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# Method development to estimate infection load in aquaculture / Þróun aðferðar til að meta sýkingarálag í fiskeldi

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## Report summary

<i>Titill / Title</i>	<b>Method development to estimate infection load in aquaculture /</b> Próun aðferðar til að meta sýkingarálag í fiskeldi		
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<i>Ágríp á íslensku:</i>	<p>Markmið forverkefnisins var að búa til DNA þreifara sem binst við erfðæfni fisksjúkdómsvaldandi bakterianna <i>Flavobacterium psychrophilum</i> og <i>Aeromonas salmonicida</i>, undirtegund <i>achromogenes</i>, sem hægt væri að skima eftir með notkun flúrljómunartækni í smásjá (FISH) og í örverugreini (flow cytometry).</p> <p>Einn sértækur DNA þreifari fyrir bakteríunni <i>F. psychrophilum</i> var búin til með samsetning tveggja og notaður með mjög góðum árangri til að skima fyrir bakteríunni með örverugreini og FISH tækni.</p> <p>Ekki var hægt að búa til sértæka DNA þreifara fyrir <i>A. Salmonicida</i>, undirtegund <i>achromogenes</i>, þar sem auðkennisgen (16S rDNA) hennar er of líkt öðrum Aeromans tegundum sem eru ekki sýkjandi. Nauðsynlegt verður að þróa nýja þreifara sem eru einstakir fyrir <i>A. Salmonicida</i>, undirtegund <i>achromogenes</i>.</p> <p>Örverugreinirinn (flow cytometry) er mjög hraðvirkt tæki til að greina bindingu sértækra DNA þreifara við örverur sem gerir tækið mjög hentugt til að greina sjúkdómsvaldandi bakteríur í vatni. Magngreining baktería með slíkri tækni er þó háð ýmsum annmörkum en hún gefur samt mjög góða vísbindingu um ástand vatnsins í eldinu svo hægt sé að meta sýkingarálagið.</p> <p>Niðurstöður þessa forverkefnis sýna að hægt er að meta sýkingarálag í fiskeldi á hraðvirkan hátt en nauðsynlegt er að þróa áfram og sannreyna aðferðafræðina við raunaðstæður í fiskeldi. Gert var ráð fyrir þessu í upphafi þessa forverkefnis og hafa þátttakendur sótt um framhaldsstyrk til AVS sem byggir á núverandi niðurstöðum og verður aðferðafræðin prófuð við raunaðstæður í bleikjueldi.</p>		
<i>Lykilorð á íslensku:</i>	<i>Aeromonas salmonicida</i> , fiskeldi, fisksjúkdómsvaldandi bakteríur, <i>Flavobacterium psychrophilum</i> , örverugreinir (flow cytometry), DNA þreifarar (FISH)		

## Report summary

<p><i>Summary in English:</i></p>	<p>The aim of this proof-of-concept study was the development and application of molecular probes for the fish pathogens <i>Flavobacterium psychrophilum</i> and <i>Aeromonas salmonicida</i> subsp. <i>achromogenes</i>, and their detection through Fluorescence In Situ Hybridization (FISH) and flow cytometry.</p> <p>A combination of two species-specific FISH probes was successfully used in combination with flow cytometry to identify and detected <i>F. psychrophilum</i> strains.</p> <p>It was not possible to find specific FISH probes for <i>A. salmonicida</i> subsp. <i>achromogenes</i>. The bacterium is too similar to other <i>Aeromonas</i> species in its 16S rRNA gene sequence and does not contain suitably unique regions that could have been used to develop a species-specific FISH probe.</p> <p>Flow cytometry offers a fast detection system for FISH probes, although technological limitations make reliable quantification difficult. The system is therefore best suited as a semi-quantitative early warning system for emerging fish pathogens in water samples from aquaculture tanks.</p> <p>The results of this preliminary project show that it is possible to estimate the infection load for certain pathogens in aquaculture rapidly but it is necessary to develop the methodology further and test it under real aquaculture conditions. The participants have applied to AVS for new funding based on these results; to develop our rapid methodology further, expand it to more pathogens and test it under real aquaculture conditions.</p>
<p><i>English keywords:</i></p>	<p><i>Aeromonas salmonicida</i>, aquaculture, fish pathogen, <i>Flavobacterium psychrophilum</i>, flow cytometry, fluorescence <i>in situ</i> hybridization (FISH), molecular probes</p>

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## Introduction

Multiple bacterial pathogens consist threats to the growing Icelandic aquaculture, in which fish is contained at high density and therefore especially susceptible to infections. Two such bacteria are *Flavobacterium psychrophilum* and *Aeromonas salmonicida* subsp. *achromogenes*. *F. psychrophilum* is responsible for the so-called Bacterial Cold Water Disease (BCWD) and the Rainbow Trout Fry Syndrome (RTFS) in salmonids, causing lesions at skin, mouth and gills, respectively infecting liver and spleen, and causing high mortality in cultured fish stocks. *A. salmonicida* causes the disease furunculosis in fish, causing large boils on the side of the fish, as well as external and internal haemorrhaging, swelling of the vents and kidneys, ulcers, liquefaction, and gastroenteritis. In contrast to *F. psychrophilum*, a vaccine exists that helps to prevent outbreaks of *A. salmonicida* infections.

With the constant threat of bacterial pathogens in aquaculture, the establishment of a suitable monitoring system, that detects these bacteria before mass infection occurs, is paramount. The methodology of detection should be fast, reliable and cost-efficient, so that a threat can be identified quickly and counter-measurements can be applied. One possible method that theoretically fulfils these requirements is flow cytometry coupled with Fluorescence *In Situ* Hybridization (FISH) to detect pathogenic bacteria directly in the water of aquaculture tanks.

