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Assessing the presence and spread of *Renibacterium salmoninarum* between farmed and wild fish in Sweden

David B. Persson (ID) | **Anna Aspán** | **Paulina Hysing** | **Eva Blomkvist** | **Eva Jansson** | **Ludvig Orsén** | **Hampus Hällbom** | **Charlotte Axén** (ID)

National Veterinary Institute, Uppsala, Sweden

Correspondence
Charlotte Axén, National Veterinary Institute, Ulls väg 2B, 751 89 Uppsala, Sweden.
Email: charlotte.axen@sva.se

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Abstract

Bacterial kidney disease (BKD) can be a devastating bacterial infection in salmonids, and it is present in aquaculture throughout the world. BKD is caused by the Gram-positive facultative intracellular bacterium *Renibacterium salmoninarum* (*R. salmoninarum*) that is spread both horizontally and vertically. Disease signs include external ulcerations and blisters and internal signs such as organ swelling, granulomas, petechiae and ascites. In Sweden, BKD accounts for a significant income loss in aquacultures due to expensive decontamination of the facility and increased disease susceptibility for the immunocompromised fish leading to higher mortality rates. In addition, uncontrolled spread in aquaculture may threaten the survival of wild fish populations. The aim of our study was to investigate the prevalence of *R. salmoninarum* in wild salmonids caught in Swedish waters where net pen farms with a recent history of BKD are present. Four rivers with at least one BKD-positive or recently BKD-positive farm were selected. In addition, we evaluated the use of environmental DNA (eDNA) for surveillance and monitoring of ongoing infections at these locations. In total, 1058 fish were sampled from four different river systems, and of them 52 (4.9%) were positive for *R. salmoninarum* by antigen ELISA. Surprisingly, these fish were not evenly distributed between the four river systems, but 50 were caught in the same river (Ljungan). This accounts for an alarmingly high rate of 17% *R. salmoninarum*-positive samples in wild salmonids in this area. This number is far above what was expected and clearly shows the risk with an open farming system as well as the importance of effective health monitoring programmes to avoid an uncontrolled spread of the disease. The use of eDNA for monitoring BKD is somewhat difficult to evaluate. Few of the water samples analysed were PCR positive for *R. salmoninarum* (2 of 38) and those were collected where no ELISA positive fish were identified. In addition to water, sediment samples were collected under a net pen farm that had recently slaughtered all fish due to ongoing *R. salmoninarum* infections. Sediment samples are more promising than water as 4 of 5 samples at one farming facility where positive for *R. salmoninarum*. Thus, sediment samples may be valuable for monitoring potential ongoing BKD in farms, without the need to sacrifice valuable fish.

1 | INTRODUCTION

Bacterial kidney disease (BKD) is caused by the intracellular Gram-positive bacterium *Renibacterium salmoninarum* (*R. salmoninarum*) and is one of the oldest known bacterial pathogens in fish (Delghandi et al., 2020). Salmonid fish species in temperate climate zones, that is, cold water areas, are prone to infection and disease occurs at approx. 7–15°C. The disease can be observed both in fresh- and salt-water and is endemic in most areas of the world where salmonid fish is farmed (Eslamloo et al., 2020; Faisal & Eissa, 2009; Jia et al., 2020; Sanders & Barros, 1986; Wallace et al., 2017; Yoshimizu, 1996). Wild fish often acts as a reservoir for pathogens and the infection is present in both farmed and wild fish. The infection spreads both horizontally from fish to fish and vertically from female to offspring (Armstrong et al., 1989; Brynildsrud et al., 2014; McKibben & Pascho, 1999; Yoshimizu, 1996). In nature, the disease is normally spread by contaminated water, whereas in aquaculture it is more common to spread the disease through infected fish or eggs, contaminated equipment or historically through contaminated feed (Brynildsrud et al., 2014). Based on observations from the 1980s, shortly after introduction of the pathogen to Sweden, the most sensitive wild species for infection are Arctic char (*Salvelinus alpinus*) and Atlantic salmon (*Salmo salar*), were as much as 80% of the farmed fish could succumb to disease in acute outbreaks (unpublished data). Brown/sea trout (*Salmo trutta*) is more resistant to disease development, whereas grayling (*Thymallus thymallus*) and whitefish (*Coregonus lavaretus*) are poorly studied (Chambers et al., 2008; Rimaila-Pärnänen, 2002; Starliper et al., 1997). Rainbow trout (*Oncorhynchus mykiss*), the dominating species in Swedish aquaculture, is prone to infection but relatively resistant to disease (Dale et al., 1997; Sakai et al., 1991; Sanders et al., 1978). Disease development is slow with up to 6 months from infection to signs of disease (Kaattari & Piganelli, 1996). Thus, once detected, the pathogen is likely well spread within a facility and in open cage systems may also have spread to the wild fish population.

