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Multiplex droplet digital PCR assay for detection of *Flavobacterium psychrophilum* and *Yersinia ruckeri* in Norwegian aquaculture

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ABSTRACT

We report the development of ddPCR assays for single and simultaneous detection of the bacterial pathogens *Flavobacterium psychrophilum* and *Yersinia ruckeri* in water from land-based recirculation aquaculture systems (RAS), producing Atlantic salmon (*Salmo salar*) smolt. The method was tested and verified for use in water analyses from RAS production sites, and proved to be specific and with sensitivity 0.0011 ng DNA for *F. psychrophilum* and 1.24 ng for *Y. ruckeri*. These bacteria are important fish pathogens that have caused reoccurring salmonid infection disease in RAS. Monitoring pathogen levels in water samples could be a useful alternative surveillance strategy to evaluate operational risk assessment connected to stress factors. Water quality is essential for fish health and growth in RAS production in general, and high or increasing levels of these pathogens in the RAS water may generate an early indication of unfavourable conditions in the RAS environment, and give directions to operational actions. This approach may reduce fish mortality, reduce production loss, and offer more effective and targeted preventive measures within RAS production.

1. Introduction

Norway is the world's largest producer of farmed Atlantic salmon, *Salmo salar* (Falconer et al., 2020), and an increasing part of the land-based hatcheries and smolt production facilities applies recirculation aquaculture system (RAS) technology. RAS has several advantages with regards to disease prevention, since these closed systems minimized the risk of the introduction of pathogens and contaminants from the environment (Xue et al., 2017). However, good microbiological water quality is still crucial for successfully operation of RAS. Although the inlet and outlet of water in RAS are strictly regulated according to Norwegian legislation (FOR-1997-02-20-192, *Regulation on disinfection of inlet and outlet water from aquaculture related activity*), pathogens may enter RAS via raw water, feed, fish roe or insert of fish. Both poor raw water quality and deterioration of the water in the recirculation system may lead to unwanted episodes of disease in RAS, thus disinfection and control can be problematic because microbes may establish in biofilm or sludge in tubes and cages (Hjeltnes et al., 2019).

Important fish pathogens that have caused reoccurring salmonid infection disease in RAS are the bacterial species *Yersinia ruckeri* and *Flavobacterium psychrophilum* (Hjeltnes et al., 2019). *F. psychrophilum* is the causative agent of bacterial cold-water disease (BCWD) and rainbow trout fry syndrome (RTFS) in freshwater salmonid fish worldwide (Nematollahi et al., 2003). This bacterium is problematic in RAS due to its biofilm forming properties. *Yersinia ruckeri* causes enteric red mouth disease (ERM) (Dear and Road, 1988), a systemic infection of yersiniosis occurring mostly in farmed rainbow trout (*Oncorhynchus mykiss*), but also in farmed Atlantic salmon (*Salmo salar*). Several stress factors are important determinants in the development of this infection, and it is suspected that disease outbreaks at sea may be related to stress-induced activation of infection carried by healthy fish from the freshwater phase (Gulla et al., 2018; Onuk et al., 2010). Both yersinosis and flavobacteriosis are increasing problems in Norwegian aquaculture, especially in salmonid fish hatcheries in the middle and north of Norway (Hjeltnes et al., 2019). In recent years, genotyping assays have revealed the occurrence of genetically distinct clonal complexes connected to disease outbreaks of both *Y. ruckeri* and *F. psychrophilum* in the Nordic countries (Gulla et al., 2018; Nilsen et al., 2014), which shows the need for highly specific detection and quantification methods.

Quantitative PCR (qPCR) of fish tissue samples and histology are the most commonly used methods for detection of pathogens in Norwegian

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aquaculture. Both when disease is suspected, and for regular monitoring, samples are collected by veterinarians associated with the aquaculture companies, and analysed by commercial diagnostic labs, generating results available after several days. Though, well established and reliable, currently applied diagnostic routines are often too time consuming to allow application of directed and effective disease response actions at an early stage. Multiplex qPCR, multilocus sequence typing (MLST), variable tandem repeats (VNTR) and multilocus variable-number tandem-repeat analysis (MLVA) genotyping assays have been previously established (Altinok et al., 2008; Apablaza et al., 2015; Gulla et al., 2018; Nilsen et al., 2014) but there are few sensitive and rapid molecular identification assay that detects both *Y. ruckeri* and *F. psychrophilum* simultaneously (Daneshamouz et al., 2019; Del Cerro et al., 2002; Onuk et al., 2010), and no known reports of application of digital PCR technology.

