Rapid quantitative monitoring method for the fish spoilage bacteria *Pseudomonas*

Eyjólfur Reynisson,*[ab] Hélène Liette Lauzon,[a] Hannes Magnusson,[a] Guðmundur Óli Hreggvidsson[ab] and Viggó Thor Marteinsson[a]

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Pseudomonas spp. is a group of microorganisms commonly found in fish and other fresh foods and is involved in their spoilage process. The aim of this study was to develop a rapid and accurate quantitative assay for *Pseudomonas* spp. in fish using real-time PCR. The assay targets the carbamoyl phosphate synthase gene (*car*A) with SYBR green based real-time PCR. The selectivity of the assay was confirmed using 24 *Pseudomonas* strains and 55 non-pseudomonad strains. A linear quantification was established over seven orders of magnitude, from $40 - 4^7$ copies reaction^{-1}. The assay was validated on cod samples collected during two shelf life trials and showed a high degree of correlation to the plate count method ($rP = 0.891$) where the difference between the methods was 0.04 $\log_{10}$ CFU g^{-1} on average. The study shows that it is possible to quantify accurately the specific spoilage organisms belonging to the genus *Pseudomonas* in fish using real-time PCR. The method takes less than 5 h from sampling to results. The short detection time of the method can provide the fish industry with an important tool for quality control and processing management.

Introduction

The spoilage process of fish initiates soon after catch by autolytic activity of endogenous enzymes, followed by microbial deterioration and lipid oxidation. [1–3] The bacterial flora associated with fish can be of various origins; from its environment, skin or digestive tract or simply from handling and processing. As soon as fish has been processed and packaged the *in situ* bacterial composition will undergo changes that depend on the storage conditions.[4,5] Until now, the process of fish spoilage through storage has been investigated intensively with regard to sensory evaluation, chemical changes of volatile compounds and microbiological growth by cultivation methods.[6–12] The microbiological methods applied have been used for estimation of bacterial growth on selective media targeting specific groups of microorganisms or the total bacterial count. [13,14] A large amount of data has been generated and three bacterial species have been proposed to be the main specific spoilage organisms (SSO) in marine fish from temperate waters, namely *Shewanella putrefaciens*, *Pseudomonas* spp. and *Photobacterium phosphoreum*. [15] The present paper is focused on *Pseudomonas* spp. since they are frequently used as bacterial indicators for spoilage and are present in different food types such as fish, meat and chicken. [10,16,17]

Selective media for the growth of these bacteria have been reported, [18–20] but methods based on cultivation need at least 2–5 days before the result can be obtained. Using real-time PCR, results can be expected in 4–5 hours after receiving the sample.

When developing a real-time PCR assay, the selection of target gene has to be considered carefully. It has to be present in all strains and conserved enough to enable the design of priming sites specific for the genus or species targeted. In *Pseudomonas* spp., the *car*A gene (carbamoyl phosphate synthase, subunit A) provides glutamine amidotransferase activity necessary for removal of the ammonia group from glutamine in the biosynthesis of pyrimidines and purines. This gene is present in all *Pseudomonas* spp. and it has specific and conserved regions possible to use as genetic markers.[21]

The aim of the present study was to develop a rapid quantitative assay specific for *Pseudomonas* spp. in marine fish using real-time PCR approach.

Experimental

Bacterial strains

Bacterial strains used in this study were isolated from refrigerated cod products during two storage trials, as well as from strain collections. In situ isolates from cod products were picked from modified cephaloridine fucidin cetrimide (CFC) agar[19] where *Pseudomonas* spp. form pink colonies on the medium incubated at 22 °C for 3–4 days. *Photobacterium phosphoreum* isolates were obtained following Malthus incubation at 15 °C in PPDM (Dalgaard *et al.*, 1996[20]), cultivation of positive samples at 16 °C onto modified Long and Hammer's medium[22] containing 1% (w/v) NaCl and selection of typical translucent colonies. *Shewanella putrefaciens* isolates were obtained from black colonies on spread-plated iron agar[18] supplemented with 1% (w/v) NaCl and following incubation at 16 °C for 5 days. Identification of isolates was confirmed by partial 16S rRNA gene sequencing.[23]

Vibrio strains were isolated from mussels and identified using the API-20E Enteric Identification System (bioMérieux, Inc., Hazelwood, MO).

[a]*Matís—Icelandic Food Research, Reykjavík, Iceland. E-mail: eyjolfur@matis.is*
[b]*University of Iceland, Reykjavík, Iceland*

Sample preparation and DNA extraction

DNA from bacterial isolates was extracted by suspending a loopful of bacterial cultures in 200 µl 5% Chelex solution by vortexing. The suspension was incubated at 55 °C for 15 min, vortexed again, boiled for 10 min and then placed on ice for 3 min. The samples were centrifuged at 11.000 *g* for 7 min and the supernatant containing the DNA recovered. The DNA extracts were diluted ten-fold prior to PCR.