Renibacterium salmoninarum has a temperature optimum at 7–15°C (Benediktsdóttir et al., 1991), and outbreaks rarely occur at temperatures above 15°C. Clinical manifestations include external signs such as irregular swimming behaviour, exophthalmia, ocular lesions, epidermal blisters, haemorrhages or petechiae around fins and the lateral line, skin discoloration/dark pigmentation, abdominal swelling and anaemia (Delghandi et al., 2020; Richards et al., 2017). Internally, swelling of several organs as well as formation of granulomatous lesions frequently occurs. Occasionally, haemorrhages may be observed in combination with bloody ascites (Bruno & Munro, 1986; Kent et al., 2013). A rare manifestation is invasion of the central nervous system (CNS) that results in brain lesions (Speare, 1997). On a cellular level, following adhesion, the bacterium invades host cells and survives intracellularly, an important

mechanism to evade the immune system. Infection is often associated with chronic inflammation leading to local tissue damage through necrosis (Delghandi et al., 2020). The granulomatous structures often consist of cellular debris and invading immune cells such as eosinophils, neutrophils and macrophages; the latter is also the cell type most frequently being infected by *R. salmoninarum* (Bandín et al., 1995; Grayson et al., 2002). In addition, an increase in the number of several populations of blood cells, including neutrophils, monocytes/macrophages and thrombocytes, have been associated with infection (Grayson et al., 2002). The ability to specifically target, or modulate, immune cells is likely an important mechanism to suppress the immune system and allows other opportunistic pathogens to take advantage of the weakened host, leading to secondary infections. Treatment options during outbreaks are limited to antibiotics, which is problematic due to the slow onset of disease and because available antibiotics work poorly against *R. salmoninarum* due to its intracellular location (Fairgrieve et al., 2005; Fetherman et al., 2020). Thus, the most effective way to control the infection is to slaughter the fish and disinfect the facility. In Sweden, because of the poor efficiency, treatment of BKD using antibiotics is not allowed. There have been several attempts to develop a vaccine, but there is currently no vaccine available.

A debate within the Swedish aquaculture industry is the question whether it is necessary to eliminate the disease as raised rainbow trout is not very prone to disease development. Latent carriers of *R. salmoninarum* can therefore easily go undetected without surveillance and keep the infection in the area. However, one major problem with ignoring infection in farmed fish is that the pathogen may spread to native wild fish populations that are more likely to develop severe disease. Therefore, the aim of this study was to investigate to what extent *R. salmoninarum* infection detected in pen farmed rainbow trout spreads to the wild fish population and to, if possible, assess the impact of net pen farming for the survival of the wild fish population. In addition, by collecting a large number of wild fish of different species, we hoped to better understand the susceptibility for *R. salmoninarum* in grayling and common whitefish. This study is important to develop guidelines for protection of our wild fish populations against the steadily increased demand for farmed fish and a growing interest in establishing fish farms.

2 | MATERIALS AND METHODS

2.1 | Collection of field samples

The fish was caught by netting during the time from mid-May to the beginning of August at temperatures between 7 and 15°C. In total, 10 sampling points each in four different river systems were

selected. In each river, farms that were classified as infected with *R. salmoninarum* within the last 7 years were located. One of the ten sampling points per river was selected as a negative control point. These were lakes separated from the respective river either completely or by migration barriers. In addition, no known movement of fish to the lakes should have been performed during these years. All remaining points per river were located up- and downstream of the farming facilities. The water temperature was measured at a depth of 1m during fishing. All fish and water samples were frozen at −20°C as soon as possible. At each sampling point, 30 fish were to be caught and two 5 L surface water samples collected at one or two occasions. If more than 30 fish had been caught at one sampling point, the smallest fish were excluded. Species and any abnormalities were noted, and a 1 g kidney sample was cut and placed in a small plastic bag. A swab was pulled throughout the full length of the kidney for confirmation by PCR in case of a positive ELISA.

In addition, sediment samples were collected at five different points in a farm in Umeälven recently emptied of *R. salmoninarum*-infected fish as well as one control point upstream of the site, for eDNA extraction. At three of the locations, the pens were emptied a week before sampling, and two of the locations a month before sampling. A follow-up sample was also collected at one of the points 3 months later. The sediment samples were collected using a grab sampler. Upon sampling, ~1 kg of sediment was collected. All samples were frozen at −20°C as soon as possible after sampling to preserve the DNA. All analyses were performed within 2–4 weeks after sampling.

