Absolute quantification of priority bacteria in aquaculture using digital PCR

Roman Netzer [a,*], Deni Ribičić [a], Marianne Aas [a], Laura Cavé [b], Trisha Dhawan [b]

[a] *SINTEF Ocean, Brattørkaia 17C, Trondheim 7010, Norway*
[b] *Stilla Technologies, Biopark, 1, Mail du Professeur Georges Mathé, Villejuif, 94800, France*

A B S T R A C T

Modern aquaculture systems are designed for intensive rearing of fish or other species. Both land-based and offshore systems typically contain high loads of biomass and the water quality in these systems is of paramount importance for fish health and production. Microorganisms play a crucial role in removal of organic matter and nitrogen-recycling, production of toxic hydrogen sulfide (H_2S), and can affect fish health directly if pathogenic for fish or exerting probiotic properties. Methods currently used in aquaculture for monitoring certain bacteria species numbers still have typically low precision, specificity, sensitivity and are time-consuming. Here, we demonstrate the use of Digital PCR as a powerful tool for absolute quantification of sulfate-reducing bacteria (SRB) and major pathogens in salmon aquaculture, *Moritella viscosa*, *Yersinia ruckeri* and *Flavobacterium psychrophilum*. In addition, an assay for quantification of *Listeria monocytogenes*, which is a human pathogen bacterium and relevant target associated with salmonid cultivation in recirculating systems and salmon processing, has been assessed. Sudden mass mortality incidents caused by H_2S produced by SRB have become of major concern in closed aquaculture systems. An ultra-sensitive assay for quantification of SRB has been established using *Desulfovibrio desulfuricans* as reference strain. The use of TaqMan® probe technology allowed for the development of multi-plex assays capable of simultaneous quantification of these aquaculture priority bacteria. In single-plex assays, limit of detection was found to be at around 20 fg DNA for *M. viscosa*, *Y. ruckeri* and *F. psychrophilum*, and as low as 2 fg DNA for *L. monocytogenes* and *D. desulfuricans*.

1. Introduction

The production of fish in aquaculture systems is constantly increasing worldwide. To meet challenges associated with intensification of fish farming, especially of high value salmonid species, different systems have been developed including net pens, sea cages, raceways and recirculating aquaculture systems (RAS). In all these systems, microbial communities play an important role for the biological water quality, and fish health and welfare are closely associated with certain microorganisms.

Especially in closed systems, such as RAS, bacteria are crucial for the water quality. Besides their vital ecosystem functions, such as nitrification, denitrification and degradation of organic material, microbes can also have a direct effect on the health of fish. In the here presented work, we targeted at analyzing bacteria belonging to three different categories, all highly relevant for commercial salmonid production: (i) fish pathogens, (ii) a human pathogen that can be transferred from (sea) food products to consumers, and (iii) bacteria threatening fish welfare by compromising the rearing environment.

While some bacteria can impact fish health positively (e.g. probiotic bacteria), pathogenic and opportunistic microbes represent an eminent threat. *Yersinia ruckeri* and *Flavobacterium psychrophilum* are major pathogens involved in infections of intensively farmed salmonids and responsible for significant economic losses (Hjeltnes et al., 2019). *F. psychrophilum* is the causal agent of bacterial cold-water disease and rainbow trout syndrome, responsible for major losses in salmonid aquaculture industry worldwide (Nematollahi et al., 2003). Beside rainbow trout (*Oncorhynchus mykiss*) as the most affected species, the pathogen has become a severe problem also in Atlantic salmon (*Salmo salar*) hatcheries in Scotland and Chile (Hoare et al., 2019). *Y. ruckeri* is the causative agent of the highly contagious yersiniosis or enteric red mouth disease which can infect different fish species including salmon, eel, sole, sturgeon and turbot (Chapela et al., 2018). Winter ulcer is a major problem for Atlantic salmon aquaculture in Norway. This ulcerative disease is mainly associated with the bacterium *Moritella viscosa* and despite widespread vaccination continuously reported in

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* Corresponding author.
E-mail address: roman.netzer@sintef.no (R. Netzer).

Norwegian coastal aquaculture (Karlsen et al., 2017).

Beside fish pathogenic bacteria, the human pathogen *Listeria monocytogenes* is of major concern in the salmon processing industry but as a typical member of biofilms in cold environments, it is also relevant in closed aquaculture systems producing market size coldwater species, such as Arctic charr (*Salvelinus alpinus*). By consuming contaminated products, this gram-positive, psychrophile, facultative anaerobic bacterium can provoke listeriosis, a potentially life-threatening illness in humans with a relatively high mortality reported to be up to 30% (Lomonaco et al., 2015).

In closed aquaculture systems with high recirculation level, also non-pathogenic microbes, such as sulfate-reducing bacteria (SRB), can negatively affect fish health by converting sulfate (SO_4^{2-}) to toxic hydrogen sulfide (H_2S) under anaerobic conditions (Kiemer et al., 1995). Sudden mass mortality has become one of the major risks in salmon smolt RAS and in the past years, an increasing number of such incidents has been reported and most cases have been associated with H_2S (Hjeltnes et al., 2019). Specific and accurate quantification of such priority bacteria in aquaculture systems is crucial for identifying problematic trends, ideally before fish health is affected, and allow implementation of targeted counteractions.

Up to date, microbiological diagnostics in the aquaculture industry are still often limited to the detection of certain fish pathogens or total bacteria numbers using methods such as (semi-)quantitative real-time PCR (rt-qPCR), cell counting, agar plating (Crane and Hyatt, 2011), and low-coverage characterization methods (e.g., DGGE, clone libraries) are still standard to describe the taxa present (Bartelme et al., 2017).

In recent years, a third generation PCR technology, named digital PCR (dPCR), has emerged rapidly as an alternative to rt-qPCR and other quantification methods, and several systems have entered the market. All systems share the common concept of partitioning a sample into a large number of nanoscale partitions and performing end-point PCR reactions in all partitions, with some of them ideally containing one or few copies of the target DNA fragment. Distribution of DNA targets in combination with Poisson statistics allows the calculation of initial target numbers in the sample (Lievens et al., 2016). In contrast to well-established rt-qPCR, dPCR allows absolute target quantification and does not require standards, references or calibration curves for precise quantification of gene targets. This is particularly advantageous when no reference material is available, e.g. due to cultivation issues. Since dPCR technology is based on quantification of positive and negative incidents after end-point PCR amplification, results are also independent of variations in amplification efficiency. This makes dPCR potentially more accurate, reproducible, and less prone to inter-laboratory variations than rt-qPCR (Huggett et al., 2013), and more resilient to inhibitors as suggested in recent studies (Morisset et al., 2013; Doi et al., 2015; Powell and Babady, 2018). So far, dPCR has been successfully used for quantification of virus (e.g. Rački et al., 2014; Huang et al., 2015), bacteria (e.g. Rothrock et al., 2013; Porcellato et al., 2016), animal/human cells (e.g. Miotke et al., 2014; Momtaz et al., 2016) and environmental DNA of fish (e.g. Rothrock et al., 2013; Doi et al., 2015). A more comprehensive overview of dPCR applications and its advantages compared to qPCR has recently been described (Cao et al., 2020).