Flow cytometry is a biophysical technology for cell characterization and enumeration, in which cells are transported at high speed in a fluid stream (sheath fluid) and hit by a laser beam. The laser light that either passes the cell or is reflected at a certain angle is measured by electronic detectors, as well as the light that the cell might emit at different wavelengths. These parameters allow to classify cells into different groups and count them. The advantage of this methodology is the high analytical speed and sample throughput, for example, the FACS Aria II flow cytometer (Becton Dickinson) is capable of analysing up to 70.000 cells per second.

Flow cytometry is though not capable of distinguishing different bacteria species based on their shape and size alone but requires in addition information from species-specific fluorescence. As heterotrophic bacteria rarely possess any autofluorescence, a way to introduce species-specific fluorescence into the method needs to be employed. A method to do this is FISH, in which short, species-specific oligonucleotides bind to the ribosomes inside a cell and can be detected through a covalently-bound fluorochrome in either fluorescence microscopy or flow cytometry. The oligonucleotides, so-called molecular probes, are designed by comparing sequence databases and searching for short (ca. 20 bp long) regions of the ribosomal RNA that are unique for the target species of interest but differ by at least one base pair to all other known species. Under the right experimental conditions, molecular probes bind only to the ribosomes of the target species, but not to any other organism, therefore they are capable to specifically detect their target even in mixed community samples. A more detailed account of the principles of probe development and FISH in general can be found in Pernthaler et al. (2001).

## Materials & Methods

### Probe development

Molecular probes based on the small subunit of the 16S rRNA gene were supposed to be developed for the fish pathogenic bacteria species

- *Flavobacterium psychrophilum*
- *Aeromonas salmonicida* subsp. *achromogenes*.

Strategies for finding suitable probes for the two species were twofold, a) a literature and database search for already existing probes that could be used in the project, and b) the design of new probes based on the available 16S rDNA sequence of the species.

#### *Literature & database search*

The most current online version of the literature database “Web of Science” (<https://apps.webofknowledge.com/>) was searched for all publications containing the keywords “*Flavobacterium psychrophilum*” or “*Aeromonas salmonicida*” in combination with “probe\*”. Available probes were also searched for in ProbeBase, the most comprehensive repository for published and unpublished rDNA probes available (<http://probebase.csb.univie.ac.at/node/8>), using the bacteria genus names as search terms (Greuter et al., 2016).

All probes found through these searches were checked for specificity using the TestProbe function on the SILVA website (<https://www.arb-silva.de/search/testprobe/>).

#### *De novo probe design*

Probe design was conducted using the software ARB (Ludwig et al., 2004; Westram et al., 2011) in combination with the SILVA database version 119 (Pruesse et al., 2007; Quast et al., 2013). In ARB, the sequences for *Flavobacterium psychrophilum* and *Aeromonas salmonicida* were marked in the phylogenetic tree included in the database, followed by automatic identification using the PROBE\_DESIGN tool at default setting. Software and database can be found and downloaded at <https://www.arb-silva.de/>.

### Samples

To establish the combined method of molecular FISH probes with flow cytometric detection, pure cultures of established species need to be used. As it was not possible to obtain these species / strains of *F. psychrophilum* (positive control) and related, non-target bacteria (negative controls) from Icelandic waters, cultures were bought from the Leibniz-Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Table 1).

Bacterial cultures were grown in liquid R2A medium ([https://www.dsmz.de/microorganisms/medium/pdf/DSMZ\\_Medium830.pdf](https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium830.pdf)) at 20°C for 2-3 days.

Table 1: Bacterial cultures used in this project

Species	Strain	Type strain	Origin
<i>Flavobacterium psychrophilum</i>	DSM 3660	yes	USA; coho salmon <i>Oncorhynchus kitsuch</i>
<i>Flavobacterium psychrophilum</i>	DSM 21280	no	France, kidney of a diseased rainbow trout
<i>Flavobacterium frigidimarit</i>	DSM 15937	yes	Antarctica, sea water
<i>Flavobacterium columnare</i>	DSM 18644	yes	USA; kidney of diseased salmon

### Fluorescence *In Situ* Hybridization (FISH)

FISH was done according to the protocol described by Strepparava et al. (2012) with modifications to adapt it for flow cytometric analysis. One bacterial colony was transferred from the plate into 200 µl sterile water and resuspended. Alternatively, 1 ml of the liquid culture was used. Cells were pelleted by centrifugation at 10.000 rpm in a microfuge for 5 min and the supernatant discarded. Cells were then sequentially dehydrated in 50%, 70% and 96% ethanol by resuspending the cells in the ethanol, incubating them for 3 min at room temperature, pelleting them by centrifugation and discarding the supernatant. After the last ethanol step, cells were resuspended in 50 µl of hybridization buffer (0.9 M sodium chloride, 20 mM Tris/HCl pH 7, X% formamide, 0.01% SDS, water) containing 50 ng of the respective oligonucleotide probe (Table 2). Formamide concentration differed for the different probes; samples hybridized with Flavo285, FlavoP77 and FlavoP477 used 30% formamide, while samples hybridized with Univ.bact16S-341 or without any probe used 0% formamide. According to Strepparava et al. (2012), the best results for the detection of *F. psychrophilum* with the species-specific probes (FlavoP77 & FlavoP477) were obtained when both probes were used together on the same sample, therefore these two probes were also used in these experiments in tandem, with 50 ng of each probe added to the hybridization buffer. Samples were incubated over night at 46°C in the dark to protect the light-sensitive fluorochromes of the probes. Afterwards, cells were centrifuged, the supernatant discarded and the pellet resuspended in 200 µl of washing solution (150 mM sodium chloride, 100 mM Tris/HCl pH 7, 5 mM EDTA pH 8, 0.01% SDS, water). Samples were then incubated for 20 min at 48°C. Finally, samples were again centrifuged and the pellet resuspended in 1 ml of sterile distilled water. Samples were analysed by flow cytometry on the same day and kept at 4°C in the dark until then.