2.2 | ELISA and PCR analysis

A sandwich ELISA was performed to detect *R. salmoninarum* protein p57 according to Jansson et al. (Jansson et al., 1996, 2008). Briefly, the collected sample of kidney was diluted 1:4 in PBS + 0.08% Tween20 and was homogenized using a paddle blender. After homogenization, the lysate was transferred to Eppendorf tubes and heat treated for 20 min at 120°C. Ninety-six-well ELISA plates were coated using an in-house generated rabbit IgG polyclonal antibody in coating buffer (50 mM $NaCO_3$, pH 9.6) for two nights. The coating solution was then discarded, and the plates washed with 1 mM PBS + 150 mM NaCl + 0.5% Tween20. Once washed the plates were blocked using 1% BSA diluted in SuperQ water for 30 min. During the blocking, the homogenized samples were centrifuged at 2,500 *g* for 20 min to pellet cellular debris. Each sample was then diluted 1:2 in PBS + 0.5% Tween20 when added to the coated and blocked ELISA plate. In addition to the isolated samples, a positive as well as negative control were included. Once applied, capture was allowed to run for 2 hr at RT before washing and addition of a goat anti-renibacterium HRP-conjugated antibody diluted 1:1,000 (VWR) in PBS + 0.5% Tween20, for 60 min at RT. After washing, detection was performed using tetramethylbenzidine (TMB) in a 100 mM sodium acetate buffer, pH 6.0, 0.006%

H_2O_2, for 6–10 min before stopping the reaction using 1 M H_2SO_4. The signal was then quantified using a Magellan plate reader at 450 nm. Samples were considered as positive if the absorbance was ≥0.1 after background subtraction.

For the PCR analysis and DNA extraction, the swab was first placed in 540 µl Buffer G2 from EZ1 Tissue kit (Qiagen) prior to extraction using an EZ1 robot. Both proteinase K and lysozyme were used to ensure sufficient cell lysis. After DNA extraction, *R. Salmoninarum* DNA was detected using a probe-based qPCR with an PerfeCTa qPCR Toughmix (Quanta Biosciences) in an ABI 7500 Fast PCR machine (Thermo Fisher Scientific) following the protocol described by Jansson et al., 2008 (Jansson et al., 2008).

2.3 | eDNA analysis

Water samples were filtered through a 2 µm pore, 47-mm-diameter, borosilicate glass fibre filter (Thermofisher Siences) using a peristaltic pump (Easy-Load MasterFlex Cole-Parmer) at a speed of 650 ml/min. Filters were aseptically removed, cut in two pieces and stored at −20°C. One piece was used for DNA extraction and the second piece as back-up. DNA extraction using the EZ1 DNA tissue kit, and the bacterial protocol, on an EZ1 Advanced XL robotic workstation according to the manufacturer's instructions (Qiagen Inc.) and qPCR was performed as described by Jansson et al., 2008 (Jansson et al., 2008), with the modification that a bead-beating step was included before sample lysis.

For DNA extraction of sediment samples, a DNeasy PowerSoil kit (Qiagen) was used, according to the manufacturer's instructions. From each 1 kg sample, eight subsamples of 0.25g were randomly collected after a thorough mix. All replicates were analysed by qPCR for *R. salmoninarum* (Jansson et al., 2008). In addition, two subsamples per sediment sample were analysed for the presence of rainbow trout DNA by qPCR (DNA extraction control). This analysis was performed by the Museum of Natural History, Stockholm, Sweden, following the protocol by Rusch et al., 2018 (Rusch et al., 2018).

3 | RESULTS

3.1 | Sample collection and clinical manifestation of BKD

In total 38 of 40 sampling points could be included in the study (Figure 1). One of the sampling points in Umeälven had to be excluded as no salmonid fish was caught, and one sampling point in Faxälven was excluded as the water temperature was consistently above 15°C. Of the 1058 included fish only one showed typical signs of BKD. Several others had small white granulomas in the kidney, and some in the heart that were probably associated with parasitic infections but could be a sign of BKD (Figure 2c, d). The dominating species was whitefish (*n* = 601), followed by trout (*n* = 236), Arctic char (*n* = 144) and grayling (*n* = 77) (Figure 2a).
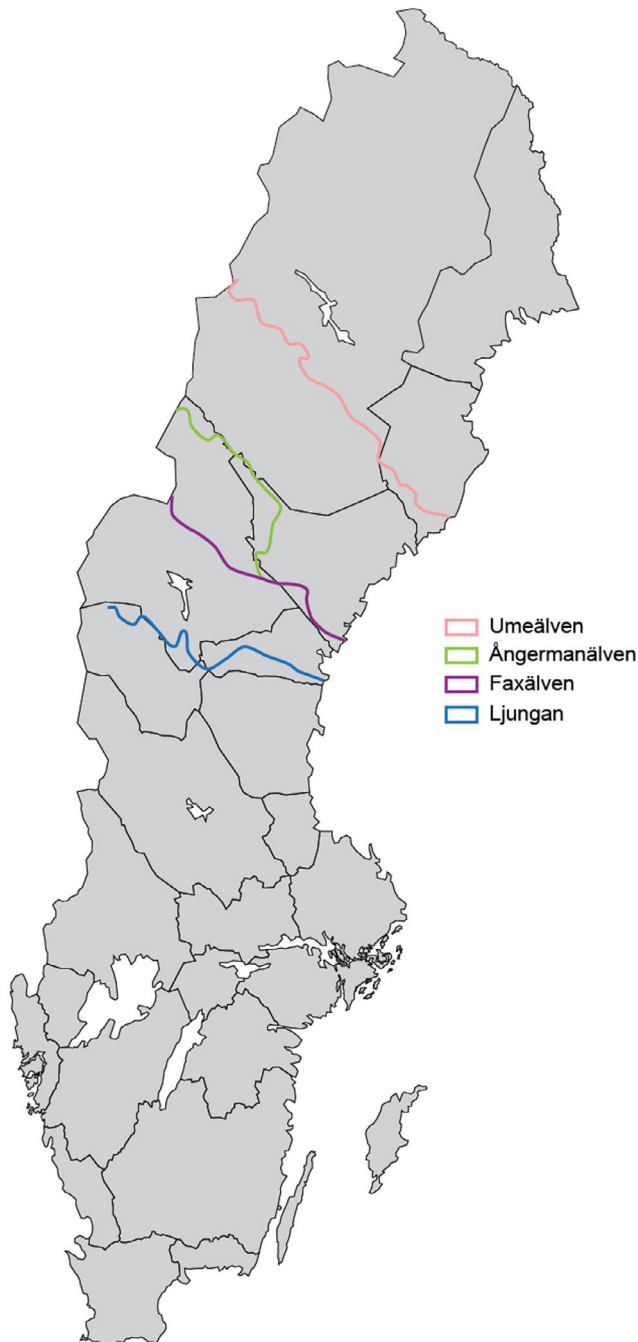
FIGURE 1 Overview of the sample locations and observations during sample collection. The rivers from north to south, Umeälven (pink), Ångermanälven (green), Faxälven (purple) and Ljungan (blue) were sampled from the mid of May until the mid of July 2021