Popular dPCR systems on the European market include the digital droplet PCR systems QX100/QX200 from Bio-Rad, based on sample partitioning into nanoliter-sized droplets (Mazaika and Homsy, 2014), and the chip-based PCR system QuantStudio 3D from Thermo Fisher Scientific, employing arrays with micrometer-sized reaction wells (Conte et al., 2015). The naica™ system for Crystal digital PCR™, recently commercialized by Stilla Technologies, elegantly combines above mentioned droplet and array-based technologies in the micro-fluidic Sapphire chip (Madic et al., 2016). The naica™ system is composed of two instruments, Geode and Prism3. Sample partitioning, thermal cycling (in the Geode), and fluorescence analysis (in the Prism3) is conducted in the planar Sapphire flow cell chip, which minimizes hands-on time and potential carry-over contamination. The Prism3 can analyze 3 distinct fluorescence channels, providing advanced options for multiplex dPCR assays superior to two-color technology used by most of the other currently available systems. The number of studies using the naica™ system is continuously growing, yet due to its novelty in the market most of them focus on medical research. A complete overview can be found on the Stilla Technologies homepage (https://www.stilla technologies.com/bibliography).

To the best of our knowledge, we here demonstrate for the first time the use of the naica™ system in single- and multi-plex mode for absolute quantification of priority bacteria relevant for aquaculture fish production and processing.

2. Materials and methods

2.1. Reference bacterial strains and DNA

Reference strains *F. psychrophilum* DSM 3660 and *M. viscosa* DSM 25798, as well as DNA from reference strains *Y. ruckeri* DSM 18506, *L. monocytogenes* DSM 20600 and *Desulfovibrio desulfuricans* DSM 642 were purchased from the German collection of microorganisms and cell cultures GmbH (DSMZ). *F. psychrophilum* DSM 3660 and *M. viscosa* DSM 25798 were cultured in R2A and marine broth medium as recommended by DSMZ.

2.2. DNA extraction and mock microbial community (MMC) standard

Genomic DNA (gDNA) of *F. psychrophilum* DSM 3660 and *M. viscosa* DSM 25798 was isolated from 300 µl culture using the Zymo Biomics DNA miniprep kit (Zymo Research) in combination with the FastPrep24 instrument (MP Biomedicals) according to manufacturer's protocol. Extracted and purchased DNA was subjected to quantification and quality control using Qubit 3.0 and Nanodrop 1000 instruments (Thermo Fisher Scientific).

A mock microbial community (MMC) DNA sample was prepared to allow quantification of priority bacteria in a realistic microbiota background. For this purpose, 200 ml of tank water from an operational salmon smolt RAS facility was filtrated through a 0.22 µm Sterivex filter (Millipore). The filter membrane was manually removed from the filter cartridge, cut in small pieces and subjected to total DNA extraction using the Zymo Biomics DNA miniprep kit (Zymo Research) according to manufacturer's protocol. For quantification of priority microbes, this MMC DNA sample was diluted to a concentration of 1 ng/µl, spiked with different concentrations of gDNA isolated from pure cultures of each target strain (either purchased or isolated as described above) and used as template in dPCR assays.

2.3. Oligonucleotides

All oligonucleotide sequences were retrieved from previously published studies and are listed in Table 1. Oligonucleotide primers and TaqMan® probes were synthesized by Eurofins Genomics in HPLC grade.

2.4. Crystal digital PCR™ assays

As template for dPCR assays, ten-fold serial dilutions (0.2–0.000002 ng/µl) of DNA extracts from all priority bacteria were prepared in 1 ng/µl MMC-DNA standard background. The dPCR workflow started with preparing 25 µl reaction mixtures containing 1× concentrated PerfeCTa Multiplex qPCR ToughMix (Quanta Biosciences, Gaithersburg, MD, USA), 1 µM fluorescein (Saint Louis, MO, USA), 1 µM of primers, 250 nM of corresponding TaqMan® probe, and an appropriate amount of DNA template. Up to four PCR reaction mixtures of 25 µl each were then loaded on a Sapphire chip and up to three chips were placed in the Geode instrument for partitioning into 20,000 to 30,000 droplets and performing PCR. PCR conditions were adapted from corresponding

Table 1
Sequences of primer and TaqMan® probe oligonucleotides used in dPCR assays.

Primer/ Probe ID	Sequence 5′ − > 3′	Bacteria (target gene)	Reference
DSR-1F+	ACSCACTGGAAGCACGGCGG	*Desulfovibrio desulfuricans* DSM 642 (*dsrA*)	Bourne et al. (2011)
DSR-R	GTGGMRCCGTGCAKRTTGG		
DSRtaq-CY3	CY3-CCGATAACRCYGCCGCCGTAACCGA-BHQ1		
HLY-F	TGCAAGTCCTAAGACGCCA	*Listeria monocytogenes* DSM 20600 (*hlyA*)	Paul et al. (2015)
HLY-R	CACTGCATCTCCGTGGTATACTAA		
HLYtaq-HEX	HEX-CGATTTCATCCGCGTGTTTCTTTTCG-BHQ1		
GLN-F	TCCAGCACCAAATACGAAGG	*Yersinia ruckeri* DSM 18506 (*glnA*)	Chapela et al. (2018)
GLN-R	ACATGGCAGAACGCAGATC		
GLNtaq-HEX	HEX-AAGGCGGTTACTTCCCGGTTCC-BHQ1		
SIG-F	GGTAGCGGAACCGGAAATG-3	*Flavobacterium psychrophilum* DSM 3660 (hypothetical protein)	Chapela et al. (2018)
SIG-R	TTTCTGCCACCTAGCGAATACC		
SIGtaq-FAM	FAM-CGCTTCCTGAGCCAGA-BHQ1		
TON-F	CGTTGCGAATGCAGAGGT	*Moritella viscosa* DSM 25798 (*tonB*)	Grove et al. (2008)
TON-R	AGGCATTGCTTGCTGGTTA		
TONtaq-CY5	CY5-TGCAGGCAAGCCAACTTCGACA-BHQ2		