Monitoring pathogen levels in water samples could be a useful surveillance strategy to evaluate operational risk assessment connected to stress factors. A challenge in this regard is that pathogen concentrations tend to be low in environmental samples such as water (Gorski et al., 2019), and inhibition by the RAS microbiota and chemical parameters calls for the development of a method that is less affected by such factors. There is a need for a simple, reasonable and less invasive method to monitor pathogen levels in the RAS-environment, that may generate an early indication of unfavourable conditions and give directions to operational actions.

Digital droplet PCR (ddPCR) is a third generation PCR technology that is unique with respect to sensitivity and specificity, and it gives an absolute quantification of a targeted genetic sequence (Hindson et al., 2011). ddPCR can be used to detect low DNA concentrations and has e.g., been used to detect low or not-detectable (with standard qPCR) levels of HIV in blood plasma, with a quantification limit of only 7 gene copies/ml (Ruelle et al., 2014). Another advantage with ddPCR is that this approach has lower variability than qPCR and are to a less degree affected by inhibition in environmental samples and competitive effects in multiplex assays (Te et al., 2015). Compared to qPCR, ddPCR has been reported as more accurate and sensitive (Li et al., 2018), and ddPCR were reported as faster and half as costly as the qPCR analyses in analyses of aquatic invasive species (Nathan et al., 2014). Therefore, ddPCR analyses has the potential for rapid screening of low levels of fish pathogens with respect to both sensitivity and accuracy, as well as costs.

The aim of the present study was to develop a sensitive and rapid method for simultaneous analysis of the bacterial pathogens *Y. ruckeri* and *F. psychrophilum* in water from commercial RAS for salmon smolts (*Salmo salar*) production. Detection of infective agents prior to disease outbreaks may generate an early indication of unfavourable conditions in the RAS environment, and give directions to operational actions. This approach may reduce fish mortality, reduce production loss, increase fish welfare, as well as offer more effective and targeted preventive measures within RAS production.

2. Material and methods

2.1. Bacterial isolates

Reference strains of *Yersinia ruckeri* (ATCC29473, serotype O1), *Flavobacterium psychrophilum* (NCIMB 2282) were used as positive controls. To verify the primer functionality against several subspecies, *Yersinia ruckeri* NCIMB1315, NCIMB1316 and NCIMB13282 as well as *Flavobacterium psychrophilum* NCIMB13383, NCIMB13384 and NCIMB1947 were also included in the study, and treated as described for the other strains below. *Yersinia enterocolitica* (CCUG 4586) and *Flavobacterium branchiophilum* (NCIMB 12904) were used as negative controls. The *Yersinia* strains were cultured in nutrient broth (Oxoid ltd., UK). *Y. ruckeri* was incubated at 20 °C and *Y. enterocolitica* at 37 °C, both with shaking at 200 rpm for 3–5 days. *Flavobacterium psychrophilum* was cultured in Anacker-Ordal medium (Anacker and Ordal, 1959) supplemented with 5% horse serum at 12 °C, and *F. branchophilum* was cultivated in Anacker-Ordal medium supplemented with 10% fetal bovine serum at 15 °C, both with shaking at 200 rpm for 5–7 days

2.2. Sample material

Samples from three different commercial salmon smolts RAS facilities in Norway were collected at six different timepoints. 150–200 ml of inlet water and rearing water was filtered (0.22 µm Sterivex filter (Millipore), and filters stored at −20 °C until used for eDNA isolation. Experimental sample material (mock-samples) used to develop the method was made using isolated genomic DNA spiked in sterile water at different concentrations, making a genomic DNA (gDNA) dilution series for determination of the assay sensitivity. In addition, isolated genomic DNA was spiked into real environmental DNA samples containing total RAS microbiota DNA to obtain a realistic background, to verify both specificity and sensitivity of the method in samples with real microbiota background.

2.3. DNA isolation

Genomic DNA was isolated from cultivated bacterial strains using 6 ml liquid culture and the Wizard Genomic DNA Purification Kit (Promega). For isolation of total DNA from water samples, ca. 200 ml tank water was filtrated through 0.22 µm Sterivex filters (Millipore). Filter membranes were manually removed from the filter cartridges, cut in small pieces and used for total DNA extraction employing the ZymoBIOMICS DNA miniprep kit (Zymo Research) in combination with the FastPrep24 instrument (MP Biomedicals). Concentration and quality of extracted DNA was analysed using Qubit 3.0 (Thermo Fisher Scientific) and Nanodrop 1000 (Thermo Fisher Scientific), respectively.