3.2 | Prevalence of *Renibacterium salmoninarum*

Each of the 1058 individuals were tested in the laboratory by an antigen ELISA. In total 52 fish (4.9%) were positive for the p57 antigen. In addition, 17 fish had elevated OD 450 nm values (OD 0.05–0.1) but failed to reach the OD threshold of 0.1. All ELISA positive samples were also tested by PCR (Jansson et al., 2008), but only one sample turned out positive by this method (data not shown). Interestingly, 50 of the ELISA positive fish were all from the

same river system (Ljungan), where a total of 294 fish were sampled (Figure 3a,b). The dominating species sampled was whitefish, a species where the susceptibility to *R. salmoninarum* has been poorly investigated. Of the 50 *R. salmoninarum*-positive fish in Ljungan, 43 were whitefish. Grayling is also a species where the susceptibility to *R. salmoninarum* has been little investigated. Looking at the overall percentage of *R. salmoninarum*-positive fish per species in Ljungan, grayling dominated 46.2% (6/13), followed by whitefish (18.2%, 43/236), trout (3.8%, 1/26) and char 0% (0/19). Positive fish were caught at water temperatures of 10°C to 15°C (Figure 3c). The prevalence of BKD in wild fish is likely to be influenced by water flow through rates and volumes, affecting pathogen concentration and dispersion. Unfortunately, no data on water turnover could be found. Instead, we compared the areas and depths of the sampled lakes/pools in the four river systems (Figure 3d). We could not obtain data to compare the total volumes of water as the depth is only categorized as 'shallow' (<3 m), 'intermediate' (3–15 m) or 'deep' (>15 m) in the Swedish Water Information System VISS (http://viss.lansstyrelsen.se) (Länsstyrelsen, 2021). Size wise, the lakes/stretches sampled in Umeälven covered the largest area (319 km^2), followed by Faxälven (233 km^2) and Ångermanälven (212 km^2). Ljungan by far had the smallest sampled area (79 km^2). The sampled parts of Ångermanälven and Faxälven were the deepest; 87% and 82% of the area, respectively, fell into the category 'deep', whereas 42% the sampled parts of Umeälven and 36% of Ljungan fell into the 'deep' category. In Ljungan, 6% of the sampled area is 'shallow'.

