were, on arrival, killed with an overdose of benzocaine and methanol, examined, and samples taken for diagnostic testing.

Before use in cohabitation challenge experiments, rainbow trout (average ca. 40 g), previously determined to be free of obvious diseases following a health check, were kept in the biosecure experimental facility at CWL in flow-through 1000 l tanks. The naïve fish were all from the same stock of rainbow trout that had been reared at CWL from 5 g and came from a hatchery with no previous experience of CWSD. The source of the freshwater supplied to all tanks for the experiments was dechlorinated at 10 ± 0.5°C and supplied at 1 to 3 l min^{-1} from the laboratory's potable water supply, taken from chalk and limestone boreholes 10 km from the laboratory. All tanks were maintained as separate flow-to-waste systems (with effluent water treated by ozonation prior to discharge). As part of CWL-standardised procedures, strict biosecurity was implemented to minimise the risks of transfer of potentially infectious material among tanks (e.g. the tanks had lids and there were dedicated nets and tank cleaning equipment for each tank).

Cohabitation challenges. Naïve rainbow trout were challenged by exposure to fish displaying CWSD-characteristic lesions from Farms B and C (see Table 1). Circular tanks of 300 l capacity were bisected using mesh screens and 15 CWSD-affected fish placed into one side of the screen and 40 stock naïve fish placed into

the other side (indirect cohabitation). Four different cohabitation challenges were set up, in duplicate, using Farm B or C fish as the cohabitant source, with or without a surface-draining standpipe. The surface-draining standpipe allowed feed and faeces to accumulate in the tanks, better mimicking farm conditions. The CWSD-affected fish were held for 6 (first replicate) or 8 (second replicate) days after delivery from the farms before being put into indirect contact with naïve fish. A negative control tank containing 40 naïve fish, from the same stock as those used for the challenges, was also included. This tank was stocked at the same time as the challenge tanks were stocked, with naïve fish situated next to challenge tanks, and was also bisected by a mesh screen under the standpipe regime. The CWSD-affected fish were removed from the challenge tanks 62 (first set of replicates) and 72 d (second set) after challenge initiation and visually examined for signs of CWSD. At the end of the 104 to 106 d post-challenge observation period, the survivors of each of the challenged groups were killed using an overdose of benzocaine/methanol (>40 ppm benzocaine), examined and sampled.

Bacteriology. Swabs were taken from the edge of lesions, or any other abnormal tissue, from a range of samples collected as part of the outbreak investigations and infection trials, and spread onto Tryptone Soy agar (TSA), Coomasie Blue agar (COO) (Markwardt et al. 1989) and a modified version of Anacker and Ordal agar (RIVAOA, Lorenzen 1993) prepared with river water (chalk stream). Samples from the initial field investigations were also plated onto other media, including TSA 8% defibrinated sheep blood (Haem). For the infection trials (including the initial examination of fish used as an infection source, see Table 2) a particular effort was also made to sample from only the interior of the lesions, following swabbing and cleaning of the surface with ethanol.

Plates were incubated aerobically at 15°C for up to 28 d. Representative colonies of dominant bacteria were subcultured onto TSA, from which subsequent identification tests were performed. Isolates were initially grouped on the basis of morphology, Gram stain, motility, catalase, cytochrome oxidase activity and glucose oxidation or fermentation (primary tests, Buller 2004). For outbreak investigations, isolates were initially identified on the basis of phenotypic tests (API 20 NE, Biomerieux), following the manufacturer's instructions and comparing resultant profiles to both the API 20 NE database and profiles of other bacteria isolated in the laboratory from diseased rainbow trout. Isolates were stored on Protect® beads (Technical Service Consultants Limited) at −80°C, according to the manufacturer's instructions.

Molecular bacteriology. ***General DNA extraction and PCR conditions:*** Bacterial cells were lysed by heatshock (94°C for 5 min then immediately placed on ice) to obtain template DNA. Template DNA from tissue samples was prepared as follows: 50 ± 5 mg tissue was weighed into 2 ml microtubes containing lysing matrix D (MP Biomedical), 1.5 ml sterile phosphate buffered saline (pH 7.2) added and samples homogenised using a Fastprep FP120 (MP Biomedical). DNA was extracted from a 0.5 ml volume using DNAzol (Life Technologies), according to the manufacturer's instructions, and washed twice in ethanol (99 and 70%, respectively). Pellets were redissolved in 50 µl sterile distilled water, heated in a hotblock for 5 min at 65°C and vortexed for 30 s. DNA templates were then stored at −20°C.

Unless otherwise described, amplifications were carried out as follows: 50 µl PCR reaction mixtures contained sterile molecular-grade water, 1× reaction buffer, 1.5 mM magnesium chloride, 1.25 U (0.25 µl) Go Taq polymerase (Promega), 0.25 mM deoxyribonucleotide triphosphate (dNTPs) and 50 pmol of each primer. Then 2.5 µl of template was added to the reaction mixture, and samples were heated at 94°C for 5 min in a PTC-225 Peltier thermocycler (MJ Research). Subsequently, 40 cycles of the following were carried out: denaturation at 95°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1 min, with a final elongation at 72°C for 10 min.

Culture-dependent bacterial DNA analysis: A selection of the organisms from the outbreak investigations and isolates from cohabitation challenges (80 in total) were also characterised genetically using Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP) analysis after Lebaron et al. (1998), with minor modifications. Approximately 1300 bp partial 16S rRNA gene sequences were PCR amplified, as described by Pond et al. (2006) for cloning from gut samples, with the general conserved eubacterial primers 63F (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1387R (5′-GGG CGG WGT GTA CAA GGC-3′) (Marchesi et al. 1998), with the exception that the reaction mixtures contained Go Taq (Promega). The resultant PCR products were digested with the restriction enzyme *Hae*III (Promega). The digested products were then resolved on 2% TBE agarose gels. Banding patterns were visualised under UV light and a digital image taken. Their RFLP patterns were then compared visually (samples with identical profiles were grouped together and a further gel was run with grouped samples in adjacent lanes) using the Bionumerics software package (Applied Maths). Isolates from each sample were then placed into groupings based on their colony morphologies, RFLP patterns and results of primary testing (Gram stain, motility, oxidation/fermentation, oxidation cytochrome oxidase and catalase produc-

tion, Buller 2004). Confirmatory 16S rRNA sequence analysis (Pond et al. 2006) was then carried out on representatives of the distinct *Hae* III RFLP morphotype groupings identified. All colonies resembling *Flavobacterium* sp. (orange-yellow pigmented colonies) were subjected to *F. psychrophilum* PCR, as described later.