Table 2: FISH probes used in this project

Name	Target	Sequence [5'-3']	Labeling	Citation
Flavo285	<i>Flavobacterium</i> spp.	GACCCCTACCCATCRTH	Cy3	Strepparava et al., 2012
FlavoP77	<i>F. psychrophilum</i>	AGTGTGTTGATGCCAACTCACT	Alexa488	Strepparava et al., 2012
FlavoP477	<i>F. psychrophilum</i>	ACTTATCTGGCCGCTACG	Alexa488	Strepparava et al., 2012
Univ.bact16S-341	Bacteria	CCTACGGGNGGCWGCAG	Alexa647	Herlemann et al., 2011

## Flow Cytometry

Flow cytometric analysis was done using a FACS Aria II cell sorter (Becton Dickinson) with three lasers and a filter combination that was suitable to detect the fluorochrome labels used in these FISH experiments. Data acquisition and analysis was done using BD FACSDiva software. Highly concentrated samples were diluted in sterile distilled water to obtain cell numbers that could be analysed in 1-2 min per and run with distilled water as sheath fluid. For quantification, Flow Check Microparticles (Polysciences Inc.) were used.

## Results

### Probe development

The search in the literature database found two publications with potentially useful probes.

Strepparava et al. (2012) described the development and testing of two specific probes for *F. psychrophilum* that were used in Fluorescence *In Situ* Hybridization (FISH) analyses. While the paper described the visualization of the probes through fluorescence microscopy, the same could be done through flow cytometry instead. Therefore, these probes were very likely to be useful in this project. However, it is necessary to check the specificity of probes and primers from time to time again after their publication, as new sequences are constantly added to databases and might show that a probe is not as specific as originally thought, e.g. through the detection of a new species that cross-reacts with the probe. The probes (Table 2) were therefore checked using the TestProbe function at the SILVA website and were all found to be still specific only for their designated target taxa.

Warsen et al. (2004) developed and tested molecular probes for 14 different fish pathogens, including *F. psychrophilum* and *A. salmonicida*. These probes have been developed for microarrays and PCR-based detection methods, but could possibly still be used in this project. A specificity check of the two probes for *F. psychrophilum* and *A. salmonicida* revealed though that they are not specific for their target organisms. The probe for *A. salmonicida* showed not only a 100% match with *A. salmonicida* subsp. *achromogenes*, but also with multiple other species of the genus *Aeromonas*, e.g. *A. bestiarum*, *A. hydrophila* and *A. piscicola*. Due to this, the probe is unable to identify the target subspecies or even the species reliably. The probe for *F. psychrophilum* showed better specificity in the ProbeTest check, however it also showed a total match to a non-target bacterium, *F. swingsii*. It should be noted, that the paper by Warsen et al. (2004) only contained a very limited number of positive and negative targets, and that therefore the testing in that publication was sub-par.

The two probes published by Warsen et al. (2004) were also the only ones for *F. psychrophilum* and *A. salmonicida* that could be found in the ProbeBase database.

Additional publications were found in Web of Science that also described probes or primers for the two target species of this project (e.g. Beaz-Hidalgo et al, 2013; Strepparava et al, 2014; Long et al, 2015; Gulla et al, 2016), but those were developed for real-time PCR assays and normally relied on combined primer & probe specificity for detection or targeted different genes than the 16S rRNA and were therefore not directly usable for this project.

*De novo* probe design using the ARB software package in combination with the SILVA database was also not able to find any probe that could discriminate between *A. salmonicida* and other, closely

related bacteria. This is due to the fact that the 16S rRNA gene of this species, which is used for this analysis, does not differ enough in its nucleotide sequence in relation to closely related species. The use of alternative genes and detection methods, e.g. PCR-based methods using specific primers, might be possible for the specific detection and identification of *A. salmonicida* subsp. *achromogenes*, but the combination of FISH and flow cytometry, as envisioned in this project, requires the use of the rRNA gene to work. Therefore, the development and application of probes for *A. salmonicida* subsp. *achromogenes* was not possible in this project.

ARB was able to detect a number of potential probes that should be specific for *F. psychrophilum* and able to distinguish it from closely related species. None of those probes showed though potentially higher discriminatory power (i.e. a higher number of mismatched nucleotides between target and non-target organisms) than the probes published by Strepparava et al. (2012). Therefore, the focus for the testing was put onto these probes with their already established hybridization conditions for FISH (Table 2).

### Probe specificity

The *Flavobacterium psychrophilum* specific probes P77 and P477 were used in combination as described by Strepparava et al (2012) and tested versus two strains of their target species and two strains of related non-target species (Table 1). Cells that were hybridized to a probe would show an increased signal strength due to the fluorochrome of the probe in comparison to the negative control that contained no probe during the FISH treatment. Therefore, if a probe does not hybridize to a cell, fluorescence signal strengths for a sample with or without probe would be identical.

The experiments showed that probes P77 & P477 specifically hybridized only to their targets but showed no increased fluorescence signals with the two non-target species (Figures 1 & 2). While the detection and quantification by flow cytometry, i.e. the chosen sensitivity of the detectors or the gate that defines the signals that are considered “positive”, are to some extent arbitrary, using the same flow cytometer settings for all samples allow for a clear comparison between them (Figure 1).