3.3 | Detection of *Renibacterium salmoninarum* in eDNA

The importance of eDNA for ecological and pathogen monitoring is increasing, since it is a powerful way to obtain a lot of data on, for example, species distribution. Therefore, we investigated if eDNA could be used to determine if there is an ongoing BKD outbreak in a farm or water system. At each of the 38 locations where fish were caught, a water sample was also collected and eDNA isolated. Only 2 of 38 samples were positive for *R. salmoninarum*, but no ELISA positive fish were found in those locations (data not shown). However, in one of the spots positive by eDNA, a farm situated approximately 3 km upstream tested positive a few weeks after the sampling for this study. We also sampled sediment at one infected farm that recently slaughtered infected fish. In total six different spots were selected; three where pens had been emptied a week ago (O1–O3), two where the pens had been emptied a month ago (O4–O5) and one control point a few km upstream where no pens had been located (O6) (Figure 4a). It was obvious that net pen farming affects the local environment while fish is present, as sediment from O1–O3 had a muddy consistency and a foul smell. Each of the 1 kg sediment samples were further sampled by taking eight 0.25 g sub-samples by random and eDNA was isolated. From the isolated eDNA, a qPCR was performed to detect *R. salmoninarum* DNA. In addition, from each location two sub-samples were also tested for rainbow trout
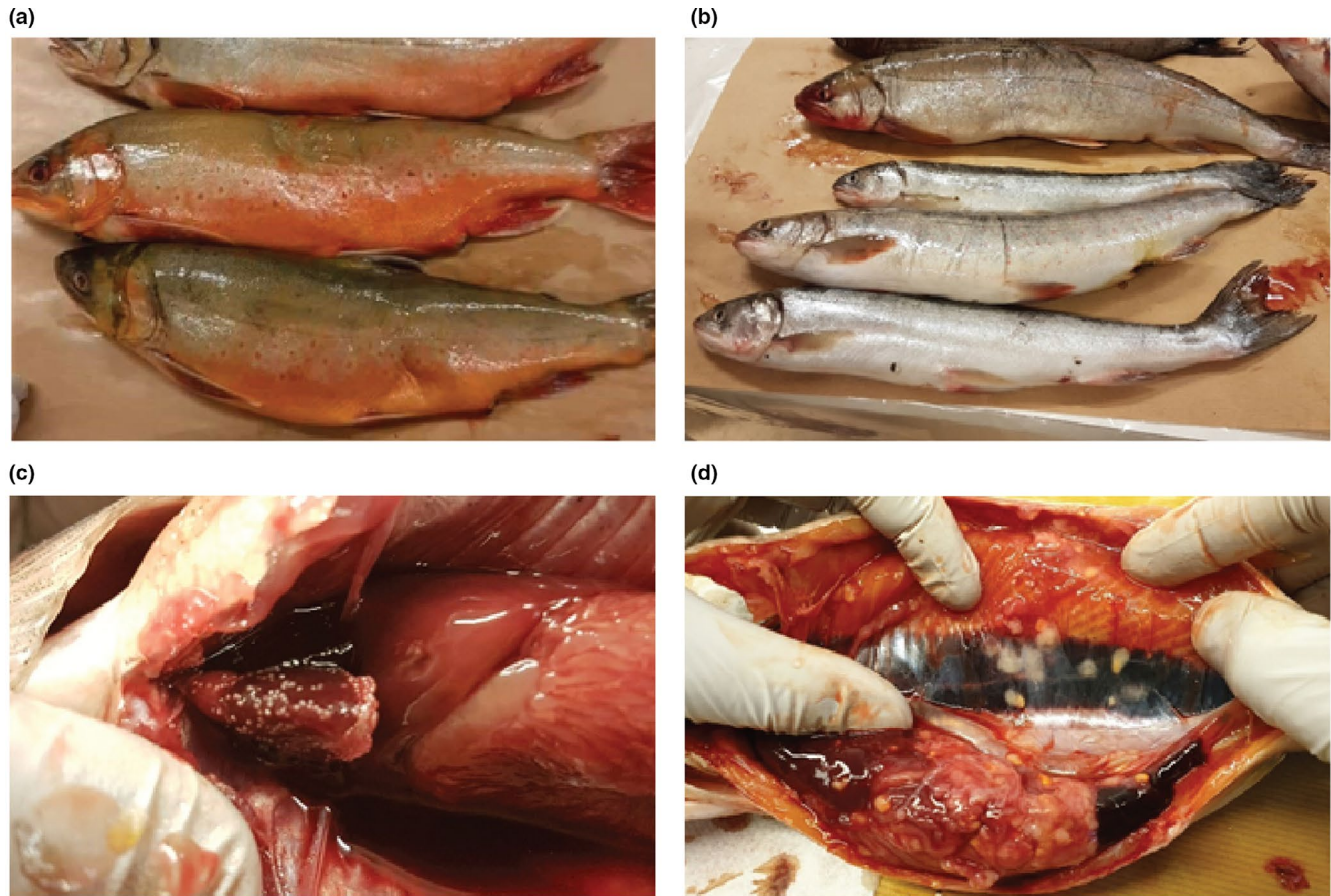
FIGURE 2 Examples of necropsy findings during the study. (a and b) Arctic char from two different locations in Ångermanälven with different coloration and physical condition. (c) Whitefish heart covered with parasite cysts/granulomas. (d) *Renibacterium salmoninarum*-infected female trout with a swollen, granulomatous kidney. The orange dots in the abdomen are trout eggs

as a control (Figure 4a). From four of the five sampled farm spots at least one sub-sample was positive for *R. salmoninarum*, in one case all eight were positive (Figure 4b). There was no correlation between the number of positive sub-samples and the time passed since pen farming occurred. When spot O3 was revisited 3 months later, the sediment sample was not smelly anymore and *R. salmoninarum* DNA could not be detected. All tested samples from O1–O5 were positive for *rainbow trout*. Control point O6 was negative for both *R. salmoninarum* and rainbow trout DNA.