Culture-independent bacterial DNA analysis: Eight samples of skin and muscle tissue from lesions or from unaffected tissue (negative control), previously stored in absolute ethanol at 4°C and representing groups from the cohabitation challenges, were processed for culture-independent bacterial DNA analysis (see Table 4).

Partial 16S rRNA genes were amplified from the resultant DNA templates and cloned into competent *Escherichia coli* cells (Promega) using the procedures described by Pond et al. (2006). Plasmid DNA was isolated from up to 30 resultant clones per sample (198 total) and RFLP analysis carried out following digestion with *Hae*III. Restriction digests were run on a 2% agarose gel (120 V, 25 min, 100 bp ladder). At least 2 representatives from each group were sequenced and compared with database reference sequences using the Sequence Match software from the Ribosomal Database Project II website (rdp.cme.msu.edu/ index.jsp).

Flavobacterium psychrophilum PCR: Single-round and nested *F. psychrophilum* PCR was carried out on a range of lesion and skin samples and a selection of bacterial colonies isolated from fish with lesions at the end of the cohabitation trial. Nested PCR was carried out using a modified version of the protocol described by Ferguson et al. (2006) for use with wax-embedded fixed material and using 16S rRNA universal primers (as used for PCR-RFLP) in the first round, with 16S rRNA primers specific to *F. psychrophilum*, designated PSY1 (5′-CGATCCTACTTGCGTAG-3′) and PSY2 (5′-GTTGGCATCAACACACT-3′) (Toyama et al. 1994) for the second round.

The National Collection of Industrial and Marine Bacteria (NCIMB) type strain of *Flavobacterium psychrophilum* (NCIMB 1947) was used as a positive control. *F. psychrophilum* PCR was carried out on DNA extracted from any bacterial colony with a similar morphology to *F. psychrophilum* (orange-yellow) along with a selection of others (negative controls). The tissue samples tested included skin and muscle taken from Farm B and C fish populations used for cohabitation trials, as well as lesions and unaffected skin samples taken from negative control and previously naïve fish showing lesion samples at the end of the trial. The tissue samples were stored in either absolute ethanol at 4°C (samples collected at the end of the trial) or at –70°C (samples collected from affected fish at the beginning of the trial) prior to DNA extraction as described earlier for culture-independent bacterial analysis.

Mycology. Swabs were taken from the edge of a lesion or any other abnormal tissue and spread onto Isolation Media (IM) (OIE 2006) to detect oomycetes. All media and components were purchased from Oxoid and prepared in-house. Plates were incubated aerobically at 7°C for up to 28 d.

Virology. Samples from skin, brain, spleen and/or kidney, were extracted using standard techniques, diluted at 1:100 and 1:1000 and inoculated onto the following cell lines: fat head minnow (FHM), bluegill fin (BF-2), epithelial papilloma carp (EPC), chinook salmon embryo (CHSE-214), salmon head kidney (SHK), rainbow trout gonad (RTG-2) and salmon kidney (TO). After 14 d incubation at 15°C and at 10°C (FHM, CHSE, SHK, RTG and TO) or 7 d incubation at 15°C (BF-2 and EPC), monolayers showing no cytopathic effect (cpe) were diluted to 1:10 and 1:100 and subcultured onto fresh cells and incubated for a further 14 or 7 d, respectively. Cell monolayers were examined daily for evidence of a cytopathic effect using a Nikon inverted microscope.

Histopathology investigations. Tissue samples were obtained from gill, skin/muscle, heart, liver, spleen, kidney, gut (stomach, pyloric caecae and pancreas) and brain, fixed in 10% neutral buffered formalin and embedded in wax using standard techniques. Sections were stained with haematoxylin and eosin (H&E), Gram, Periodic Acid Schiff and Giemsa.

Transmission electron microscopy (TEM). Skin and muscle samples were taken from 4 fish with clinical signs of CWSD and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature. Fixed tissue samples were rinsed in 0.1 M sodium cacodylate buffer (pH 7.4) and post-fixed for 1 h in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Specimens were washed in 3 changes of 0.1 M sodium cacodylate buffer before dehydration through a graded acetone series. Specimens were embedded in Agar 100 epoxy (Agar Scientific, Agar 100 pre-mix kit medium) and polymerised overnight at 60°C in an oven. Semi-thin (1 to 2 µm) sections were stained with Toluidine Blue for viewing with a light microscope to identify suitable target areas. Ultrathin sections (70 to 90 nm) of these areas were mounted on uncoated copper grids and stained with 2% aqueous uranyl acetate and Reynolds' lead citrate (Reynolds 1963). Grids were examined using a JEOL JEM 1210 transmission EM and digital images captured using a Gatan Erlangshen ES500W camera and Gatan Digital Micrograph™ software.

Scanning electron microscopy (SEM). Skin and muscle samples were taken from 4 fish with clinical signs of CWSD and fixed in 2.5% glutaraldehyde in

0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature. Fixed tissue samples were rinsed in 0.1M sodium cacodylate buffer (pH 7.4) and post-fixed for 1 h in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Specimens were washed in 3 changes of 0.1 M sodium cacodylate buffer before dehydration through a graded acetone series. Samples were critical point dried using an Emitech K850 critical point dryer and mounted onto aluminium stubs. Samples were coated in 5 nm of gold using an Emitech K550 sputter coater. Stubs were examined using a JEOL JSM 5200 scanning electron microscope and digital images captured using JEOL SemAphore software.