Bacterial DNA from fish products sampled during the shelf life trials was prepared as follows: At each sampling time two cod loins were aseptically minced, 25 g diluted with 225 g of chilled Maximum Recovery Diluent (Oxoid, Hampshire, UK) and mixed for 1 min using a stomacher (Seward Limited, Norfolk, UK). One millilitre of the ten-fold diluted fish samples was frozen at −20 °C for later DNA extraction while further serial dilutions were done as required and plated on modified CFC agar. For the DNA extraction, the diluted samples were centrifuged at 11.000 *g* for 7 min to form a pellet. The supernatant was discarded and DNA was recovered from the pellet using the Promega Magnesil KF, Genomic system (MD1460) DNA isolation kit (Promega Corporation, Madison, USA) in combination with KingFisher magnetic beads automatic DNA isolation instrument (Thermo Labsystems, Waltham, USA) according to the manufacturers' recommendations.

Primer design

Primers were designed from the *car*A gene using sequences available on NCBI and sequences obtained by *Pseudomonas* strains isolated from fish products in the present study. Multiple alignments were made in order to find appropriate regions for primer design using BioEdit (version 7.0.1). Evaluation of primer quality was performed using NetPrimer, an online primer analysis software (www.premierbiosoft.com).

Real-time PCR analysis

All PCR reactions were done using the Mx3000p instrument and Brilliant SYBR green II mastermix (Stratagene, La Jolla, CA, USA). Primers were synthesized and purified with HPLC (MWG, Ebersberg, Germany). The reaction volume was 25 µl with 200 nmol l^{-1} for primer concentration. The thermal profile was as follows: 95 °C for 10 min followed by 35 cycles at 95 °C for 30 s, 57 °C for 60 s and an extension step at 72 °C for 30 s. After the PCR a dissociation curve was carried out where the instrument went at 2 °C min^{-1} from 55 °C to 95 °C with continuous fluorescence readings.

TaqMan PCR reactions were performed in 25 µl reaction volume with the *Teg* polymerase (Matis-Prokaria, Reykjavík, Iceland) and 250 nmol l^{-1} probe concentration, 500 nmol l^{-1} primer concentration, 1 mg ml^{-1} BSA and 1.5 mmol l^{-1} $MgCl_2$ concentration. The same thermal profile was applied with the exception of 60 °C annealing temperature.

Sensitivity and linearity

An amplified *car*A PCR product was cloned using TOPO4 vector cloning kit (Invitrogen, Paisley, UK) according to the manufacturer's recommendations. The plasmid containing the insert was isolated from the cells using the GenElute plasmid Miniprep kit (Sigma-Aldrich Inc, MO, USA). The DNA concentration was measured using the Nanodrop spectrophotometer (Nanodrop Technologies, DE, USA). The copy number was calculated from the plasmid size as described by Josefson *et al.*[24] Ten-fold serial dilutions were prepared for determination of the linearity and minimal concentration for a positive response (amplification above threshold line and correct peak in melting curve analysis).

Specificity

The assay was tested on a panel of 22 *Pseudomonas* strains and 47 non-pseudomonad strains of various origins. A response was regarded as positive if the amplification curve crossed the fluorescence threshold line which was positioned by background-based algorithm (background fluorescence between cycles 5 and 9 and a sigma multiplier factor of 10) calculated by the software.

Twenty putative *Pseudomonas* isolates from cod products stored in modified atmosphere (MA) at −1.5 °C were isolated from CFC agar and characterized with 16S rRNA gene sequencing to obtain the fraction of correctly identified *Pseudomonas* species by conventional cultivation enumeration.

Calibration of standard

The accuracy of absolute quantification of the qPCR method was estimated by serially diluting an overnight culture of *Ps. fluorescens* and subjecting it to quantification using both plating method and real-time PCR. For cultivation enumeration, 100 µl fraction of the culture dilution was spread on Plate Count Agar (Oxoid, Cambridge, UK), cultivated for 5 days at 22 °C and CFU (colony forming units) ml^{-1} determined by colony counting. For real-time PCR quantification, 100 µl fraction of the culture was used for extraction. The DNA was eluted in 50 µl of water and 5 µl was used as template in the PCR reaction.

The results from the plate counting were used as an absolute value of the number of *Pseudomonas* and the results from the qPCR were adapted to that number.