2.4. Primer design and ddPCR

For specific detection of the two fish pathogens, primer sequences were designed for the reference strains, Table 1, using Primer3Plus (https://primer3plus.com/). For *Flavobacterium psychrophilum*, primers were designed to target the *dnaN* gene (GeneID 5,299,071, NCBI Reference Sequence: NC_009613.3; (Duchaud et al., 2007) producing an amplicon of 185 bp, and for *Yersinia ruckeri* the *yruR-I* gene was targeted, resulting in an amplicon of 196/194 bp, primer sequence was based on sequence information in Temprano et al. (2001). In addition, the primer sequences were blasted against other genomes of *Flavobacterium* spp. and *Yersinia* spp., respectively, giving alignment scores below 40% when aligned against other species than the targeted ones. ddPCR was performed on the QX200™ Droplet Digital™ PCR system (Bio-Rad) with the ddPCR™ Supermix for probes with no dUTP kit (Bio-Rad). Reactions were set up in a final volume of 20 µl at 900 nM for

Table 1
Primers designed for specific detection of *Y. ruckeri* and *F. psychrophilum* including information on target genes as well as the probes used.

Bacterial strain	Target gene	Forward (5′-3′)	Reverse (5′-3′)	Probe (5′-3′)
Y. ruckeri	*yruR/yruI*	CGATTGTTATCATATTTACGGCCATAC	TATGGAAATTGCGAGTCAAGCTGC	*HEX*-ATGTATTGACTACCCTATCGCCAATGGATTGTCACA- *BHQ1*
F. psychrophilum	*dnaN*	GCATCGGTAGCCACAAAAAT	GGTGACGAATTTCCAAAAGC	FAM- ATC CTT CTG GCG AAA ACT GA -BHQ1

each primer and 250 nM for the probe (primer sequences and probe information in Table 1) and droplet generation was carried out according to manufacturer's protocol. Amplification was initiated by enzyme activation at 95 °C for 10 min, followed by 40 cycles of amplification (94 °C for 30 s, 58 °C 1 min) and enzyme deactivation (98 °C 5 min), temperature ramp 2.5 °C/s. Fluorescent intensity was then measured in a QX200 Droplet Reader (Bio-Rad) and the signal data analysed (QuantaSoft, Version 1.5.38, Bio-Rad).

2.5. Experimental design

For verification of the ddPCR methodology and the primers designed, a set of test experiments were performed, using reference strains of two known fish pathogens *Flavobacterium psychrophilum* (NCIMB 2282) and *Yersinia ruckeri* (ATCC29473, Serotype O1) as proof-of-principle species. These were cultivated as liquid culture and gDNA was isolated and used for detection. Genomic DNA (gDNA) from both species was diluted stepwise in sterile water (water background samples) or diluted and spiked in at given concentrations into real total DNA samples (5 ng eDNA isolated from RAS samples), verified to lack the relevant species using 16S rRNA amplicon sequencing (i.e., 'metagenome background'). gDNA from the two species was also used in a similar manner for multiplex experiments, with simultaneous detection of both species in the same experiment (using different gDNA inputs). Apart from these model experiments, real RAS samples (n = 132) in which one of these species was previously detected or not detected based on 16S rRNA sequencing, were used for analyses of the pathogenic species, verifying detection in real samples.

3. Results

3.1. Sensitivity and specificity of the ddPCR method

The designed primers for detection of *F. psychrophilum* and *Y. ruckeri* were tested according to the experimental design described in Materials and Methods. Both primer sets were found to work for detection of the respective species, with sensitivity down to 0.0011 ng input for *F. psychrophilum* and down to a 1.24 ng input for *Y. ruckeri* (Figs. 1A and 2A, respectively). In experiments in which isolated gDNA from the respective strains was spiked in a sample of eDNA isolated from a RAS sample with no detection of the species, i.e., in a metagenome background, sensitivity was found to be at similar levels (0.0011 ng and 1.24 ng input DNA for *F. psychrophilum* and for *Y. ruckeri*, respectively) as in water background samples (Figs. 1B and 2B). To verify the primer functionality against several subspecies of *F. psychrophilum* and *Y. ruckeri*, detection experiments with the same design were run using isolated gDNA from subspecies *Y. ruckeri* NCIMB1315, NCIMB1316 and NCIMB13282 as well as *F. psychrophilum* NCIMB13383, NCIMB13384 and NCIMB1947, resulting in positive gene counts for all subspecies (for number of gene copies counted, see Table 2), with similar counts monitored for DNA diluted in both water as well as in metagenome background. The method and the primers designed were shown to be more sensitive for the detection of *F. psychrophilum* compared to *Y. ruckeri*, differing by a magnitude of 1000. Sterile water did not result in any gene copy counts using either of the primers sets. Further, specificity of the primers was analysed using isolated gDNA from *F. branchiophilum* (NCIMB 12904) as well as from *Y. enterocolitica* (NCIMB 12904), both serving as negative control strains and none resulting in any gene copy counts in the analysis (Figs. 1A and 2A), verifying the species specificity and accuracy of the primers designed and the analysis.