While positive signals were only detected in significant numbers for the target species *F. psychrophilum*, negative signals were also observed when this species was hybridized with probes P77 & P477 (Figure 1, black signals). There are multiple possibilities while this can happen during FISH based on experimental conditions and/or the physiological status of the cell. While every care was taken during the experiments and proper hybridization conditions were already established by Strepparava et al (2012), it is still possible that some cells and their ribosomes were not accessible for the probes, e.g. cells clumped together or broken apart during treatment, cell walls and membranes not made permeable by the ethanol treatment, etc.). While these aspects can be partially reduced through modifications in the experimental conditions of the FISH, the second main reason for non-labeled cells, their physiological state, will still mean that never all cells will show a proper fluorescent signal with their targeted probe, especially under field conditions, respectively in samples from aquaculture tanks. The principle of FISH detection of species-specific molecular probes depends on the binding of these probes to the ribosomes inside the cell. The sometimes ten thousands of ribosomes represent a huge number of targets for the probe molecules and together allow for a signal that is strong enough to be detected by either fluorescence microscopy and the human eye, or the detectors of a flow cytometer. However, if cells are at a low physiological state, e.g. in stationary growth phase or generally stressed by adverse environmental conditions, for example low nutrient levels, then the number of ribosomes can be considerably lower and therefore signal strength of the fluorescent probe will also be lower, possible below level of detection. Under the conditions of aquaculture, for which this project aims to

test the application of molecular probes, it can never be considered that all target bacteria are in perfect physiological condition and will give strong probe signals. Instead, bacteria might be present but dormant and the signal strength of the probes would not be sufficient for them to be identified.

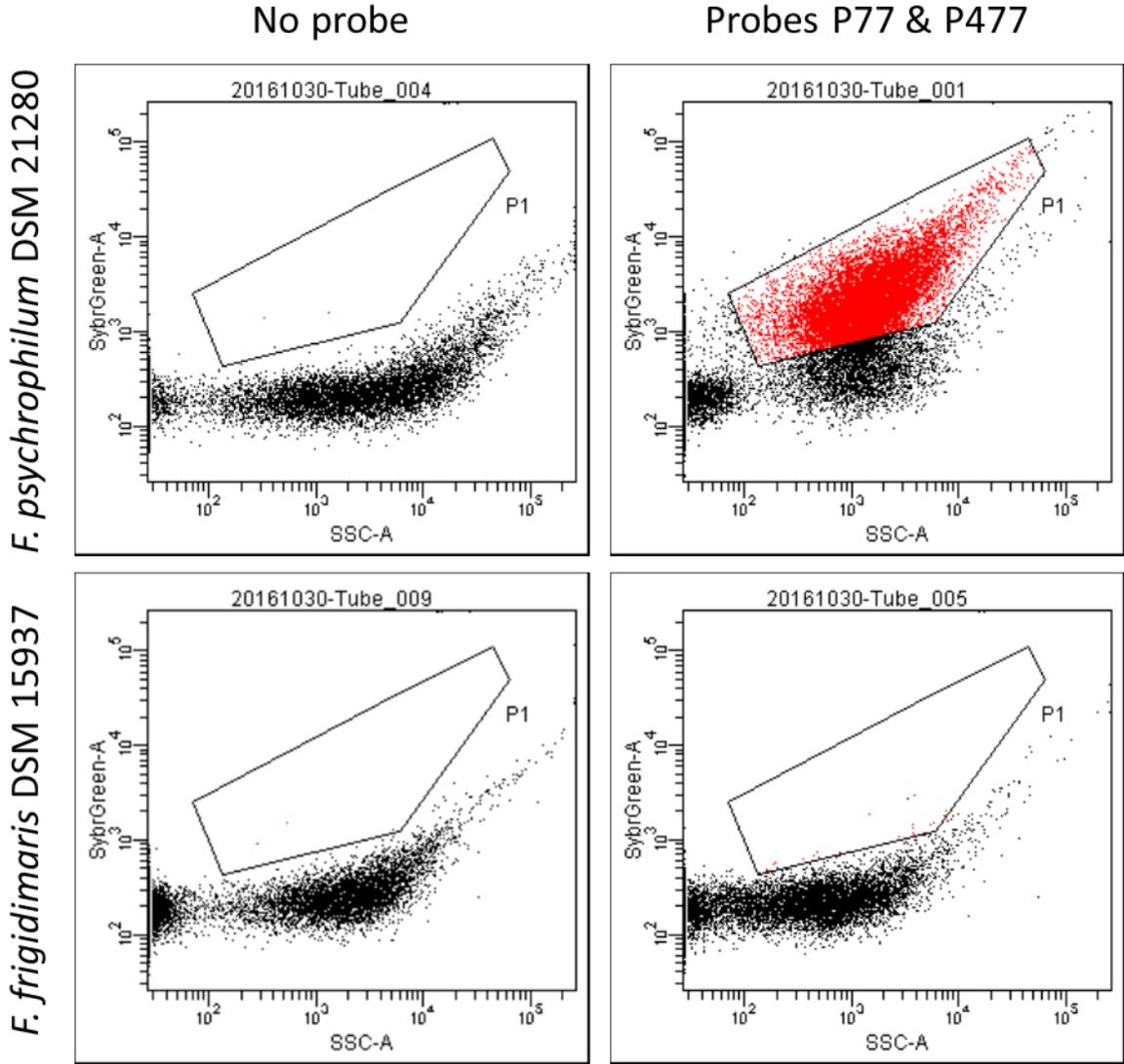
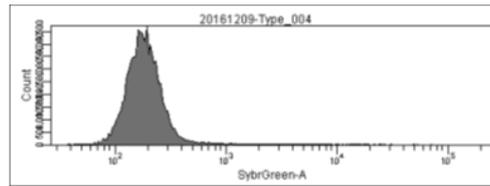
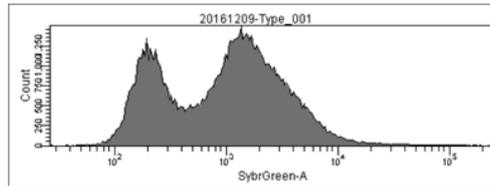


Figure 1: Example of flow cytometry dot plots (fluorescence versus side scatter) for *F. psychrophilum* and *F. frigidimaris* hybridized with *F. psychrophilum*-specific probes P77 and P477. The gate (P1) shows the region in which cells with increased fluorescence were detected. Signals (in black) below this region mainly represent cells that did not hybridize with the probes.