Data analysis. Stata version 9.2 (StataCorp) was used to display and analyse data from the cohabitation trial, using bar, box and scatter graphs and ANOVA. As the main results (see Table 1) were clear cut, formal analysis using, for example, a generalized linear model, was rendered superfluous.

Database sequence submissions. The partial 16S rRNA gene sequences of a number of bacterial isolates, as well as partial 16S rRNA genes of bacteria amplified directly from tissue samples, were deposited in EMBL under the following accession numbers: AM779863 to AM779887.

RESULTS

Outbreak investigations

Clinical signs

Affected fish displayed dermatitis with lesions of varying sizes from 5 mm to several centimetres in diameter. Early-stage lesions appeared as pale yellow/grey patches with reddening in the central regions, which subsequently increased in area and became bright red in colour. If lesions were multifocal, they were present on the flanks, dorsal and ventral surfaces, as opposed to the head or fins. Eventually they were raised above the surrounding tissue.

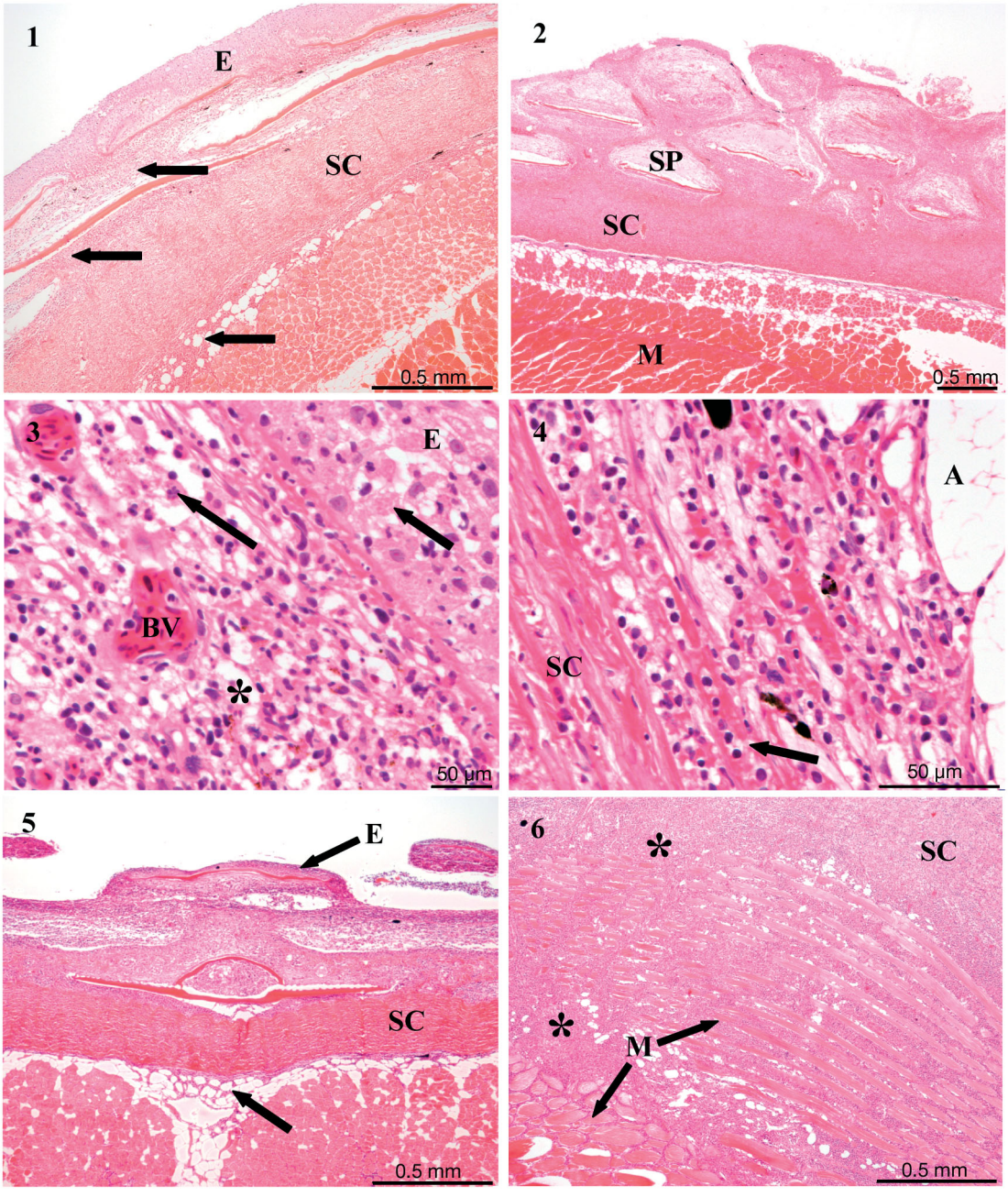
Histopathological features and ultrastructure

Skin and muscle sections taken from affected fish tissue displayed a range of severity in the degree and extent of dermatitis. In mild cases, where there was minimal discolouration or lifting of the scales, the epidermis remained intact and inflammatory cells, predominantly lymphocytes, were observed in scale pockets and below the stratum compactum (SC) of the dermis (Fig. 1). Relatively few lymphocytes were present in the SC itself. In more severe cases, there was extensive inflammation and thickening of the dermal layers, including the SC. Scale pockets were enlarged and filled with pale eosinophilic, presumably proteinaceous, fluid and inflammatory cells (Fig. 2). Frequently there was dissolution of the scales associated with the presence of multinucleate osteoclast cells. In advanced cases phagocytic cells, particularly neutrophils, increased in number amongst the inflammatory cells in the connective tissues immediately below the epidermis (Fig. 3) and between the SC and underlying adipose tissue (Fig. 4). In both locations, disintegration of connective tissue and cellular necrosis was evident (Fig. 4). In many cases, lymphocytic infiltration of the underlying musculature was seen, where individual muscle fibres became isolated and in some cases necrotic. Inflammatory lesions were seen in other organs, including the heart (epicarditis and cardiomyositis), intestinal smooth muscle and connective tissues. Ultrastructure of affected skin samples from severe cases confirmed the presence of cellular necrosis, both at the surface of the lesion (SEM, not shown) and within the dermis, where disassociated connective tissue fibres were also noted. No evidence of infectious agents was seen by EM or light microscopy.