4 | DISCUSSION

Bacterial kidney disease is not only a topic of discussion but also a major problem within the aquaculture industry (Bayliss et al., 2018). Sweden has had a BKD screening programme initiated in the early 1990s, according to the Swedish regulation on mandatory health monitoring of farmed fish (SJVFS 1994:94). This programme was further supported by an eradication programme approved through additional guarantees accepted by the European Union where facilities are routinely sampled by official veterinarians. An identified BKD infection leads to several restrictions, including slaughter of all

fish and no new fish can enter the farm before slaughter and disinfection has been performed. For the farmer, this often leads to large financial losses that are not paid for by the authorities, thus a delayed slaughter has been allowed to minimize losses. Unfortunately, these regulations have worked against eradication, since the infection has time to become manifest in the wild fish, allowing reinfection once new fish is introduced in the farm. This makes the task of eliminating the bacterium impossible as the pathogen will repeatedly circulate between the two populations. It is therefore critical to study the prevalence of the bacterium in wild fish to assess what role net pen farming has for the spread and establishment of local *R. salmoninarum* infection in wild fish. By acknowledging the current situation, we can provide better guidelines for a sustainable way of net pen farming without risking the spread of BKD to the wild fish populations.

In the Swedish river systems, we have five major native species of salmonid fish: Arctic char, Atlantic/Baltic salmon, Sea/brown trout, whitefish and grayling. Regarding BKD sensitivity, char and salmon are considered the most sensitive species followed by trout. For whitefish and grayling, limited data are available. It is known that they can become infected, but how sensitive they are to infection and disease is largely unknown (Chambers
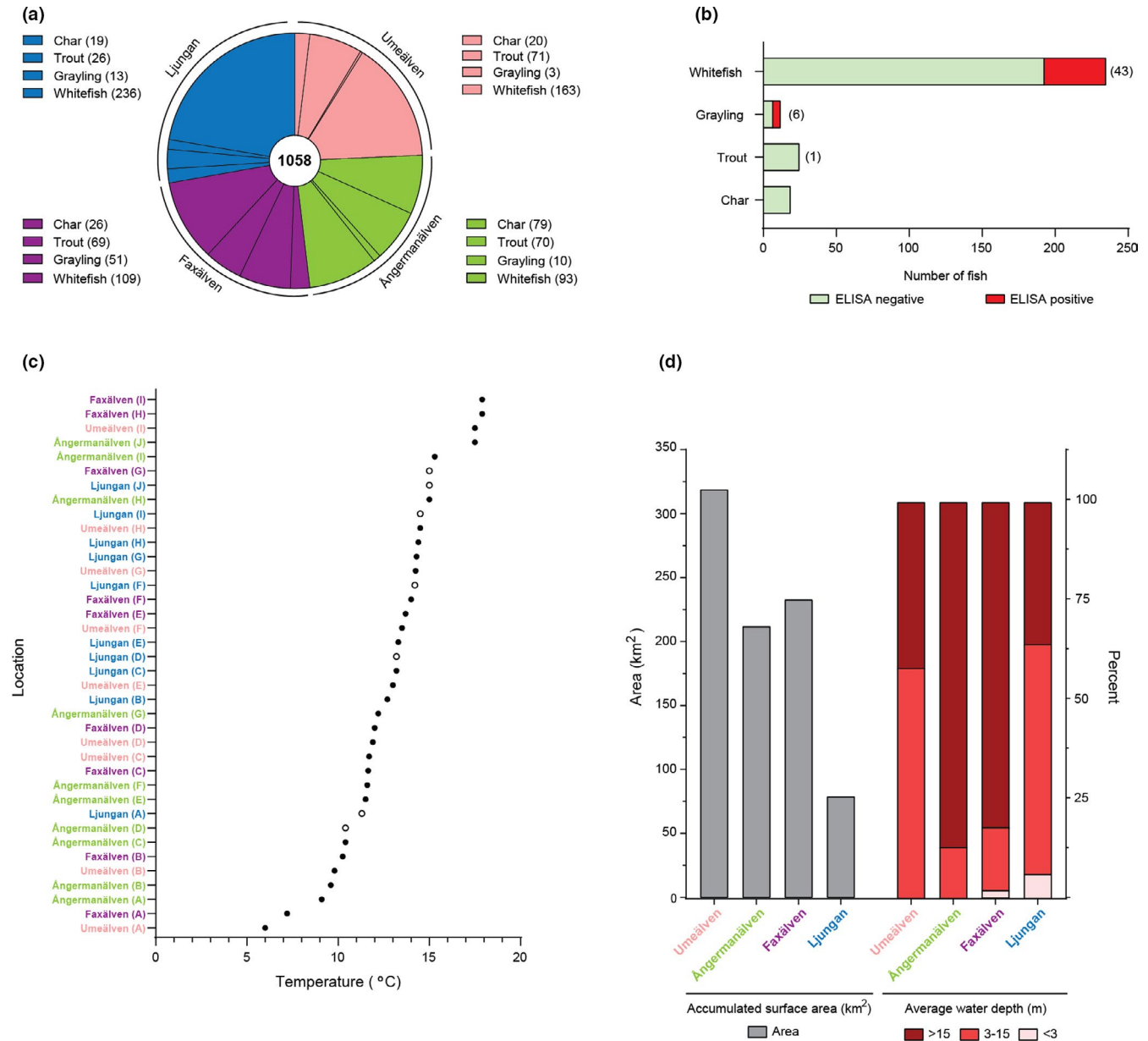
FIGURE 3 Collective data from the BKD analysis. (a) Summary of species distribution in relation to capture location, of the 1058 fish sampled in the four river systems. The different rivers are indicated using the same colour scheme as in Figure 1 with Umeälven (pink), Ångermanälven (green), Faxälven (purple) and Ljungan (blue). (b) Data showing the percentage of *Renibacterium salmoninarum*-positive samples for the different species of fish collected in Ljungan. Given within parentheses is the actual number of positive individuals. Results from the ELISA for samples from Ljungan where 17%, for example, 50 individuals, of the sampled fish were positive for *R. salmoninarum*. (c) Recorded temperatures at the different fishing locations from the mid of May to the beginning of August. Open circles indicate spots/ temperatures where *R. salmoninarum*-positive fish were caught. To differentiate between different fishing locations in the respective river system, a capital letter is used within parenthesis. (d) Comparison of the size and depth of the four investigated river systems with area in grey and depth in different shades of red