3.2. Multiplex analyses

To verify that multiplex analyses, i.e., simultaneous detection of two species, do not affect the sensitivity of the assay, a mixture of gDNA from *F. psychrophilum* and *Y. ruckeri* (0.055 ng and 6.3 ng, respectively) was analysed using primers for both species separately, or simultaneously, both in a water background as well as in a metagenome background (Fig. 3). Results show that the sensitivity was similar for both species regardless of if they were analysed separately or simultaneously (multiplexing), or if they are analysed in a water background or in a metagenome background. To look further into detail of the multiplex experiment, gDNA from the two species, *F. psychrophilum* and *Y. ruckeri*, were mixed at different concentrations (Fig. 4, panel C), and the different samples analysed simultaneously both in water as well as metagenome background (Fig. 4, panels A and B, respectively). The presented data indicate, that even though the method is more sensitive for detection of *F. psychrophilum* than of *Y. ruckeri*, the results identifying both species are reproducible, following the respective dilution of the gDNA spiked into the samples, and do not differ when analysed in water or metagenome background (Fig. 4).

3.3. RAS water samples

Both species could also be detected in real RAS water samples (Table 2), with established detection of *F. psychrophilum* or *Y. ruckeri*, as determined by 16S amplicon sequencing (results not shown). This multiplex experiment analysed both species simultaneously. *Y. ruckeri* was detected in samples 1–3, where *F. psychrophilum* was not detected. *F. psychrophilum* was detected in samples 4–6, where *Y. ruckeri* was not detected. Two positive controls were included in the same run (gDNA isolated from pure culture of *F. psychrophilum* and *Y. ruckeri*, respectively), verifying the functionality of the assay. Altogether 132 RAS samples were analysed by the multiplex ddPCR method to demonstrate the applicability. *Y. ruckeri* was detected in 3 samples from one RAS production sites, while *F. psychrophilum* was detected in 18 samples from 2 RAS production sites, ranging from 1 to 240 gene copies / ml sample water for *F. psychrophilum* and 1–40 gene copies / ml water sample for *Y. ruckeri* (Table 2).

4. Discussion

A multiplex ddPCR method for simultaneous detection of *Y. ruckeri* and *F. psychrophilum* has been successfully developed. The method has been tested and verified for use in water analyses from RAS production sites. The results establish high specificity and sensitivity in detecting both fish pathogens, including 4 subspecies of each pathogen, and the method allows for low input of DNA in the analysis.

Comparison and calculation of DNA gene counts correlations to colony forming units (CFU) are challenging due to several uncertain factors, since the methodology measures DNA rather than colonies. The number of gene copies of a targeted sequence does not reflect the actual number of live cells in the sample, this has also been assessed in the literature (Gorski et al., 2019). Factors such as sample preparation, DNA isolation procedures, DNA shearing as well as cultivation challenges can make such comparisons unreliable. The ddPCR methodology measures gene copies of amplification-able DNA, and hence highly degraded DNA will not give signal in the analysis. Detection methods based on isolated DNA will also capture bacterial cells not culturable on agar plates, which for many bacteria is limited and unsuitable as a detection methodology due to low growth recovery. Therefore, comparing ddPCR results using isolated DNA to methodologies operating with CFU as detection limits is challenging, and since DNA input has been used in this study, attention has not been made to compare the results with CFU of cultivated strains. Some studies however, such as the one by Onuk et al. (2010) based on multiplex PCR (m-PCR) do also operate with DNA input as measure for sensitivity, and the established ddPCR methodology can therefore be compared to these. Onuk and colleagues reported sensitivity, in a mock sample in sterile water, to be 0.035 ng of DNA input for both *F. psychrophilum* and *Y. ruckeri*. In comparison, the DNA input amounts reported here were 0.001 ng and