Epidemiology

On Farms A and B the condition first emerged in January to April 2005 and originated in stocks of fish sourced from the same Scottish hatchery the previous September to October. This hatchery had, itself, suffered CWSD outbreaks in the past. It was also reported to Fisheries Research Services (FRS) Aberdeen by industry representatives (D. Pendrey pers. comm.) that at least 10 other sites in Scotland supplied with fry by the same Scottish hatchery subsequently experienced outbreaks of CWSD. Farm C in Wales was first affected in September 2005. There were indications that the condition spread from imported, infected stock to other batches of fish on Farms A and B and to stocks on 2 other farms supplied with fish from Farm C. Farm C reportedly allowed no live fish movements onto the site and used externally supplied batches of trout eggs that were disinfected (hydrogen peroxide- and iodophore-treated) upon arrival. It was also reported that there had been an introduction of farmed brown trout from southern England the previous April into the neighbouring river, but there was no evidence linking these fish to any farms known to have affected fish.

On Farm C, stocks reportedly only developed clinical signs after they entered the maze system. No fish in other parts of the system (hatchery or nursery raceways that were also supplied with river water) were affected. Development of the condition within a batch



Figs. 1 to 4. *Oncorhynchus mykiss*. Clinical cases from farm situations. All sections are stained with haematoxylin and eosin. Fig. 1. Section from a fish displaying moderate clinical signs. The epithelium (E) is intact and influx of inflammatory cells can be seen between the scales and below the stratum compactum (SC) (arrows). Bar = 0.5 mm. Fig. 2. Transverse section through the skin and muscle of a rainbow trout displaying marked clinical signs of cold water strawberry disease (CWSD). Note the marked inflammation and thickening of the dermal layers, including the stratum compactum (SC) and enlarged scale pockets (SP). Portions of the epithelium remain intact. The underlying musculature (M) appears unaffected. Bar = 0.5 mm. Fig. 3. Section at the base of the epidermis (E) of a fish with clinical CWSD showing a marked influx of inflammatory cells (✻), including neutrophils (long arrow) and numerous lymphocytic cells. Blood vessels (BV) are congested and small amounts of pigment are present. Note the pale intracellular eosinophilic granules in the epithelial cells (short arrow). Bar = 50 µm. Fig. 4. Section at the basal junction of the stratum compactum (SC) and adipose tissue (A). Marked inflammation and necrosis of the connective tissue is evident (arrow). Occasional melanocytes are present. Bar = 50 µm. Figs. 5 & 6. Fish experimentally exposed by cohabitation to CWSD-affected fish from Farm C. Fig. 5. Transverse section showing the typical inflammatory reaction extending from below the epidermis (E) to the adipose tissue (arrow) below the stratum compactum (SC). Bar = 0.5 mm. Fig. 6. Section through the dermis and muscle showing extensive infiltration of inflammatory cells (✻) within the stratum compactum (SC) and into the underlying musculature (M). Bar = 0.5 mm

Table 1. *Oncorynchus mykiss* (Walbaum) cold water strawberry disease (CWSD) transmission trial results. Proportion of previously naïve rainbow trout showing CWSD-characteristic lesions 104 to 106 d after being put into contact with fish with CWSD from Farms B or C, with or without surface-draining standpipes in the tanks. Also included was a negative control group (fish from the same population of naïve fish used for the transmission treatments held in a separate tank). nd: not done; na: not applicable

Treatment Source of infected fish	Surface drain	Lesions 104 to 106 d post-challenge Replicate 1	Replicate 2	Mean %
Indirect cohabitation				
Farm B	Yes	5/34[a]	3/40	11%
Farm B	No	5/37	4/38[a]	12%
Farm C	Yes	23/40	35/39	73.4%
Farm C	No	25/40	23/40	60%
Negative control				
na	Yes	0/40	nd	0%

[a]Surviving fish in these tanks also had oedema at challenge termination

on all farms examined followed a characteristic pattern, with 10 to 60% of the fish developing clinical signs within 2 to 3 wk of first observation of the disease. The fish then appeared to recover from the syndrome and showed no signs of recrudescence, despite the observation that other stocks held on the same water supply subsequently developed the condition. The condition reportedly responded to treatment with a number of antibiotics (oxolinic acid, OTC and florfenicol). Bath treatment with Chloramine T and formalin was reportedly less effective, although rejection rates from processors were reduced (likely due to the resultant cosmetic improvement in the appearance of the fish). The affected fish on all farms presented with a range of lesions of differing severity.

Farmers reported that the condition developed when water temperatures were generally below 15°C. Water temperatures on Farm A were 6.6°C when it was visited 19 January 2005 and 11.9°C on 28 April 2005, while water temperature on Farm B was 12.2°C on 28 April 2005. Farm C water temperatures were 7.3°C when it was visited on 12 December 2005.

Cohabitation challenges

For the semi-indirect cohabitation treatments, survival in all the tanks was >85%. There were no signs of CWSD-like lesions in the naïve fish in any of the tanks until at least 65 d after the fish had been in contact with affected fish. However, by the time the trial was terminated, 18 of the surviving 151 naïve fish that had been exposed to CWSD-affected fish from Farm B (11.9%) and 106 of the 159 naïve fish that had been exposed to CWSD-affected fish from Farm C (66.7%) showed characteristic CWSD lesions (Table 1, Figs. 5–7). Buildup of feed and faeces in the experimental tanks did not appear to affect the rate of transmission, with no apparent difference in the proportion of fish displaying lesions 104 to 106 d post-challenge maintained in conventional flush-through systems, as opposed to tanks with surface-draining standpipes (Table 1).

The Farm B and C CWSD-affected fish were examined after they were removed from the tanks 62 and 72 d after introduction, respectively, and it was found that, apart from some limited superficial scarring on less than 10% of the fish, they all appeared to have recovered from the condition and were otherwise apparently very healthy. In all, only one of the fish that was used as a cohabitant disease source died (out of 120).