et al., 2008; Rimaila-Pärnänen, 2002; Starliper et al., 1997). In our study, whitefish were the dominating species caught in all four river systems, and data from the BKD ELISA showed that the species are susceptible to infection even if they lacked the common clinical signs of severe BKD (Starliper et al., 1997). A working hypothesis is that whitefish gets infected but clear the infection relatively well thus preventing development of signs. This is supported by the fact that all the ELISA positive samples in this study

were also tested by qPCR but only one was qPCR positive, suggesting that the active infection is cleared with bacterial antigens lingering in the tissue for a longer time after infection compared to bacterial DNA. For grayling, a similar trend was noticed but given the relatively small numbers of grayling caught it is hard to draw any major conclusions beyond that they were positive for *R. salmoninarum* without showing any significant signs. The relatively low prevalence in char and trout is hard to interpret in relation to

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FIGURE 4 Overview of the experimental design and eDNA analysis of sediment samples. (a) Schematic overview of the sampling procedure for sampling locations (O1-O6). Orange fish symbols are used to indicate time passed since *Renibacterium salmoninarum*-positive fish were kept in the pen. Four orange fish indicate that the pen was emptied 1 week before sampling, one orange fish indicate 1 month since sanitation and no orange fish equals control point. Each of the collected sediment samples were subdivided into eight samples and DNA extracted and analysed for the presence of *R. Salmoninarum*. (b) Results from the qPCR analysis of the sediment samples with the percentage of *R. Salmoninarum*-positive subsamples in green. The detection of rainbow trout DNA in the sediment samples acted as a control and only two of the sub-samples were analysed. The detection of rainbow trout DNA is shown in grey
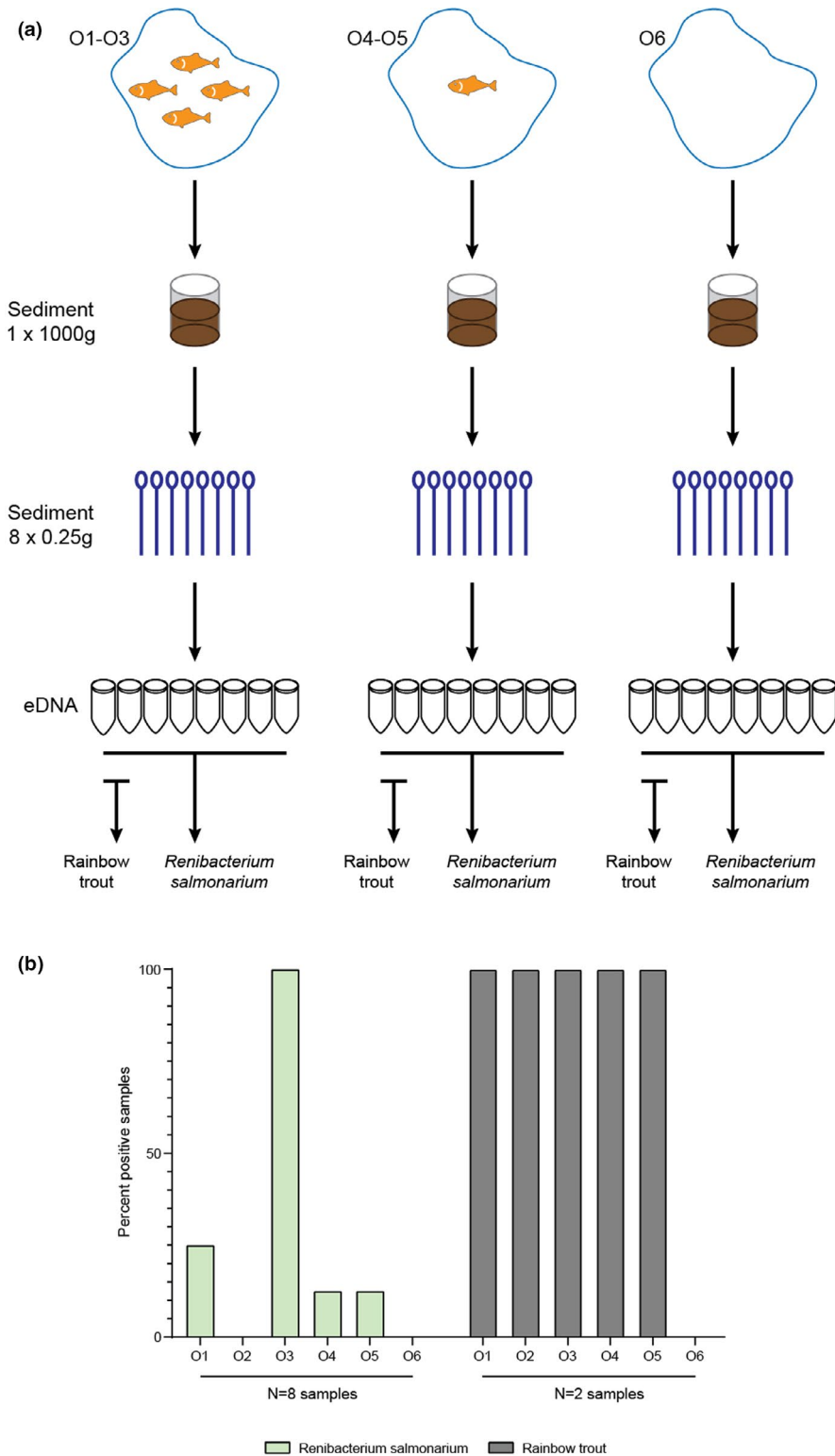
the prevalence in whitefish and grayling. The apparent low frequency could be due to several factors, such as relative hardiness compared to whitefish/grayling, or the opposite: a higher susceptibility with higher mortality rates. It could also be that more char and trout were caught further from the infected farms, thus exposed char/trout populations were not sampled at the same rate. In fact, some of the fish sampled had a reddish coloration of