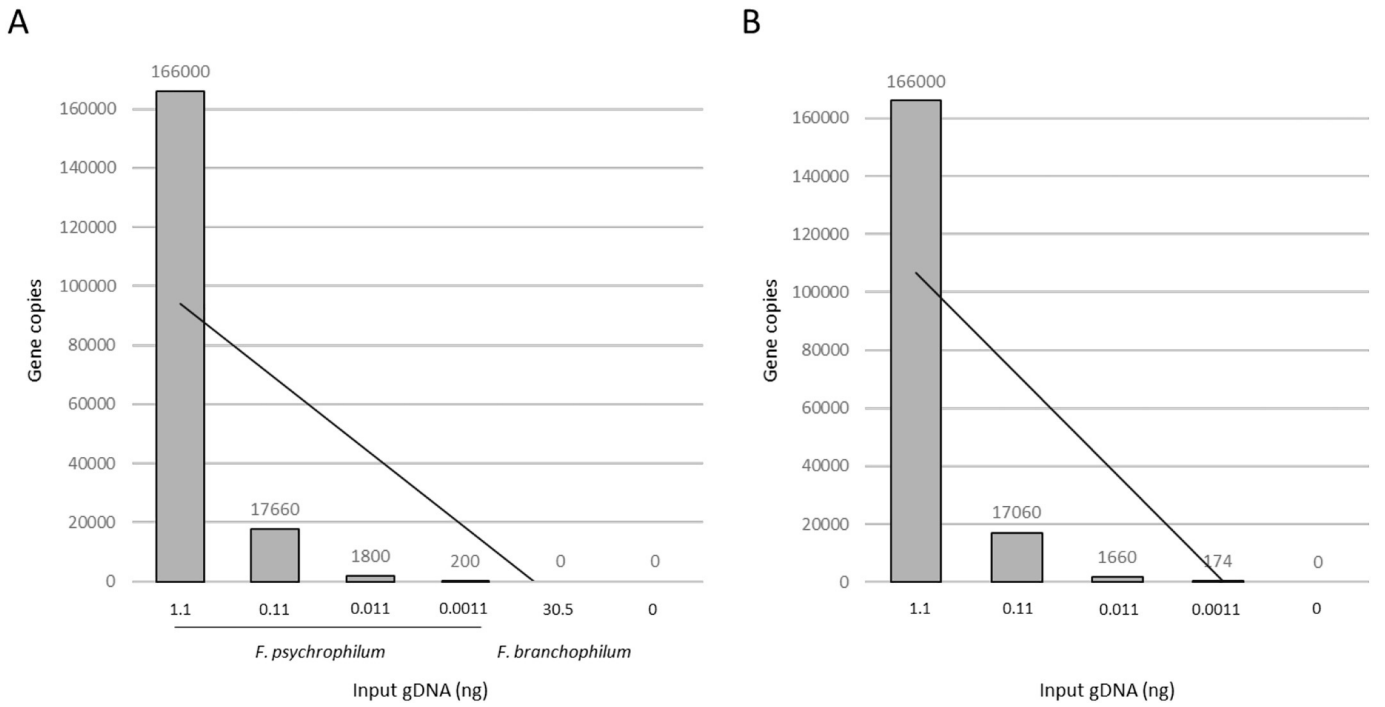


Fig. 1. A) Dilution series in sterile water of *F. psychrophilum* gDNA isolated from pure culture (4 left bars). Addition of 1.1 ng DNA gave 166,000 gene copy counts. Ten-fold dilution steps gave about ten-fold reduction in counts recorded. Input of 30.5 ng of *F. branchophilum* gDNA (negative control) gave no gene copy counts, second bar to the right, and no addition of gDNA (0), i.e., sterile water sample, did not also not result in gene copy counts, (far right bar). B) Dilution series within a metagenome background (isolated aquaculture eDNA, no *F. psychrophilum* detected) of *F. psychrophilum* gDNA isolated from pure culture spiked into the sample. Water (0) was used as blank and gave no gene copy counts. A linear regression line showing the best fit of datapoints is presented in panel A and B.

0.6 ng for *F. psychrophilum* and *Y. ruckeri*, respectively. Hence the sensitivity in detecting *F. psychrophilum* is higher using the ddPCR method established, whereas for *Y. ruckeri* the observed sensitivity is found to be lower than earlier observed. This can be explained by the efficiency of the primers designed, the gene targeted, the nature of the ddPCR methodology or a combination of these factors. Onuk et al. (2010) target the 16S rRNA gene, known to be presented by several copies in the genome, and therefore direct comparison is difficult to make when counting detected gene copies. In addition, primers designed may also have different sensitivity, which will be reflected in the observed results.