There was also evidence of another skin condition being transmitted in 2 of the tanks containing Farm B-affected fish. A high proportion of these presented with skin that was spongy in appearance and was haemorrhaging at trial termination. Histologically, there was oedema of the dermal layers, in particular of the connective tissues around the scales (not shown). Characteristic inflammation seen in fish with CWSD

Fig. 7. *Oncorhynchus mykiss*. Cold water strawberry disease (CWSD) lesions on naïve fish exposed to Farm C CWSD-affected fish at termination of infection trial (110 d post initiation of contact)

was largely absent. The fish in these tanks were also significantly emaciated (mean weight 95.6 g, SE = 3.32 g) compared to the other fish that were cohabited with Farm B-affected fish (137.2 g, SE = 3.37 g, $p < 0.001$). There was otherwise no significant difference among the weights of fish reared with or without standpipes or in contact with Farm B or C CWSD-affected fish. In comparison, average weights of the fish in the other tanks varied from 120 to 148 g.

Bacteriology

A range of different organisms was recovered from lesion material and other organs from the different samples (Table 2). Bacteria recovered on the different media used could all generally be subcultured without difficulty on TSA. Dominant morphotypes were characterised and more than 10 genetically distinct types of bacteria were isolated covering the genera *Flexibacter*, *Chryseobacter*, *Bacillus*, *Aeromonas* and *Psychrobacter* (Table 3). There was no evidence that a particular strain or species of culturable bacteria was associated with affected tissue from the range of different samples analysed, with similar organisms also recovered from the different organs sampled (data not shown). Similarly, there was wide within and between sample variation in the number of colonies cultured from swabs taken from different lesions. As would be expected for otherwise healthy fish, the majority of samples taken from internal organs were presumptively sterile (no or very few organisms recovered, data not shown). There was also wide variation in lesion samples examined, ranging from samples from which no TSA-culturable bacteria were isolated to heavy mixed growth. *Aeromonas salmonicida* subsp. *salmonicida* (confirmed by biochemical tests and latex agglutination, Bionor) was also isolated from an ulcerated lesion from a field-sampled fish from Farm A.

Culture-independent bacterial DNA analysis

Following plasmid extraction and digestion with *Hae*III, distinct RFLP profiles were identified from 198 clones. These each contained partial 16S rRNA genes PCR-amplified from DNA extracted from skin/muscle tissue from lesions or from unaffected tissue (negative control, Table 4). Sequence analysis was carried out on 40 representatives from the dominant groups (Tables 3 & 4). The majority (84%) of the cloned phylotypes from all the samples analysed were assigned to *Acidovax*-like β-Proteobacteria, *Methylobacterium*-like and *Bradyrhizobium*-like α-Proteobacteria, or *Porphyrobacter* α-Proteobacteria representatives (Table 4). There were also a number (31 out of 198) of RFLP types that could not reliably be assigned to the main 3 groupings and were apparently representatives of phylogenetically diverse organisms. Eleven were sequenced and shown to include representatives of the genera *Bradyrhizobium*, *Methylobacterium*, *Acidovorax*, *Bacillus* and *Pseudomonas*.

The dominant phylotypes recovered from lesion material from fish exposed to both Farm B and C CWSD-affected fish were also generally associated with samples of skin and muscle collected from unaffected control fish (Table 4). No DNA homologous to the partial 16S rRNA gene of *Flavobacterium psychrophilum* was identified in any of the cloned samples of PCR-amplified 16S rRNA DNA examined.

Table 2. *Onchorynchus mykiss*. Identities of bacteria isolated on solid media (TSA, AOA and Haem) from skin, head kidney and gill samples from fish showing characteristic cold water strawberry disease (CWSD) signs sampled as part of outbreak investigations and transmission trials. For all samples, representative isolates recovered on the different media were characterised on the basis of colony morphology on TSA, results of biochemical testing and PCR-RFLP pattern. Organisms were assigned to groupings as described in Table 3

Sample origin	Date sampled	No. of isolates	Bacterial identity													
			Ia	Ib	Ic	Id	II	IIIa	IIId	IV	V	VI	VII	VIII	IX	Other
Farm A	19 Jan 05	3					1							1	1	
Farm A	28 Apr 05	9		1			3					1	2	1		2
Farm B	28 May 05	14	1	2			6			1	1		1	2		
Farm C field samples	13 Dec 05	9						2		7						
Farm C fish used as infection source for trial	19 Jan 06	5						1		3				1		
Farm B fish used as infection source for trial	19 Jan 06	4						3	1							
Fish with lesions following cohabitation with Farm B fish	9 May 06	11			1				1	1	1	2	3			2
Fish with lesions following cohabitation with Farm C fish	9 May 06	12	3			5			1		1				1	1

Table 3. *Oncorynchus mykiss*. Colony morphologies and phylogenetic affiliations of a number of the strains or partial 16S rRNA genes isolated or PCR-amplified from samples of skin or cold water strawberry disease (CWSD) lesions. nd: not done