previously published methods based on rt-qPCR technology. Sapphire chips were then transferred to the Prism3 fluorescence reader for imaging (Fig. 1A). Data analysis was performed employing Crystal Miner software V2.3.5 (Stilla Technologies, Fig. 1B). For multi-plex assays, spill-over effect may occur due to spectral overlap of the chosen fluorophores. Crystal Miner allows computing the spill-over compensation from mono-color control experiments. The compensation matrices for

duplex and triplex assays were applied before data analysis with Crystal Miner. Regression analysis was performed by plotting the log of DNA concentrations against the corresponding target gene copy number. For all assays, reactions with no template (NTC) were performed to control for DNA contaminations. In addition, reactions with MMC-DNA (1 ng/μl) were performed for all assays to exclude potential false-positive dPCR events.

3. Results and discussion

Various cultivation-based and cultivation-independent methods are used for the quantification of bacteria. Cultivation-based methods will only detect bacteria that are able to replicate under the provided conditions and taxonomic differentiation is very limited. As reported previously, most microorganisms cannot be cultivated and thus not be quantified using such methods (Sharma et al., 2005). The "great plate anomaly" describes the well-known several orders of magnitude differences between bacteria numbers quantified by microscopic examination and microscopic plate counting (Staley and Konopka, 1985). Enumeration using (fluorescence) microscopy is also prone to errors because bacteria often grow in aggregates or biofilms, which makes single cell counting challenging. Such aggregation may also be problematic for quantification employing flow cytometry or fluorescent activated cell sorting. In recent years, rt-qPCR has become a popular cultivation-independent method for microbial diagnostics. Rt-qPCR is a quantitative technique employing fluorescent reporter dyes to combine PCR amplification and detection in a single tube format. Fluorescence signal monitoring during the exponential phase of PCR, when the quantity of the PCR product is directly proportional to the amount of template present in the original DNA, allows for quantitative analysis of microbes by measuring the abundance of a target sequence in DNA samples. Rt-qPCR has been proven as a powerful tool for enumeration of various bacterial species, groups and even total bacteria counts. However, a major drawback of this technology is that so-called threshold cycle (C_T)
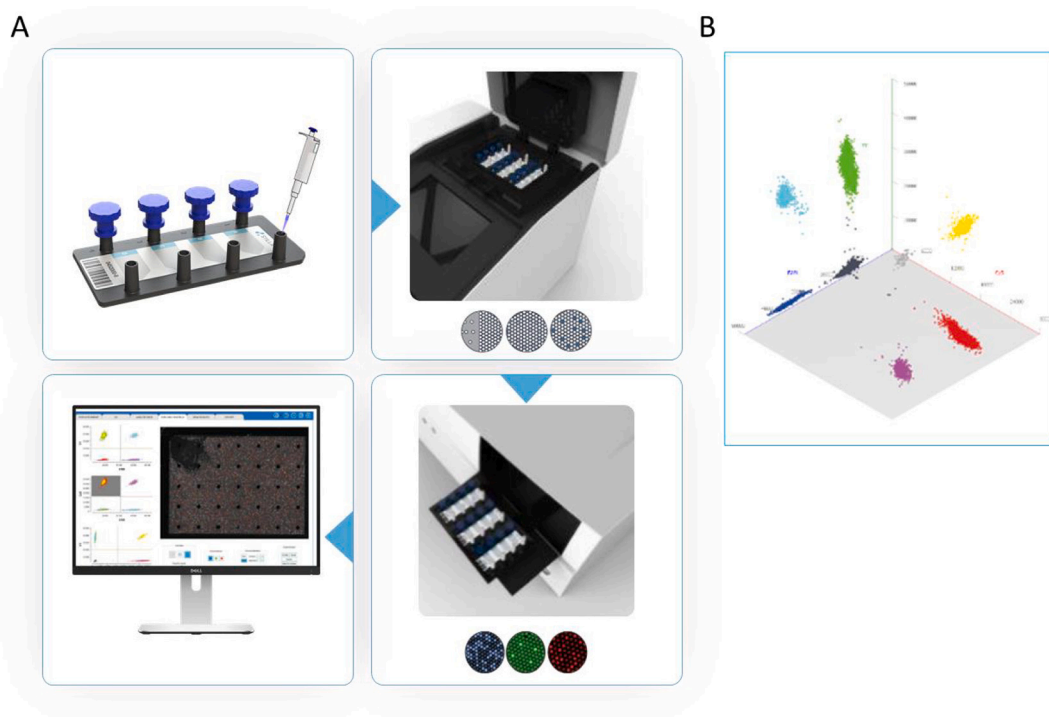

Fig. 1. A) The naica™ system workflow including pipetting 25 μl of PCR mix into the Sapphire chips and seal with cap, placing Sapphire chips into the Geode and launch the combined partitioning and thermocycling program and reading Sapphire chips with Prism3 using three fluorescent detection channels and analyze results using the Crystal Miner software. Depending on the PCR program, the entire workflow takes about 2 h and 30 min with a hands-on time of 5 min. B) Data can be visualized in a 3D dot plot to accurately visualize the populations.

values are determined in the sample and a calibration curve for a reference material of known target concentrations is needed to convert C_T's into corresponding target concentrations. Hence, rt-qPCR assays require a standard for determining absolute copy numbers of a certain gene target, which makes inter-experimental comparison and interpretation of data difficult. Digital PCR technology has overcome this limitation by partitioning a DNA sample into a large number of subsamples which are subjected to an end-point PCR amplification (Kuypers and Jerome, 2017). Consequently, dPCR allows absolute quantification of DNA target molecules in a sample, such as total DNA isolated from environmental sample material. Here, the naica™ system has been used for absolute quantification of different bacterial species which are highly relevant for the aquaculture sector. For all species, single copy genes have been selected as targets which allows direct correlation of results with the genome copy number. Assuming that bacteria typically contain one genome copy per cell, this number can be seen as equivalent with the cell number. Based on the individual genome size and DNA amount used in dPCR assays, the theoretical genome copy number for each strain at the lowest DNA concentration revealing a positive result (limit of detection, LoD) has been calculated using the online NEBioCalculator V1.12.0 (www.nebiocalculator.neb.com) and are shown in Table 2.