Apart from the detection methodology (PCR, ddPCR, TaqMan multiplex PCR) the gene target for the analysis is of relevance, both for specificity and sensitivity. For *F. psychrophilum*, the *dnaN* gene was

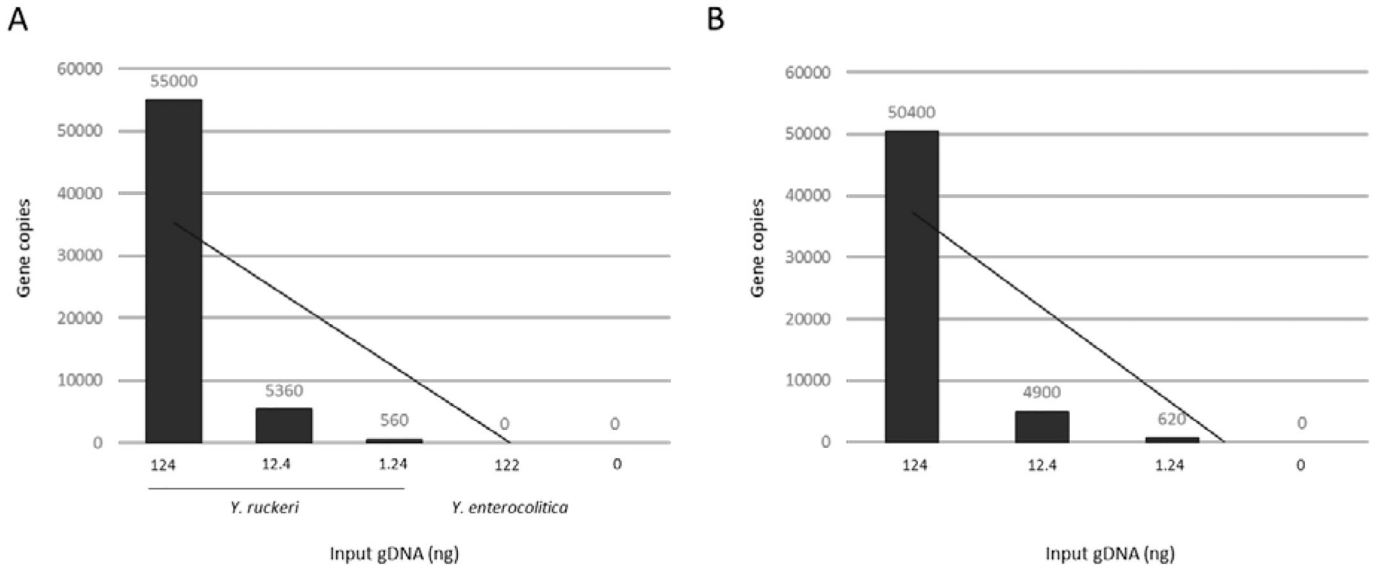


Fig. 2. A) Dilution series in sterile water of *Y. ruckeri* gDNA isolated from pure culture (3 bars to the left). Ten-fold dilution steps gave about ten-fold reduction in gene copy counts recorded. Input of 122 ng of *Y. enterocolitica* gDNA (negative control) gave no gene copy counts, second bar to the right, and no addition of gDNA (0), i.e., sterile water sample, did not result in any recorded gene copy counts (far right bar). B) Dilution series within a metagenome background (isolated aquaculture eDNA, no *Y. ruckeri* detected) of *Y. ruckeri* gDNA isolated from pure culture spiked into the sample. Ten-fold dilution steps gave about ten-fold reduction in gene copy counts recorded. Water was used as blank (0), not shown. A linear regression line showing the best fit of datapoints is presented in panel A and B.

Table 2
Results from detection of *F. psychrophilum* and *Y. ruckeri* in water samples from three RAS facilities (n = 132). Detections were run in multiplex experiment with simultaneous analysis of both species and using 1 ng metagenomic DNA isolated from aquaculture samples as input. Positive controls were run using gDNA from *F. psychrophilum* and *Y. ruckeri* in 20 µl reactions (using 1 ng and 10 ng gDNA, resulting in 6640 and 7480 counts, respectively) and water was used as blank control.

Sample	Gene copies	
	F. psychrophilum	*Y. ruckeri*
1	0	40
2	0	40
3	0	40
4	140	0
5	20	0
6	240	0
7–25	1–60	0
26–132	0	0
Pos. *F. psychrophilum* NCIMB2282 *(1 ng)*	6640	0
Pos. *F. psychrophilum* NCIMB13383 *(0.27 ng*	418	0
Pos. *F. psychrophilum* NCIMB13384 *(0.58 ng)*	7720	0
Pos. *F. psychrophilum* NCIMB1947 *(0.55 ng)*	12520	0
Pos. *Y. ruckeri* ATCC29473 *(10 ng)*	0	7480
Pos. *Y. ruckeri* NCIMB1315 *(1.2 ng)*	0	274
Pos. *Y. ruckeri* NCIMB1316 *(1.3 ng)*	0	454
Pos. *Y. ruckeri* NCIMB13282 *(3.9 ng)*	0	8180
Sterile water	0	0