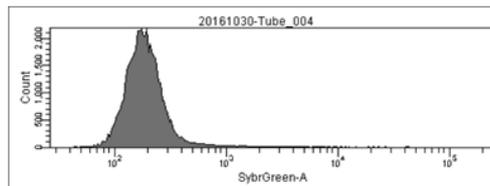
*F. psychrophilum* DSM 3660  
No probe (negative control)



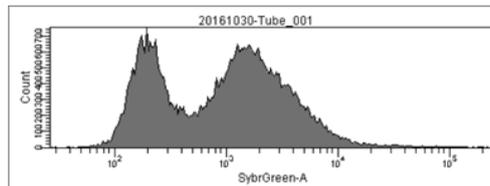
*F. psychrophilum* DSM 3660  
Probes P77 & P477



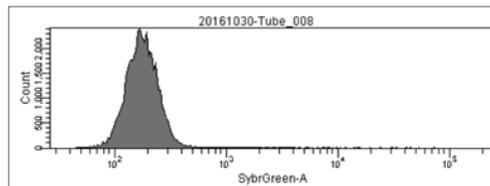
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No probe (negative control)



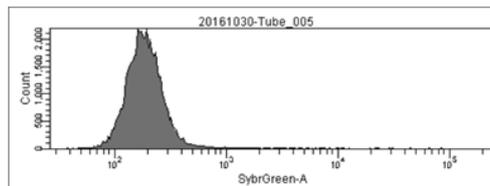
*F. psychrophilum* DSM 21280  
Probes P77 & P477



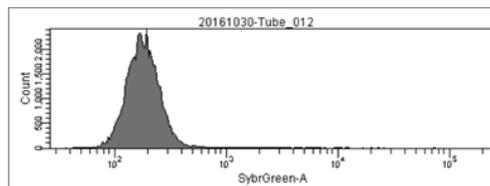
*F. frigidimaris* DSM 15937  
No probe (negative control)



*F. frigidimaris* DSM 15937  
Probes P77 & P477



*F. columnare* DSM 18644  
No probe (negative control)



*F. columnare* DSM 18644  
Probes P77 & P477

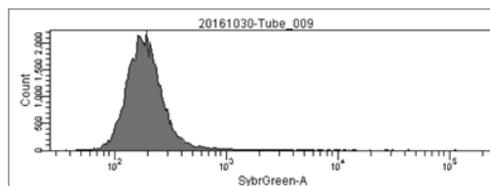


Figure 2: Flow cytometry histograms (fluorescence strength versus number of cells) of *Flavobacterium* strains hybridized with or without (negative control) probes P77 & P477. Only *F. psychrophilum* strains showed an increased number of fluorescent cells with these probes.

### Quantification of *F. psychrophilum* by FISH and flow cytometry

Flow cytometry is not a technology that was developed in the first place for quantification of cells and it is not possible to directly relate the number of detected signals to the number of cells per ml. To overcome this limitation, quantification is done by weighting the tube with the sample before and after measurement on a micro balance and calculate the number of signals in relation to the amount of liquid they were in, based on the weight loss. As this is a very laborious and time-consuming task if done regularly with real samples, micro beads of known concentration can be added to samples and the number of cells calculated in relation to those beads. However, as the beads age over time, loose their fluorescence or clump together, and therefore change their concentration, it is still necessary to regularly check their concentration by weighting them. The beads used in the quantification experiments with the probes P77 & P477 (Flow Check Microparticles, Polysciences Inc.) were last calculated at a concentration of 6 million beads per ml.

A second aspect to take into consideration when trying to quantify by flow cytometry is the partially arbitrary choice of the gates that defines what is considered beads and/or a positive signal. For example, if gates are drawn differently (see for example gate P1 in Figures 1 and 3) then the number of cells that are considered “positive” would change as well. With these caveats, cell numbers based on flow cytometry data should be taken with care.

Estimation of a rough detection limit for *F. psychrophilum* by flow cytometry and FISH probes was done through a dilution series of hybridized cells with added beads of known concentration (Figure 3). The number of signals from the beads (gate P2) was then correlated to the number of positive signals (gate P1) that were detected at the same time, meaning in the same amount of sample volume. Starting at a concentration of roughly  $2 \times 10^6$  cells/ml in the first dilution (“10X”), numbers were declining in a linear fashion according to the dilution factor (Figure 4) with roughly  $1,8 \times 10^5$ ,  $1,7 \times 10^4$  and  $2,2 \times 10^3$  cells/ml for the dilution steps down to 1:10.000. Only the lowest dilution of 1:100.000 diverted more drastically at  $4,7 \times 10^2$ . However, while cell numbers seem to be reliably detected over a range of magnitudes, data shown in histograms (Figure 5) reveal that the amount of “noise” in relation to clearly identifiable signals increases drastically at higher dilutions. Without prior knowledge about the expected results, i.e. in natural samples from aquaculture tanks where the number of positive cells is not known, this bad signal-to-noise ratio makes a reliable detection of target cells at low concentrations problematic. Due to this – and the other mentioned factors that influence flow cytometry cell counts – it would therefore not be advisable to rely on these cell counts at low concentrations. Based on these experiments though, numbers of FISH-labeled cells above  $2 \times 10^4$  cells/ml should be detectable.