RFLP pattern/ morpho-type	Representative partial 16S rRNA sequence (accession numbers)	Colony morphology	Closest RDP 9.0 16s rRNA gene match (RDP Sim score)
Ia	AM779882	Cream, smooth, regular edges	*Aeromonas sobria* ATCC 43979T x74683 (1.0)
Ib	AM779881	Cream with green tinge; smooth, regular edges	*Aeromonas sobria* X74683 (0.975)
Ic	AM779879	Orange, slightly mucoid	*Aeromonas media* (*T*); ATCC 33907T; X74679 (0.995)
Id	AM779877	Cream with green tinge; mucoid, regular edges	*Aeromonas sobria* ATCC 43979T; X74683 (*0.978*)
II	AM779885	Orange/brown, smooth	*Sejongia jeonii* AT1047; AY553294 (0.963)
III	AM779887	Orange/yellow	*Flavobacterium frigoris*; LMG 21471; AJ440988 (0.938)
III	AM779876	Orange, mucoid	*Flavobacterium* sp. WB-1.1.56; AM177392 (0.976)
IV	AM779880	Orange, mucoid	*Chryseobacterium piscium*; PTB2043; DQ862541 (0.990)
V	05062	Cream, white; Gram positive	nd
VI	AM779886	Bright yellow	*Microbacterium* sp. VKM Ac-2050; AB042084 (1.0)
VII	AM779884	Cream, smooth	*Psychrobacter* sp. ikaite c11; AJ431338 (1.0)
VIII	AM779878	Cream, buff peaked	*Rhodococcus erythropolis*; MR32W; AJ576250 (0.983)
IX	AM779883	Cream	*Pseudomonas fluorescens*; ATCC 17573; AF094730 (1.0)
X[a]	AM779866	Cloned samples only	*Acidovorax* sp. R-24667; AM084010 (0.987)
X[a]	AM779865	Cloned samples only	β-*Proteobacterium* DQ337047 (0.961)
XI[a]	AM779864	Cloned samples only	*Bradyrhizobium* sp. AF384135 (0.986)
XI[a]	AM779869	Cloned samples only	*Methylobacterium fujisawaense* AB175634 (0.971)
XII[a]	AM779873	Cloned samples only	*Sphingomonus yanoikuyae* AB120764 (0.938)
XII[a]	AM779863	Cloned samples only	*Porphyrobacter donghaensis* AY559428 (1.0)

[a]RFLP types generated from whole-digested plasmids containing cloned 16S RNA gene inserts as opposed to digested amplified 16S RNA PCR products

Table 4. *Onchorynchus mykiss.* Analysis of cloned partial 16S rRNA genes PCR-amplified from samples of skin and lesion taken from negative control and fish showing cold water strawberry disease (CWSD) at the termination of the transmission trial. RFLP grouping assignations as described in Table 3: X = β-Proteobacteria group 1 (*Acidovorax*-like); XI = α-Proteobacteria group 1 (*Methylobacterium* and *Bradyrhizobium*-like); XII = α-Proteobacteria-like group 2 (*Shingomonas* and *Porphyrobacterium*-like); NA = other RFLP groups. Identity of clones based on RDP Seqmatch homology with Genbank deposited partial 16S rRNA gene sequences, as described in Table 3

Sample	No. of sample clones analysed by RFLP	RFLP grouping X	XI	XII	NA	No. of sample clones sequenced	Identity of sequenced sample clones	No. of >99% similar sample sequences
Skin and muscle from negative control fish (n = 2)	49	32	4	10	4	14	*Methylobacterium* sp.	1
							Porphyrobacter sp.	1
							Bradyrhizobium sp.	5
							Sphingomonas sp.	1
							β-*Proteobacterium* sp.	2
							Acidovorax sp.	4
Lesion material from fish exposed to Farm B fish with CWSD (n = 3)	89	49	13	11	16	17	*Methylobacterium sp.*	3
							Bradyrhizobium sp.	2
							Sphingomonas sp.	3
							β-*Proteobacterium* sp.	3
							Acidovorax sp.	5
							Pseudomonas sp.	1
Lesion material from fish exposed to Farm C fish with CWSD (n = 3)	60	21	14	13	11	10	*Porphyrobacter* sp.	1
							Methylobacterium sp.	1
							Bradyrhizobium sp.	2
							Sphingomonas sp.	3
							Acidovorax sp.	2

Flavobacterium psychrophilum PCR

All bacterial colonies tested (n = 21/67) were negative for *Flavobacterium psychrophilum* DNA by single-round *F. psychrophilum* PCR.

Flavobacterium psychrophilum DNA was detected in 4 out of 5 of the lesion skin/muscle samples taken from Farm B fish used as the cohabitant source for the infection experiment (Fig. 8). The 5 internal control samples collected in parallel (skin and muscle from unaffected areas of the fish) were all negative for *F. psychrophilum* by PCR. However, none of the samples collected from Farm C fish used for the transmission trial (n = 5, Fig. 8), or any of the material examined from lesions or unaffected areas of fish from the second infection experiment, were positive by single-round *F. psychrophilum* PCR (n = 50). The 10 samples that were used for the preparation of PCR-amplified 16S rRNA gene clone libraries were also tested with the nested *F. psychrophilum* PCR protocol and shown to be negative using this more sensitive protocol. For all PCR tests, appropriate positive and negative controls were included.

An initial validation exercise showed both tests to be both highly sensitive and highly specific, with the single and nested *Flavobacterium psychrophilum* protocols detecting *F. psychropilum* in homogenised rainbow trout skin tissue samples spiked with serially diluted *F. psychrophilum* cultures (to 10^{-6} from an OD_{550} culture = 0.2, equating to a sensitivity of <1 cell per reaction, data not shown).

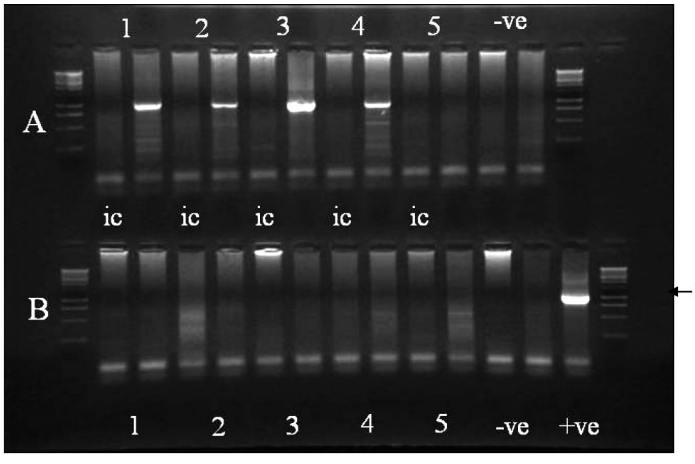


Fig. 8. Results of single-round PCR examination of lesion material of the population of rainbow trout from Farm B (row A) and Farm C (row B) used for the cohabitation challenge trial for the presence of *Flavobacterium psychrophilum* DNA. An internal control sample (ic: unaffected area of skin and muscle) from each fish was also tested. –ve = skin and muscle from a control population of unaffected rainbow trout. Control = *F. psychrophilum* NCIMB, row B lane 14). 1 kb molecular weight marker row A lanes 1 & 14, row B lanes 1 & 15)

Mycology, parasitology and virology

No fungal or oomycete agents were cultured and no parasites were observed in histological sections or in samples examined by EM. No novel cytopathic effect was observed on any cell lines tested, although Infectious Pancreatic Necrosis Virus (IPNV) was observed in 2 Farm B samples, a likely incidental finding as IPN is endemic in farmed UK rainbow trout (Cefas unpubl. data).