selected as a target gene based on it being species specific, in addition to being present in one copy only per genome (in contrast to the 16S rRNA gene). *dnaN* seems like a satisfactory performing gene target for taxa detection for *F. psychrophilum*, showing high sensitivity as well as specificity in the analysis and control experiments performed. For *Y. ruckeri*, the *yruI-R* gene was chosen as a target based on its presence as one copy per genome and the reference from Temprano et al. (2001), where this gene was used as a target with good results. From the results in this work, however, the 16S rRNA gene is seemingly more sensitive compared to the *yruI-R* gene, and therefore it might be of interest to apply primers targeting this gene in the described ddPCR method. However, when using the 16S rRNA gene as target one must be aware of the occurrence of several copies of this gene within a bacterial genome (copy number dependent on species). Furthermore, apart from the results in this study presenting the same results for *F. psychrophilum* and *Y. ruckeri* DNA both in water samples and in a metagenome background, it is generally observed in the literature that the sensitivity is lower when analysing DNA in a sample involving background (Apablaza et al., 2015; Del Cerro et al., 2002; Onuk et al., 2010). The results show that the method is minorly affected by inhibition of the background microbiota and/or chemical factors, which has been reported previously (Gorski et al., 2019).

Low DNA yields are challenging in RAS water since pathogen concentrations may be very low due to the water dilution effect, and the sampling procedures may cause loss of DNA. The ddPCR method has the potential for rapid screening of samples and detection of low levels of *Y. ruckeri* and *F. psychrophilum*, and it may also have the potential of sublevel detection of different clonal complexes of the pathogens.

Monitoring pathogen levels in water samples could be a useful alternative surveillance strategy to evaluate operational risk assessment connected to stress factors. Both biological and physio-chemical water quality is essential for fish health and growth in RAS production in general, and it is also suspected that disease outbreaks at sea may be related to stress-induced activation of infection carried by healthy fish from the fresh-water phase (Gulla et al., 2018; Onuk et al., 2010). High or increasing levels of these pathogens in the RAS water may be used as early warning and give directions to the management of the RAS.