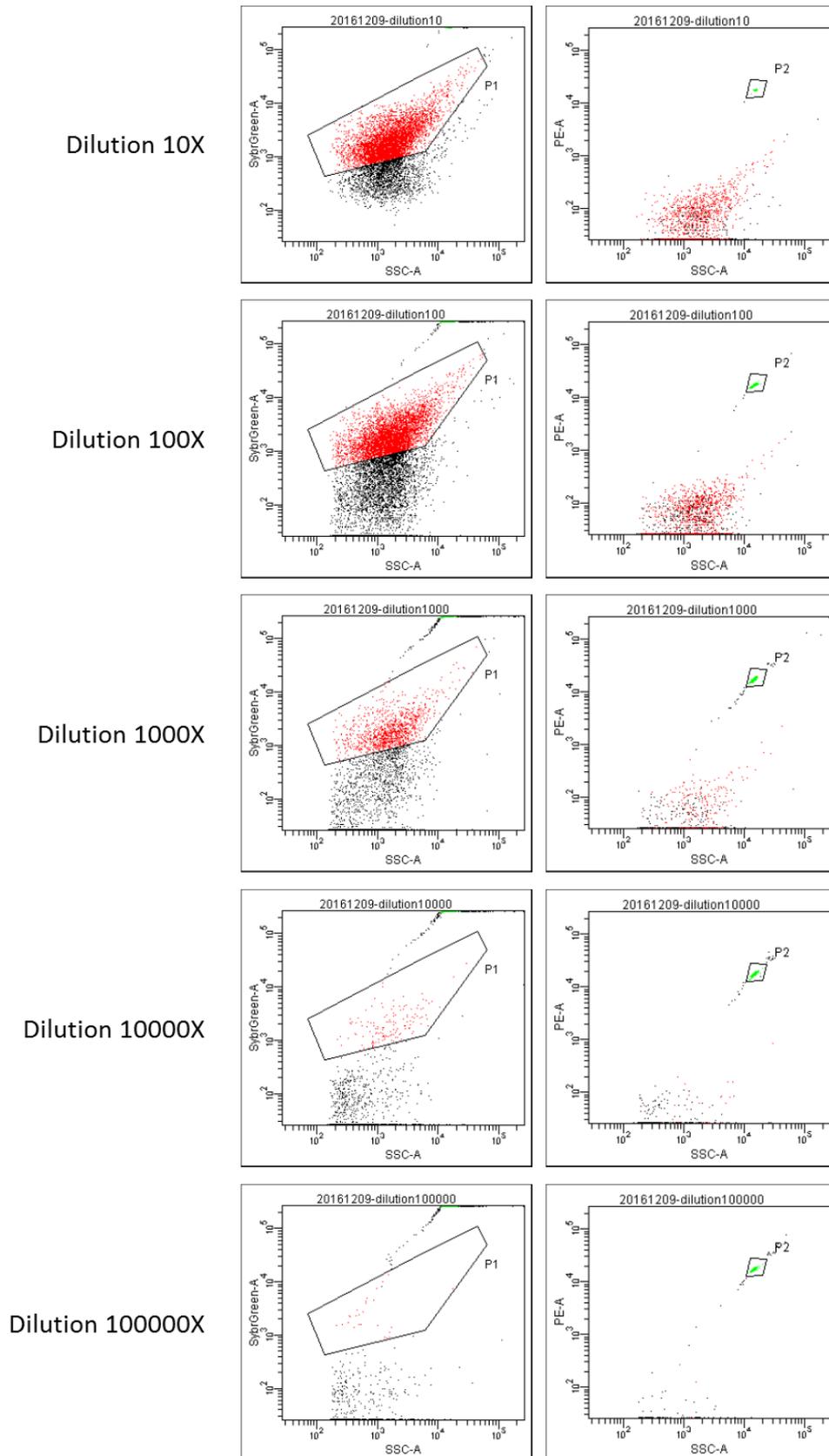


Figure 3: Flow cytometry dot plots (fluorescence versus side scatter) for different dilutions of *F. psychrophilum* hybridized with probes P77 & P477. Signals in gate P1 were considered positive and were counted. Fluorescent beads of known concentration were added to each sample, counted (gate P2) and used to calculate the number of positive signals in cells/ml.

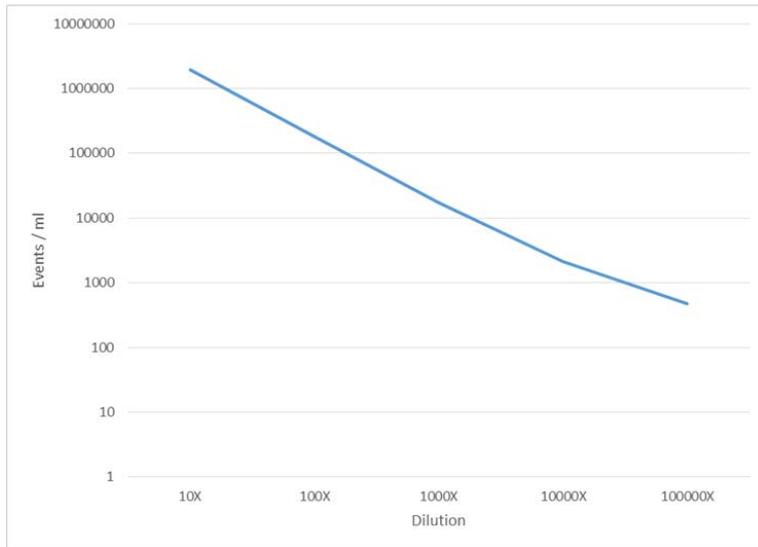


Figure 4: Number of FISH-labeled cells per ml at different dilutions.