For absolute quantification of all five selected priority bacteria, dPCR assays have been developed by adapting primers and TaqMan® probe combinations that have been reported for rt-qPCR assays previously. Main focus was on the development of single-plex dPCR assays for the five targeted priority bacteria. All assays were performed in the MMC-DNA background to ensure realistic diagnostic conditions. For all five investigated gene targets, no positive signal was detected in the absence of corresponding DNA isolates. This was verified by performing corresponding assays with non-spiked MMC-DNA background as template (1 ng/µl). Individual reactions were also performed with DNA-free water instead of MMC-DNA and no significant differences were observed (data not shown). Beside the single-plex assays, one duplex and one triplex assay have been tested to demonstrate the potential of the naica™ system for simultaneous quantification of two and three gene targets, respectively.

3.1. Single-plex dPCR assay for L. monocytogenes quantification

For the quantification of *L. monocytogenes*, the single copy gene *hlyA*, encoding the virulence factor listeriolysin O, has been reported to be an excellent biomarker for identifying *L. monocytogenes* in the presence of other *Listeria* strains (Nogva and Rudi, 2004). The HLY primer/probe combination has been validated in rt-qPCR assays previously, showing to be positive for all 65 tested *L. monocytogenes* isolates and negative for all other *Listeria* strains (16 isolates from five species tested) and several other bacteria (18 species tested) (Nogva et al., 2000). Here, the previously described rt-qPCR assay was modified and performed as dPCR assay for absolute quantification of *hlyA* gene copy numbers in the MMC-DNA background spiked with DNA isolated from the reference strain DSM 20600. Six 10-fold dilutions between 0.2 and 0.000002 ng/µl were prepared in the 1 ng/µl MMC-DNA standard and corresponding *hlyA* gene copy numbers are shown in Table 3. The assay revealed high linear dynamic range over six $\log_{10}$ dilutions with a regression coefficient of R^2

= 1 (Figs. 2 and 3). The lower limit of detection was found to be 2×10^{-15} g/µl DNA.

Calculating the genome copy number based on the genome size of the bacterium, this concentration corresponds to 0.67 genomes/µl. Assuming one genome per cell, the method reported here allows quantification of less than one *L. monocytogenes* cell equivalent in 1 µl DNA extract. In similar rt-qPCR assays recently reported, the limit of detection in food was determined to be 10 CFU (colony forming units) per g and 9.4 CFU per 25 g sample material, respectively (Russo et al., 2014; Garrido-Maestu et al., 2018). Due to different sample material and processing, data are not directly comparable and further comparative studies are needed. However, the hereby reported Crystal Digital PCR™ assay has been demonstrated to be highly sensitive for quantification of *L. monocytogenes*. As for other food products, *L. monocytogenes* contamination has been identified as severe thread in ready-to-eat salmon products. It has been reported that listeriosis is caused by the ingestion of food containing an infectious dose of about 10^3 CFU/g (FAO/WHO, 2004). Beside its role in food processing, *L. monocytogenes* is also posing an eminent risk in RAS systems used for the land-based production of fish, such as Atlantic charr. Cultivation of this fish species takes place at low water temperatures in nutrient rich environments, which represents favourable growth conditions for this pathogen (Miettinen and Wirtanen, 2006).

3.2. Single-plex dPCR assay for D. desulfuricans quantification

Production of toxic and corrosive H_2S by SRB poses considerable risks for industrial activities in aquatic environments, including land-based aquaculture, wastewater treatment and offshore oil and gas exploration. Consequently, reliable and rapid quantification of SRB in such environments is mandatory for risk assessment and taking prompt measures to anticipate or counteract H_2S associated problems (Letelier-Gordo et al., 2020). Classic quantification of SRB is based on cultivation of sample material in selective media, such as Postgate's and Baar's medium, and enumeration using most probable number (MPN) approach (Tanner, 1989). This methodology is time consuming and of limited accuracy since only SRB can be detected that are able to grow under the provided conditions. Therefore, molecular biology methods are promising approaches for precise quantification of SRB. The *dsrAB* gene fragment, encoding the key enzyme in H_2S biosynthesis, dissimilatory sulfite reductase, has been found to be a suitable phylogenetic marker for SRB identification (Klein et al., 2001). These genes are single copy genes and can be found in all sulfate reducing prokaryotes (Zverlov et al., 2005). Bourne et al. have developed a rt-qPCR TaqMan® assay for quantifying SRB in limnic environments based on a primer/probe combination targeting the *dsrA* gene and being selective for most mesophilic SRB (Kondo et al., 2008; Bourne et al., 2011). The primer combination DSR-1F+/DSR-R has previously been validated to be specific also for SRB reference strains *D. desulfuricans* DSM 642T, *Desulfobacterium autotrophicum* DSM 3382T, *Desulfobulbus propionicus* DSM 2032T, *Desulfobacter latus* ATCC 43918T and *Desulfococcus multivorans* DSM 2059T (Kondo et al., 2008). We have used the corresponding primer/probe combination for the absolute quantification of *D. desulfuricans* in the MMC-DNA standard background using the naica™ system. Six 10-

Table 2
Calculated genome copy numbers for tested strains at the LoD DNA concentration in corresponding dPCR reactions.

Organism	Strain	Genome size	Accession number	LoD (g DNA/µl)	Calculated genome copy number at LoD
L. monocytogenes	DSM 20600	2.9 Mbp	JOOX00000000	2*10E-15	0,64
D. desulfuricans	DSM 642	3.4 Mbp	NZ_ATUZ00000000	2*10E-15	0,57
F. psychrophilum	DSM 3660	2.7 Mbp	CP007207	2*10E-14	7,4
Y. ruckeri	DSM 18506	3.8 Mbp	JPPT00000000	2*10E-14	5,2
M. viscosa	DSM 25798	5.1 Mbp	not avaiable[a]	2*10E-14	3,8

[a] No Genome sequence available. Given genome size represents an average of 13 genomes published for other *M. viscosa* strains (https://www.ncbi.nlm.nih.gov/genome/genomes/36568).