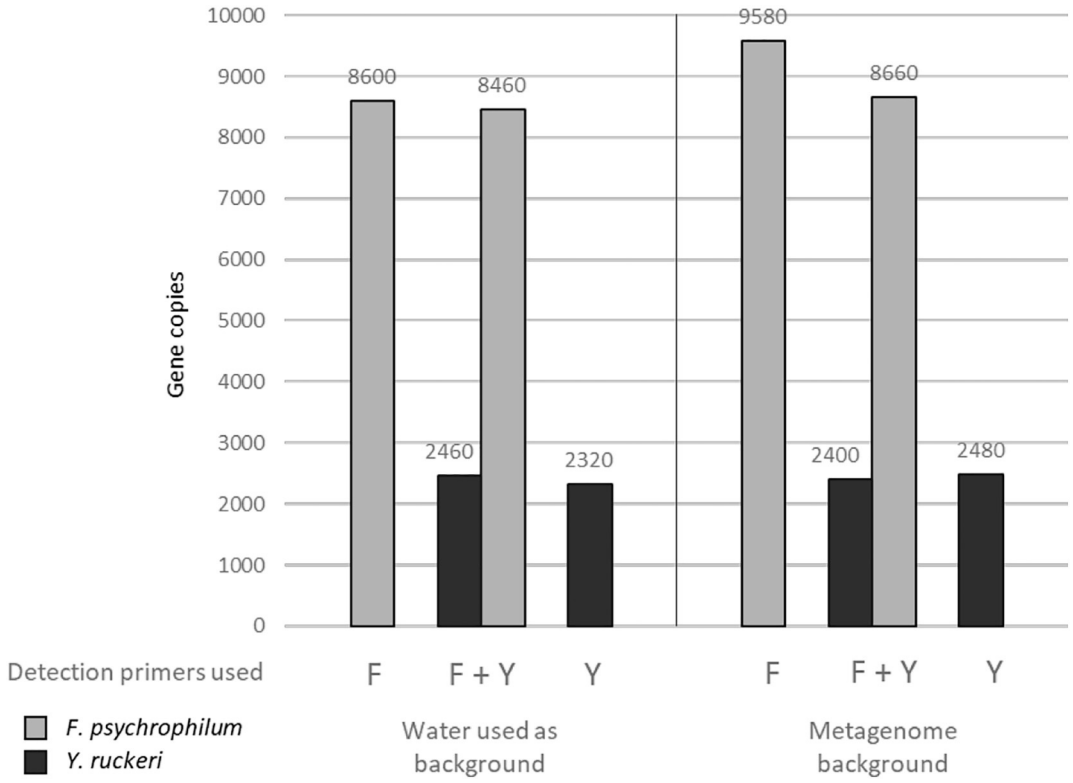
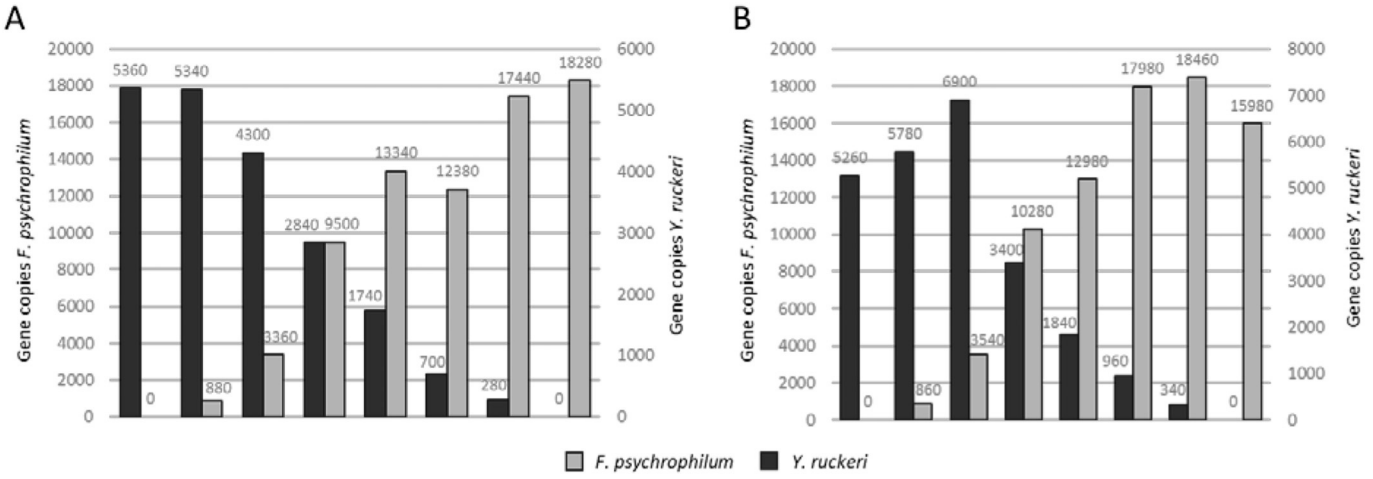


Fig. 3. Multiplex analysis and its effect on detection levels. Left panel) Detected gene copies of *F. psychrophilum* (F) and *Y. ruckeri* (Y) analysed separately as well as multiplexed (F + Y) in a water background, i.e., gDNA isolated and diluted in sterile water. Right panel) Detected gene copies of *F. psychrophilum* (F) and *Y. ruckeri* (Y) analysed separately as well as multiplexed (F + Y) in a metagenome background, i.e., gDNA isolated spiked into an eDNA sample from aquaculture (5 ng) with no previous detection of the two species. For all samples, 0.055 ng of gDNA was used for *F. psychrophilum* and 6.3 ng of gDNA from *Y. ruckeri*.



C

Sample	gDNA (ng)	
	F. psychrophilum	*Y. ruckeri*
1	0	12.4
2	0.0055	11.8
3	0.022	9.9
4	0.055	6.2
5	0.077	3.7
6	0.094	1.9
7	0.1	0.6
8	0.11	0

Fig. 4. A) Multiplex analyses for simultaneous detection of *F. psychrophilum* and *Y. ruckeri* using isolated gDNA from the two species at different concentrations and in a water background. gDNA input used is presented in table to the right, panel C. B) Multiplex analyses for simultaneous detection of *F. psychrophilum* and *Y. ruckeri* using isolated gDNA from the two species at different concentrations and in a metagenome background, i.e., gDNA spike into total DNA from aquaculture (5 ng) with no previous detection of the two species. gDNA input used is presented in the table, panel C. C) Amounts of gDNA from *F. psychrophilum* and *Y. ruckeri* used in sample 1–8, presented in Fig. 4 A and B.

Further studies are needed to establish such applications. At present, large amounts of fish are sacrificed to exclude or detect diseases. It is an aim for the aquaculture industry to minimise the use of fish for research and surveillance purposes. Analyses of water samples are a less invasive surveillance strategy in this regard and may reduce the need for investigating live fish, although further research is needed to investigate the relationship between pathogen water levels and actual disease.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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