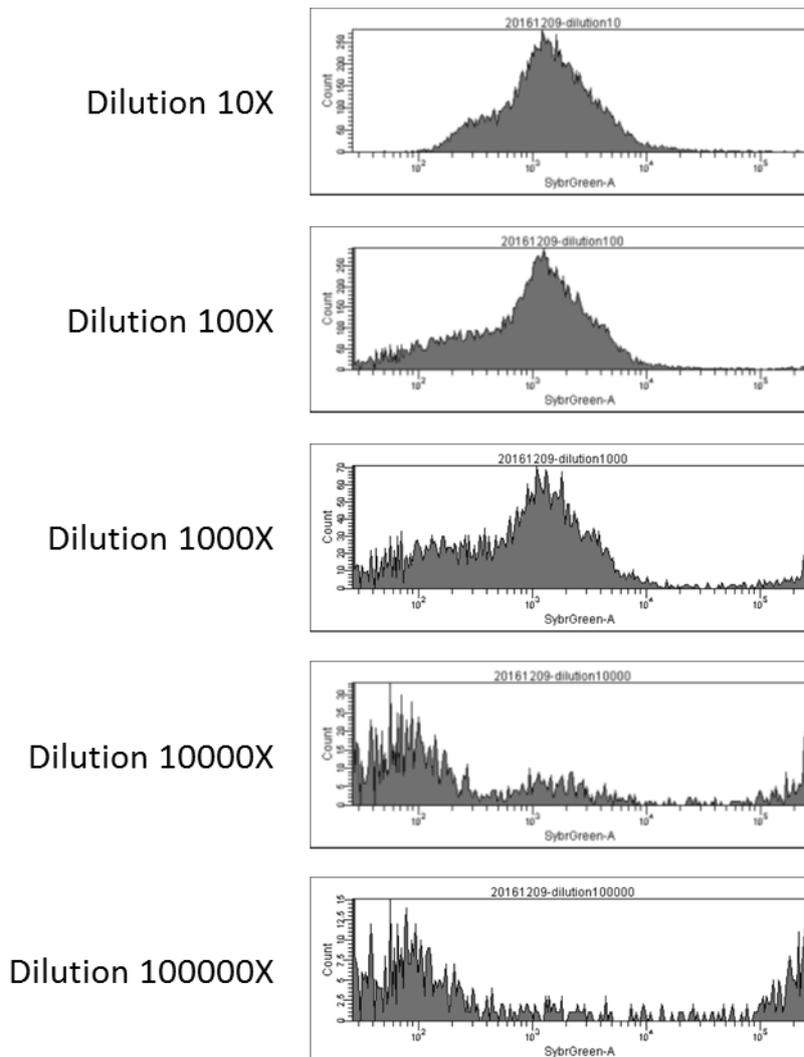


Figure 5: Flow cytometry histograms (fluorescence strength versus number of cells) for different dilutions of *F. psychrophilum* hybridized with probes P77 & P477.

## Discussion

This small proof-of-concept project was able to answer some questions regarding the usability of the application of species-specific FISH probes in combination with flow cytometry for the routine monitoring of pathogen loads in aquaculture. However, more testing and method establishment would be required in a larger, follow-up project before routine molecular detection systems for bacterial pathogens can be usefully employed in the Icelandic aquaculture.

Flow cytometry is in general a very useful tool for a routine monitoring scheme in aquaculture, as it allows for a fast screening of many water samples at low costs (after acquisition of the – expensive – flow cytometer itself). However, heterotrophic bacteria, which include the fish pathogens from this study, do not contain enough morphological features that can be used to unambiguously identify them at any useful (for the purpose of pathogen detection) taxonomic level. To achieve this task, species- or even strain-specific probes have to be added to the samples to identify their target organisms. The general possibility of this has been shown in the literature (e.g. Sekar et al, 2004) and was confirmed in this project for *F. psychrophilum* strains.

Species-specific molecular probes were successfully used in FISH experiments for the identification and characterization of a broad taxonomic range of bacteria and in many different terrestrial and aquatic environments (e.g. Groben et al., 2000; Simonato et al., 2010). Under the correctly established conditions, they can specifically detect only their target species in a mixed community and are therefore a useful tool to specifically identify pathogenic bacteria in water samples from aquaculture tanks before an infection occurs. However, this technology requires that the probes were designed based on differences in the sequence of the ribosomal RNA (rRNA) among species/strains (Amann et al, 2001; Pernthaler et al, 2001). In some cases – like with *A. salmonicida* – this is not possible as the rRNA of this species is too similar to the rRNA of other, related species, and a specific, discriminatory probe cannot be found. An alternative technology for the detection of pathogenic bacteria species in aquaculture water samples would be real-time or quantitative Polymerase Chain Reaction (RT-PCR / qPCR) (e.g. Martins et al, 2015). This methodology also uses species-specific primers and/or probes that are capable of detecting a particular species in a mixed community sample, but in contrast to FISH, those primers/probes bind to the isolated DNA of the organism and therefore the whole genome can theoretically be used to develop this specificity. In comparison to FISH/flow cytometry, RT-PCR has some disadvantages, i.e. it is more labour-, time- and cost-intensive as it requires DNA isolation of the samples and greater technical skills in its application. However, it has normally a very high sensitivity and could be expected to show a lower detection limit for pathogenic bacteria than flow cytometry, and, as mentioned before, it might be a possibility where FISH cannot be applied. If molecular detection systems are to be applied in the Icelandic aquaculture, both technologies should be tested for their capability to monitor pathogenic threats.

While flow cytometry offers many advantages for the analysis of microorganisms, one problematic aspect is the quantification of these organisms. Commercially available flow cytometers have no direct means of estimating the number of cells per ml directly but require additional steps like the use of microbeads and the weighting of samples to calculate the amount of liquid that was analysed. All these steps are potential error sources that reduce the accuracy of quantitative bacteria numbers. In addition, the definition of what is considered a positive signal, i.e. a bacterium identified by a bound

probe, is to some extent arbitrary and depends on the experience of the flow cytometry operator. All this means that quantitative results using the flow cytometry / FISH combination are not very accurate and do not have a low detection level. Despite all these aspects that need to be considered when evaluating the results of a measurement, a flow cytometer can still be set up for routine sample analysis. By using the same detector settings and gates that were established to identify the target organisms, the occurrence of these pathogens will be detectable if cell numbers are high enough. Such a set-up can at least be used as a fast early warning system for the emergence of pathogenic bacteria which might then require additional methods for more accurate identification and quantification.