Table 3

Numbers of target gene copies detected in 1 ng/μl MMC-DNA background spiked with different concentrations of target DNA isolated from corresponding bacteria and corresponding 95% confidence intervals (in parentheses). Target gene copy numbers are given in copies per μl target DNA extract.

| Target DNA conc. [ng/μl]	*L. monocytogenes* DSM 20600	*D. desulfuricans* DSM 642	*F. psychrophilum* DSM 3660	*Y. ruckeri* DSM 18506	*M. viscosa* DSM 25798
0,2	109,280 (3.7%)	136,103 (5.1%)	56,784 (1.7%)	36,114 (1. 6%)	31,689 (1.6%)
0,02	11,167 (2.3%)	15,515 (1.8%)	5,960 (2.6%)	3,224 (3.5%)	2,901 (3.6%)
0,002	1,056 (6.2%)	1,375 (5.4%)	489.6 (8.8%)	321.6 (10.8%)	306.2 (11.3%)
0,0002	101.1 (18.8%)	153.3 (15.6%)	57.2 (26.0%)	40.8 (33.1%)	31. (35.2%)
0,00002	16.9 (46.2%)	17.5 (59.1%)	4.5 (87.7%)	3.4 (113.2%)	1.5 (196.0%)
0,000002	1.4 (196%)	9.6 (69.3%)	0	0	0
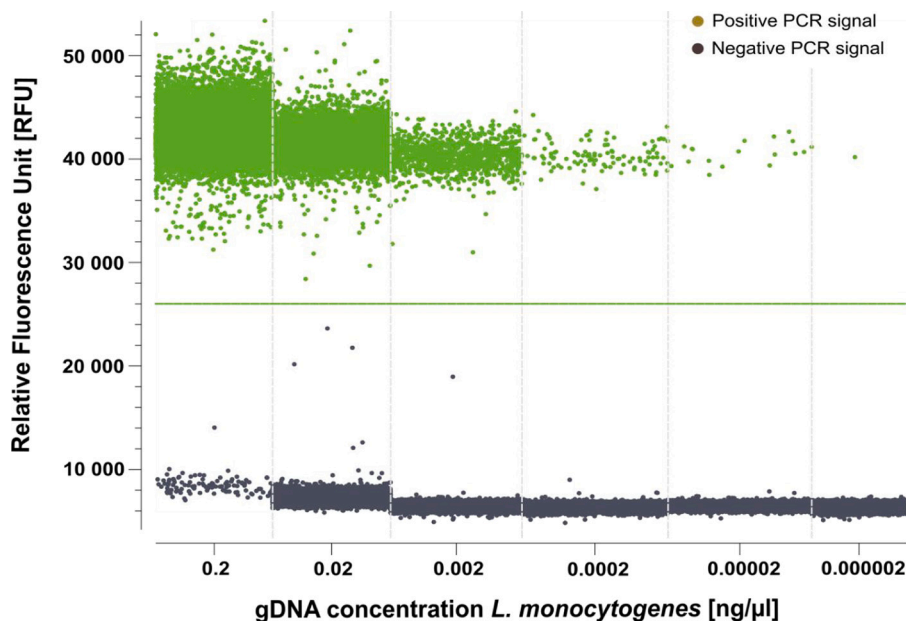
Fig. 2. Dot plot of dPCR analysis for quantification of *hlyA* gene copies in MMC-DNA background (1 ng/μl) with different concentrations of *L. monocytogenes* genomic DNA (gDNA).
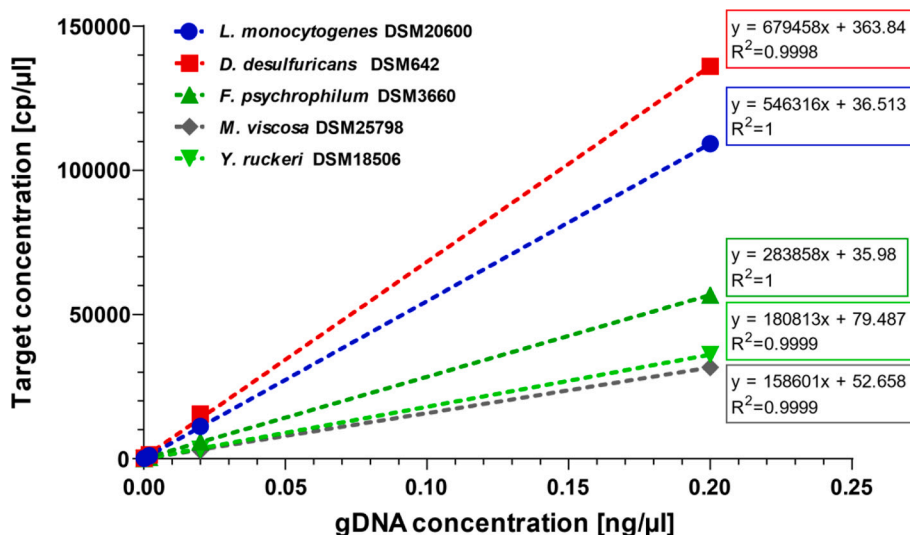

Fig. 3. Simple linear regression plot visualizing the dynamic range of the naica™ system for quantification of gene targets in total DNA derived from five bacterial strains. Corresponding equations and regression coefficients are provided.

fold dilutions (0.2–0.000002 ng/μl) were prepared and used as template in dPCR reactions. Corresponding *dsrAB* gene copy numbers are shown in Table 3. The reported assay showed a high linearity ($R^2 = 0.9998$) over the entire tested range from 0.2 ng/μl to 2 fg/μl *D. desulfuricans*

DNA (Fig. 3). The lower detection limit was found to be around 2 fg template DNA/μl used in the corresponding dPCR assay, where still 9.6 *dsr* gene copies/μl were detected. This DNA amount corresponds to ca. 0.57 genomes/μl, calculated with NEBioCalculator, which is 17-fold

lower than the measured cell equivalent number (*dsr* gene number). Previous studies have found that *D. desulfuricans* contains about 5.9 fg DNA per cell (Edgcomb et al., 1999), which is in the same range as 2.9 fg DNA calculated with NEBioCalculator assuming 1 genome per *D. desulfuricans* cell. The discrepancy between calculated genome copy number and *dsrA* copy number indicates that one SRB cell may in fact contain several *dsrA* gene copies. This could be explained if one SRB cell in reality would contain more than one genome. This is supported by previous studies and Postgate et al. (1984) reported that *Desulfovibrio gigas* and *D. vulgaris* possess in average 9.1 and 4.1 genome copies per cell, respectively. In actively growing non-N-limited *D. gigas* cultures, numbers were found to increase to 17.2 genome copies per cell. The authors assumed that these high genome copy numbers may reflect a high frequency of initiation of replication combined with a remarkably low rate of DNA synthesis, but more likely reflecting multiple genomes in these bacteria (Postgate et al., 1984). We therefore hypothesize that *D. desulfuricans* DSM 642 may also contain several genome copies per cell. Due to their anaerobic requirements, SRB are typically found to be members in biofilm communities, rather than growing planktonic (Karunakaran et al., 2016). This makes quantification with classical methods such as cultivation, fluorescence microscopy or flow-cytometry particularly challenging. We consider absolute quantification of the *dsrA* marker gene copy numbers with dPCR to be superior since the method is independent of the sample structure (e.g. bacteria located in aggregates or biofilm fragments) and background particles. However, more studies are necessary to investigate if the here employed assay can be used for quantification of various SRB in environmental samples.