DISCUSSION

The epidemiological and transmission trial data together demonstrate that CWSD is very likely caused by one or more infectious agents with a long (>500 day-degrees) latency. It was also shown that, left untreated, fish recover from the condition with limited effects on growth and mortality.

Ferguson et al. (2006) suggested that that CWSD is closely related to SD as described in the US (Olson et al. 1985). Both CWSD and SD present as a full-thickness dermatitis with extensive lymphocytic infiltration into the subdermal layer, with limited epidermal involvement. Clinical signs are also more likely to occur when water temperature is less than 16°C.

In contrast, what has been described previously in the UK, Europe and Japan (Fleury et al. 1985, Kfoury et al. 1996, St. Hilaire & Jeffery 2004) as SD appears to be somewhat different. That condition is reportedly more prevalent at higher temperatures (>15°C). An early US study also reports that SD was transmissible (Erickson 1969), but other workers have not been able to repeat these results (S. Lapatra pers. comm.).

A recent report (Ferguson et al. 2006) has suggested that *Flavobacterium psychrophilum* may be associated with CWSD. Although *F. psychrophilum* 16S rRNA DNA was PCR-amplified from the lesions of 4 out of 5 Farm B CWSD-affected fish used for the second transmission trial, no *F. psychrophilum* or *F. psychrophilum*-like DNA were cultured or isolated from any of the resultant lesion material in previously naïve fish analysed at the termination of the infection trials. Further, none of the lesion samples examined from the Farm C CWSD-affected fish used for the second transmission trial was positive for *F. psychrophilum* by PCR. The data presented here do not support the hypothesis that *F. psychrophilum* is the sole infectious agent causing CWSD. Infection with *F. psychrophilum* is very common at fish farms in England and Wales (Cefas

Fish Health Inspectorate [FHI] unpubl. data). It is possible that any association of the organism with CWSD-lesion material is a result of the organism adhering as a secondarily opportunistic pathogen rather than as the causative agent. However, in the absence of any identified alternative agent, involvement of a variant *F. psychrophilum* strain cannot be ruled out.

It is well established that the types of bacteria that dominate in the aquatic environment are not readily culturable on conventional bacteriological media (Amann et al. 1995). However, there are, to date, few general data derived from the use of non-culture-based methods on the microbial floras of freshwater fish (particularly their skin).

There was also limited overlap between the 16S rRNA gene sequences directly cloned from the tissue samples (Table 4) and the 16S rRNA genes of the bacteria cultured from these and other lesion samples (Table 2), suggesting many of the bacteria associated with skin or lesion material were not readily recoverable on standard bacteriological media. The isolation of similar phylotypes from both negative control and lesion material suggests they did not play a role in the development of CWSD-characteristic lesions. The culture-independent analysis demonstrated that the dominant types of bacteria were likely β-Proteobacteria and α-Proteobacteria-like, as has been found in a range of surveys of microbial communities associated with other freshwater environments (Alfreider et al. 1996, Weiss et al. 1996, Gich et al. 2005).

Romero & Navarrete (2006) used a combination of Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) and direct PCR to show that the microbial flora of the early-stage (egg and sac fry) microfloras of coho salmon were dominated by α-Proteobacteria representatives, as shown in the present study for rainbow trout skin and lesion samples. In contrast, the gut microflora of feeding coho salmon was markedly different, dominated instead by γ-Proteobacteria representatives. The gut microflora of feeding rainbow trout, as characterised by Fluorescent *In Situ* Hybridisation (FISH) with specific bacterial probes and cloning of PCR-amplified partial 16S RNA genes, was also shown to be dominated by γ-Proteobacteria representatives (Spanggaard et al. 2000, Huber et al. 2004) as well as a *Clostridium* sp. (Pond et al. 2006).

There was consistent transmission of CWSD from Farm C-affected fish to naïve fish. However, the results from the transmission trial experiments, where Farm B-affected fish were in indirect contact with naïve fish, were difficult to interpret. As well as the oedema skin condition noted in some of the fish indirectly cohabited with Farm B fish, undiagnosed mortality was also observed in naïve fish that were directly cohabited with Farm B fish immediately after transfer to our laboratory (Verner-Jeffreys et al. 2006 and other data not shown). This suggests the Farm B fish used in these experiments had concurrent infections that may have complicated the transmission of CWSD to naïve fish.

It cannot be assumed on the basis of these data that the conditions affecting the fish from Farms B and C had the same cause. The rates of transmission of CWSD-characteristic lesions from Farms B and C fish to the same population of naïve fish were very different. There was also no evidence linking the emergence of the condition on Farm C through live fish movements from other affected sites.

CWSD lesions are characterised by extensive lymphocyte infiltration characteristic of a hyper-inflammatory host response. It is possible that the host had already inactivated and cleared the pathogen(s) responsible for provoking this response by this late stage of the infection cycle, preventing its visualisation or isolation. Although attention has been mainly focussed on the skin lesions, cardiac lesions are also associated with the condition (Ferguson et al. 2006, the present study). Similar lesions can be caused by a number of infections including those caused by alphaviruses in pancreas disease and sleeping disease, particularly in chronic and recovered fish (McLoughlin & Graham 2007). Although there remains the possibility that an alphavirus is involved with CWSD, colleagues at FRS Marine Laboratory Aberdeen were not able to show the presence of salmonid alphavirus when they examined CWSD-affected fish in Scotland (P. Noguera pers. comm.).