One problem regarding the evaluation of the methodology for pathogen detection in the Icelandic aquaculture was the lack of available Icelandic bacterial strains in this project, which is especially problematic in cases like *A. salmonicida* where different strains/subspecies might be pathogenic or not. While some information about the specificity of the method can be obtained from testing strains from other parts of the world, the ultimate proof that the method is working should come from a test that uses pathogenic bacteria from an infected Icelandic aquaculture tank in which the bacteria have been unquestionably identified, e.g. by sequencing. However, while no Icelandic strains have been tested in this project, the two *F. psychrophilum* strains used in this project – originally isolated from the USA and France - were both successfully identified by the available probes. In addition, all other available *F. psychrophilum* rDNA sequences, which originated from isolates taken all over the world, were a perfect match to the used probes P77 and P47. Therefore, it can be reasonably expected that these probes will also be able to specifically detect Icelandic strains of *F. psychrophilum*.

To summarize, the combination of species-specific FISH probes for fish pathogenic bacteria with flow cytometric detection offers the potential for a fast early warning system that could be applied in the Icelandic aquaculture industry. The methodology is though only applicable for some pathogens (*F. psychrophilum*) but not for others (*A. salmonicida*). The establishment of a full routine monitoring system that can be used to detect multiple pathogenic threats to the Icelandic aquaculture industry would therefore require additional tests and the inclusion of further technologies, i.e. RT-PCR detection methods.

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## References

- Amann R, Fuchs BM, Behrens S (2001). The identification of microorganisms by fluorescence *in situ* hybridization. *Curr. Opin. Biotechnol.* 12: 231-236.
- Beaz-Hidalgo R, Latif-Eugenin F, Figueras MJ (2013) The improved PCR of the *fstA* (ferric siderophore receptor) gene differentiates the fish pathogen *Aeromonas salmonicida* from other *Aeromonas* species. *Vet. Microbiol.* 166: 659-663.
- Greuter D, Loy A, Horn M, Rattei T. (2016). probeBase — an online resource for rRNA-targeted oligonucleotide probes and primers: new features 2016. *Nucleic Acids Res.* 10.1093/nar/gkv1232
- Groben R, Doucette GJ, Kopp M, Kodama M, Amann R, Medlin LK (2000) 16S rRNA targeted probes for the identification of bacterial strains isolated from cultures of the toxic dinoflagellate *Alexandrium tamarense*. *Microb. Ecol.* 39: 186-196
- Gulla S, Duodu S, Nilsen A, Fossen I, Colquhoun DJ (2016) *Aeromonas salmonicida* infection levels in pre- and post-stocked cleaner fish assessed by culture and an amended qPCR assay. *J. Fish Diseases* 39: 867-877.
- Herlemann DP, Labrenz M, Jürgens K, Bertilsson S, Waniek JJ, Andersson AF (2011). Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME J.* 5:1571-1579
- Long A, Call DR, Cain KD (2015). Comparison of quantitative PCR and ELISA for detection and quantification of *Flavobacterium psychrophilum* in salmonid broodstock. *Diseases Aquat Organ.* 115: 139-146.
- Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Forster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, König A, Liss T, Lussmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH (2004). ARB: a software environment for sequence data. *Nucleic Acids Res.* 32:1363-1371
- Martins P, Navarro RVV, Coelho FJRC, Gomes NCM (2015) Development of a molecular methodology for fast detection of *Photobacterium damsela* subspecies in water samples. *Aquaculture* 435: 137-142.
- Pernthaler, JF, Glöckner, O, Schönhuber, W, Amann, R (2001). Fluorescence *in situ* hybridization. In J. Paul (ed.), *Methods in Microbiology: Marine Microbiology*, vol. 30. Academic Press Ltd, London.
- Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig WG, Peplies J, Glöckner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucl. Acids Res.* 35:7188-7196
- Simonato F, Gomez-Pereira, PR, Fuchs, BM, Amann R. (2010) Bacterioplankton diversity and community composition in the Southern Lagoon of Venice. *System. Appl. Microbiol.* 33: 128-138
- Strepparava N, Wahli T, Segner H, Petrini O (2014) Detection and quantification of *Flavobacterium psychrophilum* in water and fish tissue samples by quantitative real time PCR. *BMC Microbiol.* 14: 105.
- Strepparava N, Wahli T, Segner H, Polli B, Petrini O (2012) Fluorescent *In Situ* Hybridization: A new tool for the direct identification and detection of *F. psychrophilum*. *PLoS ONE* 7: e49280. doi:10.1371/journal.pone.0049280

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. [Opens external link in new window](#) *Nucl. Acids Res.* 41 (D1): D590-D596

Sekar R., Fuchs BM, Amann R, Pernthaler J (2004). Flow Sorting of Marine Bacterioplankton after Fluorescence In Situ Hybridization. *Appl. Environ. Microbiol.* 70: 6210-6219.

Warsen AE, Krug MJ, LaFrentz S, Stanek DR, Loge FJ, Call DR (2004) Simultaneous discrimination between 15 fish pathogens by using 16S ribosomal DNA PCR and DNA microarrays. *Appl. Environ. Microbiol.* 70: 4216-4221

Westram R, Bader K, Pruesse E, Kumar Y, Meier H, Glöckner FO, Ludwig W (2011) ARB: a software environment for sequence data. In: de Bruijn FJ (ed) *Handbook of Molecular Microbial Ecology I: Metagenomics and Complementary Approaches*. [Opens external link in new window](#) John Wiley & Sons, Inc., pp 399-406