3.3. Single-plex dPCR assay for M. viscosa quantification

M. viscosa is a major and highly infectious causative agent for winter ulcer disease in Atlantic salmon

and rainbow trout farmed in the Northern Atlantic area, and responsible for elevated mortality at low water temperatures (Grove et al., 2010). Although vaccination has become a standard precautionary measure, the efficiency may be limited and mortalities remain high (Grove et al., 2008; MacKinnon et al., 2019). For reliable risk assessment during the seawater phase, precise and rapid quantification of the pathogen is a basic requirement. The *tonB* gene, encoding for an outer membrane transport protein, has been used as target for developing a specific rt-qPCR assay for identification and quantification of *M. viscosa* previously (Grove et al., 2008). The TON primer/TaqMan® probe combination showed 100% specificity when tested with 21 other bacterial species, including *Moritella marine*, using rt-qPCR. In that study, 151 Atlantic salmon and rainbow trout tissue samples were tested for *M. viscosa*. While cultivation-based detection only demonstrated the presence of the pathogen in 39.7% of the samples, the rt-qPCR assay detected the presence of *M. viscosa* in 88.1% of the examined fish samples. In the same study, the lower detection limit of the rt-qPCR assay was found to be approximately 6.09×10^{-14} g of total *M. viscosa* DNA, equivalent to approximately 10 *M. viscosa* genomes (Grove et al., 2008). Here, we quantified *tonB* gene copy numbers in six 10-fold dilutions (0.2 and 0.000002 ng/μl), prepared in the 1 ng/μl MMC-DNA standard and corresponding results are provided in Table 3. Employing the same primer/probe combination in the reported dPCR assay here, the method was found to be more sensitive with a lower detection limit of 2.0×10^{-14} g DNA/μl, corresponding to ca. 4 *M. viscosa* genomes/μl based on the calculation using the NEBioCalculator. The assay presented here showed high dynamic linearity ($R^2 = 0.9999$) over five $\log_{10}$ dilutions (Fig. 3). Assuming the *tonB* gene to be present in a single copy per bacterial genome, we conclude the detection limit of the dPCR assay to be around 4 *M. viscosa* cell equivalents per μl. This shows that LoD obtained with both PCR methods are in the same range, however, quantification with Crystal Digital PCR™ was found to be more sensitive than for the reported rt-qPCR assay.

3.4. Single-plex dPCR assay for Y. ruckeri quantification

Y. ruckeri is the causative agent of enteric redmouth disease (yersiniosis), one of the most important diseases of salmonids and causing significant economic losses in the salmon aquaculture sector (Horne and Barnes, 1999). Different methods have been developed for analyzing *Y. ruckeri*, including culturing, serological tests and molecular biological techniques (Tobback et al., 2007). Keeling et al. (2012) developed an rt-qPCR assay based on targeting the glutamine synthase encoding single-copy gene *glnA* (Keeling et al., 2012). The assay was evaluated with 21 *Y. ruckeri* strains (field isolates and reference strains) and 33 unrelated bacterial species and found to have 100% specificity. We employed the same GLN primer/TaqMan® probe combination to establish a dPCR assay for absolute quantification of *Y. ruckeri*. Five $\log_{10}$ dilutions (0.2–0.00002 ng/μl) of reference strain *Y. ruckeri* DSM 18506 DNA were analyzed in the MMC-DNA background and corresponding *glnA* gene copies quantified (Table 3). The assay showed to be linear over the entire tested range ($R^2 = 0.9999$) and the LoD was found to be around 20 fg *Y. ruckeri* DNA (Fig. 3). Using the NEBioCalculator, we calculated that 20 fg DNA of the reference strain correspond to 5.2 genome copies or *Y. ruckeri* cell equivalents per μl DNA extract. At this maximal dilution, the dPCR assay detected 3.4 *glnA* gene copies per μl which corresponds well with the calculated genome copy number.

3.5. Single-plex dPCR assay for F. psychrophilum

F. psychrophilum is an important bacterial pathogen in salmonid aquaculture industry worldwide and described as causative agent for rainbow trout fry syndrome and bacterial cold-water disease (Ngo et al., 2017). Since the bacterium has also been identified in reproductive fluids and eggs, vertical transmission has been hypothesized, which poses a severe risk also for hatcheries and brood stock facilities (Long et al., 2015). Currently, both rt-qPCR and a capture enzyme-linked immunosorbent assay (ELISA) are frequently used for quantification of *F. psychrophilum*. Marancik and Wiens (2013) have designed a primer/TaqMan®-probe combination targeting a single-copy gene, which encodes a conserved hypothetical protein of unknown function. The corresponding rt-qPCR assay showed 100% specificity against in total 210 *F. psychrophilum* and 23 non-*F. psychrophilum* bacterial isolates cultured from various field cases (Marancik and Wiens, 2013). We have adapted the SIG primer/TaqMan® probe combination to establish a Crystal Digital PCR™ assay for absolute quantification of *F. psychrophilum* on the naica™ system. Five $\log_{10}$-fold dilutions (0.2–0.00002 ng/μl) of reference strain *F. psychrophilum* DSM 3660 DNA in the MMC-DNA background were used as template in dPCR reactions and corresponding results are presented in Table 3. We found the assay to be linear over the entire range of five log units with a regression coefficient of $R^2 = 1$ (Fig. 3). The limit of detection was around 20 fg *F. psychrophilum* DSM 3660 DNA/μl. With this amount of template DNA, 4.5 target gene copies per μl DNA extract were detected. Based on the NEBioCalculator calculation, 20 fg of the reference strain DNA corresponds to 7.4 genome copies, equivalent to cell numbers. Calculated and analyzed *F. psychrophilum* numbers are in good agreement and we conclude the LoD to be in this range.