The eggs, from which the affected fish on Farms A, B and C were grown, were all from a common source. However, as the majority of rainbow trout grown in the UK at the time of the outbreak investigations originated from the same international egg supplier, this link can only be regarded as circumstantial, particularly as the company also supplies a number of countries that have not, to date, reported the emergence of CWSD.

In terms of available control measures, it is not known the extent to which CWSD will persist on a site if only uninfected fish are subsequently introduced. It is recommended that CWSD-affected stocks are kept separate from unaffected stocks and, where possible, such affected stocks are harvested before the end of the high temperature (>15°C) summer period, as this provides a potential opportunity to remove the source of on-farm infection when the syndrome is not likely as readily transmissible to naïve stocks. In this regard, it is noteworthy that Farm C no longer has the condition. There is evidence that at least one other farm in England and Wales has rid themselves of the condition by systematic fallowing and disinfection of ponds where the disease has been observed (Cefas FHI, unpubl. data).

Currently, CWSD is managed by medication and continued growing-on of affected fish, to allow natural resolution of the condition, or harvesting for fillet production (thus avoiding downgrading losses). However there are concerns about the impact of lengthy antibiotic treatments on the general health status of the fish and susceptibility of bacterial pathogens.

CWSD is an economically important disease and further information is urgently required about its epidemiology, the nature of the responsible agent and how it should be controlled. Since the initial outbreak investigations reported in the present study, the condition has apparently spread to a number of other farms in both England and Scotland, with the FRS Marine Laboratory FHI reporting a possible 25 farms in Scotland to have suffered outbreaks from 2006 to 2007 (D. Pendrey pers. comm.). Cefas FHI have reports of up to 12 more sites in England and Wales perhaps affected over this period. It is now estimated that farms responsible for more than 50% of UK trout production are affected, or have been affected, by CWSD (R. Hughes pers. comm.).

It is recommended that a continued concerted effort be applied to uncover the responsible aetiological agent(s), as the lack of this basic information is severely hampering efforts to diagnose and control the disease. Clear case definitions for both CWSD and WWSD need to be developed and agreed to allow future studies to clearly refer to/report on one or the other condition. The identification of risk factors for expression of CWSD, through observational epidemiological studies, will support the development of on-farm control strategies.

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LITERATURE CITED

Alfreider A, Pernthaler J, Amann RI, Sattler B, Glockner FO, Wille A, Psenner R (1996) Community analysis of the bacterial assemblages in the winter cover and pelagic layers of a high mountain lake by *in situ* hybridization. Appl Environ Microbiol 62:2138–2144

Amann RI, Ludwig W, Schleifer KH (1995) Identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169

Buller NB (2004) Bacteria from fish and other aquatic animals: a practical identification manual. CABI Publishing, Wallingford

Erickson D (1969) An investigation on strawberry disease in trout. American Fishes and US Trout News

Ferguson HW, Girons A, Rizgalla G, LaPatra S and others (2006) Strawberry disease in rainbow trout in Scotland: pathology and association with *Flavobacterium psychrophilum.* Vet Rec 158:630–632

Fleury HJA, Vuillaume A, Sochon E (1985) Isolation of an adeno-like virus from two cases of strawberry disease in rainbow trout. Ann Inst Pasteur 136:223–228

Gich F, Schubert K, Bruns A, Hoffelner H, Overmann J (2005) Specific detection, isolation and characterisation of selected previously uncultured members of the freshwater bacterioplankton community. Appl Environ Microbiol 71: 5908–5919

Huber I, Spanggaard B, Appel KF, Rossen L, Neilson T, Gram L (2004) Phylogenetic analysis and *in situ* identification of the intestinal microbial community of rainbow trout (*Oncorhynchus mykiss* Walbaum). J Appl Microbiol 96: 117–132

Kfoury JR, Okamoto N, Tanaki M, Yoshimizu M, Lapatra SE, Maita M (1996) 'Rash' skin disease of rainbow trout. Fish Pathol 31:197–201

Lebaron P, Ghiglione JF, Fajon C, Betailler N, Normand P (1998) Phenotypic and genetic diversity within a colony morphotype. FEMS Microbiol Lett 160:137–143

Lorenzen E (1993) 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 13:64

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Wade WG (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbiol 64:795–799

Markwardt NM, Gocha YM, Klontz GW (1989) A new application for Coomassie brilliant blue agar: detection of *Aeromonas salmonicida* in clinical samples. Dis Aquat Org 6:231–233

McLoughlin MF, Graham DA (2007) Alphavirus infections in salmonids — a review. J Fish Dis 30:511–531

OIE (2006) Manual of diagnostic tests for aquatic animals. Chapter 2.3.7. http://www.oie.int/eng/normes/fmanual/A_00054.htm

Olson DP, Beleau MH, Busch RA, Roberts S, Krieger RI (1985) Strawberry disease in rainbow trout, *Salmo gairdneri* Richardson. J Fish Dis 8:103–111

Pond MJ, Stone DM, Alderman DJ (2006) Comparison of conventional and molecular techniques to investigate the intestinal microflora of rainbow trout *Oncorhynchus mykiss*. Aquaculture 261:194–203

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 117:208–212

Romero J, Navarette P (2006) 16S rDNA-based analysis of dominant bacterial populations associated with early life stages of coho salmon (*Oncorhynchus kisutch*). Microb Ecol 51:422–430

Spanggaard B, Huber I, Nielsen J, Appel K, Gram L (2000) The microflora of rainbow trout: a comparison of traditional and molecular identification. Aquaculture 182: 1–15

St-Hilaire S, Jeffery K (2004) Strawberry disease in rainbow trout. Trout News 37:24

Toyama T, Kita-Tsukamoto K, Wakabayashi H (1994) identification of *Cytophaga psychrophila* by PCR targeted 16S ribosomal RNA. Fish Pathol 29:271–275

Verner-Jeffreys D, Algoet M, Feist S, Bateman K, Peeler E, Branson E (2006) Studies on Red Mark Syndrome. Finfish News 1:19–22

Weiss P, Schweitzer B, Amann R, Simon M (1996) Identification *in situ* and dynamics of bacteria on limnetic organic aggregates. Appl Environ Microbiol 62:1998–2005

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