the muscle tissue that suggests that they have been feeding on carotenoid-rich feed close to net pens. However, if these fish were *R. salmoninarum* positive to a higher extent was not investigated. Feeding behaviour in relation to the development of BKD would be interesting to study but require a separate study as we did not record any detailed data on where the individual fish were caught in relation to the location of local net pen farms. For a complete

evaluation of susceptibility, a controlled infection trial including all four species would be necessary. However, it seems that whitefish and grayling are at least as susceptible to infection with *R. salmoninarum* as Arctic char.

During net pen farming, diseases are not only spread rapidly within the pen due to dense populations but also potentially to the wild fish as excess feed attracts fish in the surrounding water. Surprisingly, the only river with significant numbers of infected fish was Ljungan, where 17% of the caught fish were positive for *R. salmoninarum by* antigen ELISA. Water turnover rates (flow velocity) and depth of lakes will affect the dilution of bacteria released into the water and thereby create different infection pressures. Farm size, size of the wild fish population scouting the farms for free food and migration patterns of the wild fish will also affect the risk of spread. In Ljungan, the local infection pressure is higher as the water volumes are a lot smaller compared to the other three rivers, thus increasing probability for the bacteria to infect wild fish, given that the size and density of the pen farms are comparable at each location. The fact that almost all eDNA water samples were negative, even in sampling points with high prevalence in fish, could be due to the fact that bacteria in general aggregate to particles and sediment to the bottom (Trunk et al., 2018), or that they are simply diluted too much in the vast volumes of water. Thus, the detection of *R. salmoninarum* is likely challenging from water samples unless larger volumes (>10L) are filtered. However, when analysing eDNA from sediment samples taken below previously infected pens, we detected *R. salmoninarum* DNA at all locations but one. No *R. salmoninarum* DNA was detected at the control point. This is a promising discovery as eDNA from sediment may then be used to monitor introduction of BKD and infection pressure during outbreaks. When one of the locations were resampled 3 months later, no *R. salmoninarum* DNA was detected showing that the presence of *R. salmoninarum* DNA in the sediment is directly connected to an ongoing BKD outbreak in the pen.

In conclusion, our study shows that BKD is present in the wild fish populations at a low level. However, there is a significant risk associated with open net pen farming in these waters as the pathogen can easily be spread outside the pen if the conditions are right. In addition, we show that eDNA from sediment may be useful to determine if *R. salmoninarum* is present in a farm without scarifying valuable livestock. This, however, needs to be further verified with samples from both sediment and fish taken at the same time point.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon request.

ORCID

David B. Persson https://orcid.org/0000-0002-6799-7795
Charlotte Axén https://orcid.org/0000-0001-5729-1353

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