3.6. Duplex dPCR assay for Y. ruckeri and F. psychrophilum

Several rt-qPCR assays for individual quantification of the potent fish pathogens *Y. ruckeri* and *F. psychrophilum* have been established previously (Del Cerro et al., 2002; Mata et al., 2004; Daneshamouz et al., 2020). A novel multiplex rt-qPCR assay for simultaneous detection of these two species and in addition *Lactococcus garvieae* has been reported recently (Chapela et al., 2018). The authors compared the use of a novel multi-plex rt-qPCR method with classical culture-based methods for bacteria identification in tissue samples derived from trout aquaculture. While no absolute LoD-values were determined, results clearly showed

that the rt-qPCR method was highly specific, and no positive amplification was observed for 83 non-target bacterial strains (Chapela et al., 2018). In our here presented work, we used the same oligonucleotides as primers and TaqMan® probes and adapted the assay for the naica™ system. Results obtained in the duplex assay were in good agreement with results from corresponding single-plex assays (Fig. 4) and analysis of 10-fold dilutions from 0.02 to 0.00002 ng/µl revealed excellent linear dose-response ($R^2 = 1$) for both bacterial targets.

In a recently published study, a first dPCR assay for simultaneous quantification of the same bacteria using a BioRad digital droplet (dd) PCR QX200 system has been demonstrated. In that assay, different oligonucleotides were employed and sensitivity was found to be significantly different for both species, with 0.0011 ng input DNA corresponding to 200 and 174 gene copies and 1.24 ng input DNA corresponding to 560 and 620 gene copies for *F. psychrophilum* and *Y. ruckeri*, respectively (Lewin et al., 2020). In the here presented work, the Crystal Digital PCR™ duplex assay sensitivity was found to be 0.00002 ng input DNA for both species, corresponding to 3.5 gene copies for *F. psychrophilum* and 1.8 gene copies for *Y. ruckeri*. Consequently, normalized to input DNA amount, gene copy numbers for *F. psychrophilum* obtained with both systems were in very good agreement, while for *Y. ruckeri*, sensitivity of the Crystal Digital PCR™ assay was ca. 300-fold higher compared to the BioRad QX200 ddPCR assay. The reason for this may be due to different assay components and conditions, first of all different oligonucleotides. However, we cannot exclude that the different dPCR instruments and workflows used in both studies may influence sensitivity and comparative studies are necessary to provide detailed evidence.

3.7. Triplex dPCR assay for F. psychrophilum, M. viscosa and L. monocytogenes

Based on the previously described single-plex assays for *F. psychrophilum*, *M. viscosa* and *L. monocytogenes*, corresponding primer/TaqMan® probe combinations were also tested in a triplex assay. The MMC-DNA (1 ng/µl) was spiked with four 10-fold dilutions (0.2–0.0002 ng/µl) of DNA from all three reference strains and used as template in Crystal Digital PCR™ reactions. When plotted against the corresponding theoretical target concentrations calculated with the NEBioCalculator, the triplex assay results were in excellent agreement with target gene concentrations detected in the corresponding single-plex assays (Fig. 5). Minor variations are most likely caused by errors when pipetting very small volumes. To the best of our knowledge, we here report for the first time an ultra-sensitive and precise assay for absolute and simultaneous quantification of three priority bacteria

species with high relevance for diagnostics in aquaculture operation. The combination tested here is only an example and demonstrates clearly that the naica™ system is a powerful tool for precise quantification of three different microbial species in complex environmental samples simultaneously.

3.8. Reproducibility

In the perspective of aquaculture facilities, it is essential that quantitative data generated over a monitoring campaign time course and at different locations are reproducible and consequently directly comparable. As an end-point PCR leading to absolute quantification results, dPCR is expected to avoid errors often impairing rt-qPCR result reproducibility, such as inaccurate thermal cycling conditions, low primer efficiency or suboptimal amplification due to inhibitors in sample material. Here we assessed reproducibility of the Crystal Digital PCR™ technology by repeating the above described single-plex assay for *M. viscosa* quantification in three temporary independent experiments where four 10-fold dilutions in MMC-DNA background (1 ng/µl) were prepared individually for each experiment. For the here tested *M. viscosa* quantification assay, results showed high reproducibility with low run-to-run variations (Fig. 6). Observed variations are likely due to pipetting errors.

4. Conclusions

In aquaculture systems, accurate enumeration of certain bacteria is of eminent importance to control potential risks, especially species that are pathogenic for the cultivated fish and its final consumers. Modern quantitative PCR technology has become a powerful method for enumeration of bacteria and other biological agents in environmental samples, including water, biofilm and sediment from aquaculture operation. However, conventional rt-qPCR results need to be calculated based on standards with known target gene concentrations and thus, only provides relative quantification. In contrast, dPCR technology allows for absolute quantification of genetic targets by simultaneous PCR in a large number of partitions derived from the original sample. The technology combines end-point binary signal recognition with Poisson statistics. Since dPCR does not rely on calibration curves for sample quantification, this technology also avoids inaccuracies caused by variations in reaction efficiencies (Quan et al., 2018). The here presented study demonstrates that the naica™ system is capable of simultaneous absolute quantification of one to three gene targets in a complex DNA background, representative for environmental sample material. We have demonstrated that dPCR is a suitable technology for direct
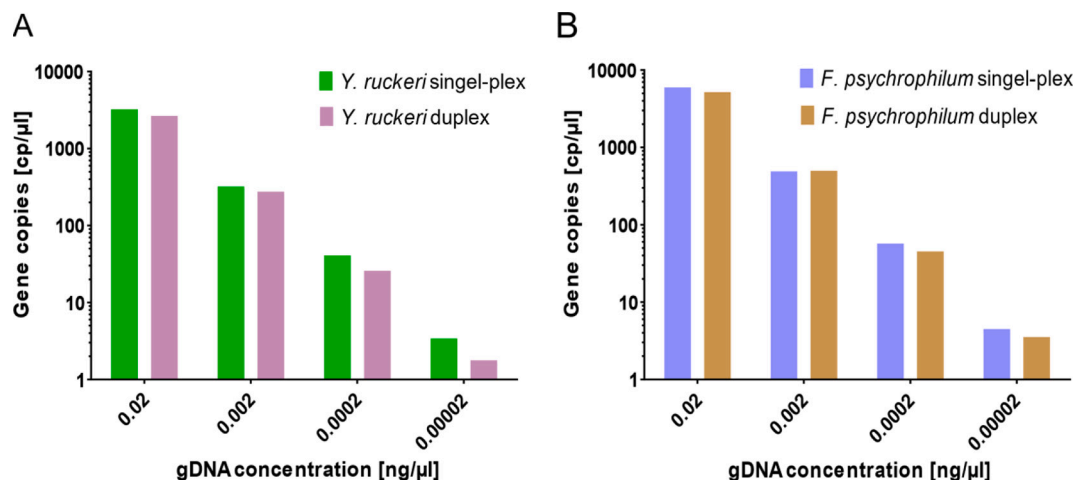

Fig. 4. Comparison of results obtained with single-plex and duplex assays for *F. psychrophilum* (A) and *Y. ruckeri* (B) quantification. Gene copy numbers were quantified in 10-fold dilutions of *F. psychrophilum* and *Y. ruckeri* gDNA spiked in MMC-DNA background (1 ng/µl).
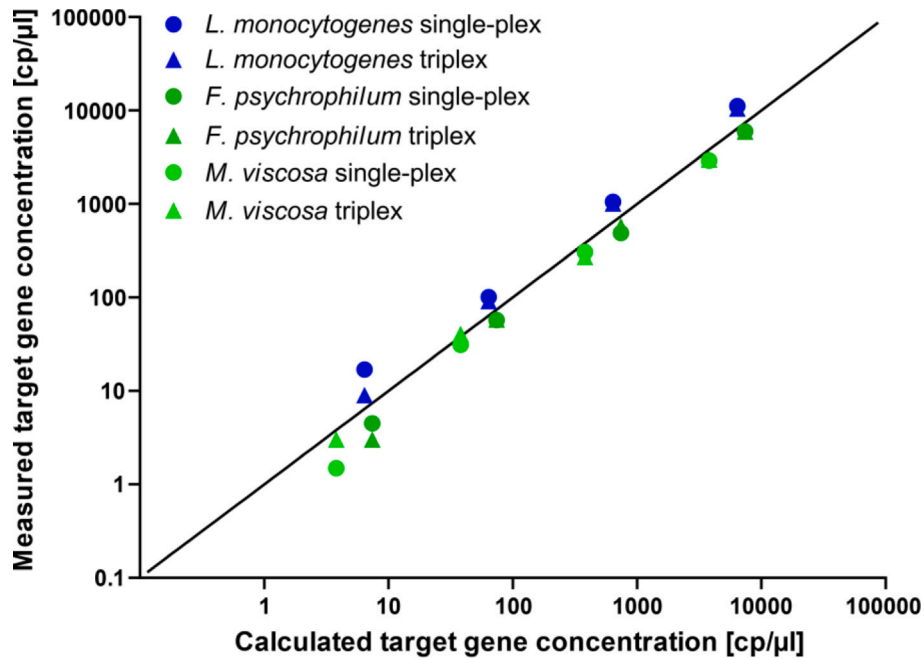
Fig. 5. Measured concentrations of target gene copies detected in single-plex (circles) and triplex (triangles) assays in 1 ng/μl MMC-DNA background plotted against the calculated target gene concentration. The line of identity indicates where every point has equal X- and Y-coordinates.
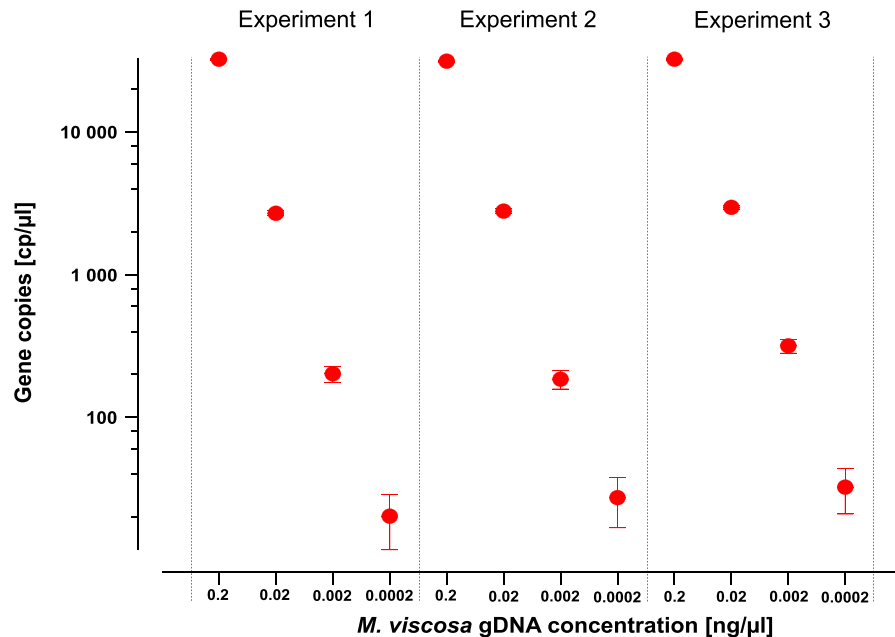

Fig. 6. Concentrations of *tonB* gene copies and corresponding 95% confidence intervals detected in 0.2–0.0002 ng/μl total *M. viscosa* DNA in 1 ng/μl MMC-DNA background analyzed in three independent experiments.

quantification of bacteria relevant in salmonid aquaculture. Obtained results showed that the Crystal Digital PCR™ has a linear dynamic range over at least five orders of magnitude, exhibits high sensitivity and reproducibility. Moreover, the system offers a streamlined workflow with low hands-on time and minimizes risks of cross-contamination. It is noteworthy that the price per sample and target using dPCR systems is higher than using conventional rt-qPCR, due to often higher investment and consumable costs (e.g. chips or arrays). Still, considering typically high hourly rates for scientific personnel, the integrated and time-efficient workflow of dPCR systems may compensate these costs. Moreover, dPCR systems may also be used in combination with and for

calibration of rt-qPCR assays to reduce costs per sample for routine analyses.

In conclusion, these advantages make the naica™ system a powerful tool in microbial diagnostic applications. In this study, we presented a collection of single-plex, duplex and triplex assays for absolute quantification of five selected bacterial species with high relevance for salmonid aquaculture operation, adapted from previously reported rt-qPCR protocols. Further expansion of the dPCR assay portfolio, by development of new or adapting existing rt-qPCR protocols, would improve the microbial diagnostics toolbox and provide reliable quantitative data for decision making in aquaculture facilities.

Funding

Transparency declaration

Co-authors from Stilla Technologies contributed with suggestions that were key to technical resolution, assay-optimization and manuscript preparation. They were not involved in conducting the experimental work or data treatment presented in this manuscript. No financial aid has been provided by Stilla Technologies for the preparation of this manuscript or the associated work.

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. Co-authors from Stilla Technologies contributed with suggestions that were key to technical resolution, assay-optimization and manuscript preparation. No financial aid has been provided by Stilla Technologies for the preparation of this manuscript or the associated work.

Acknowledgements

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