Shelf life trials

Cod samples were collected in two independent shelf life trials during autumn 2006 and 2007. The cod loins were stored in retail packs (FPP trays: 985 ml, FÆRCH Plast, K71-51W - 71051413; film: Cryovac EOP 240 mm) in air or modified atmosphere (CO_2/O_2/N_2: 50/5/45) at superchilled temperatures (−4 to 0 °C) for up to 28 days with periodic sampling which resulted in 32 samples in total. At each sampling day the specimens were plated directly on agar medium for viable counts and 1 ml fractions were frozen at −20 °C for later DNA extraction for real-time PCR analysis.

Statistical analysis of data

Statistical difference between cultivation and real-time PCR data was analysed by linear regression of a correlation curve and by Pearson correlation coefficient in Excel. The data used for the statistical analysis was from the shelf life trials.

Results

Primer design

Conserved *Pseudomonas* spp. specific region was found in the *car*A gene as shown by multiple alignment (Table 1). The amplicon of the PCR is 165 bp. Blasting of the forward primer resulted in perfect hits with the following *Pseudomonas* species: *fluorescens*, *putida*, *mendocina*, *syringae*, *chlororaphis*, *aureofaciens*, *aurantiaca*, and one base mismatch hits on *Ps. resinovorans*, *tolaasii* and *marginalis*. Blasting of the reverse primer resulted in perfect hits on *Ps. fluorescens*, *putida*, *chlororaphis*, *aureofaciens*, *aurantiaca*, *lundensis*, *marginalis*, and 1 base mismatch hit on *Ps. agarici*. The primers are able to detect *Pseudomonas* groups I and II according to the classification of Shewan *et al.*[25] The design of an internal probe used for TaqMan based assay was also made which can distinguish between groups I and II by one base pair. Locked nucleic acids (LNA) were inserted in the probe in order to increase the melting temperature (T_m) above the primers' T_m. The LNA probe was gCCaGtTGctCGc where capitals represent LNA bases. This probe has a T_m of 78 °C (http://lna-tm.com/).

PCR optimization

The SYBR green assay was optimized with regards to annealing temperature. In some occasions false positive responses were obtained at 55 °C annealing temperature but increasing it to 57 °C eliminated the cross-reactivity. Primers were kept constant at 200 nM and using this setup along with the current thermal profile, a satisfactory specificity and sensitivity was established.

Specificity

Using the SYBR green system, the strains tested up to date showed a high degree of specificity against *Pseudomonas* (Table 2). Four *Ps. fluorescens* strains, one *Ps. fragi* and one *Ps. aeruginosa* strain obtained from strain collections also reacted to the assay. *Ps. aeruginosa* showed however a delayed response compared to other *Pseudomonas* strains indicating variability in the priming sequences (data not shown). In some cases, a weak response was observed in non-pseudomonad strains with a Ct value around 35. Using the Taqman probe based assay, *Ps. fluorescens* (group I) was distinguished from *Ps. fragi* (group II) and strains belonging to group I were distinguished from other bacterial groups. *Pseudomonas* spp. belonging to the Shewan groups III–IV did not respond to the assay. The 16S rRNA gene sequence of these strains showed that they have high 16S similarity to *Pseudoalteromonas* spp. and are therefore named *Pseudoalteromonas* spp. in our study (Table 2).

Sensitivity and linearity of real-time PCR assay

A broader specificity towards *Pseudomonas* spp. was obtained with the SYBR green system than the TaqMan (Table 2) and therefore, further developments were concentrated on the SYBR green system. Linear quantification was achieved over seven orders of magnitude ranging from 40 up to 4×10^7 copies per reaction using SYBR green detection system on pure DNA target in a TOPO vector. A standard curve with $R^2 = 0.994$ and calculated efficiency of 96.7% (slope of −3.404) was achieved. A thermal dissociation curve showed that the melting point of the product was 85.7 °C.

Accuracy with reference to cultivation

The concentration of the DNA standard used for quantification was determined by calculating the copy number from the known plasmid size and spectrophotometric measurements on purified DNA. When compared against the enumeration (plate count method) of pure cultures, a difference of 1.36 logarithmic unit (SD = 0.40) was observed on average between the methods but a high degree of linearity ($R^2 = 0.9218$). For correction of the standard against the plate count method, this number was subtracted from all values obtained by real-time PCR (Fig. 1). The best linearity was achieved between 2 and 6 logarithmic units with real-time PCR. Quantitative analysis was done at all dilutions in the real-time PCR but with the plate count method, it was only possible to count directly colonies in the two lowest dilutions, *i.e.* 11 ± 1 and 118 ± 34 CFU (average of three replicates).

Assay validation on fish samples

The assay was performed on samples collected during two cod shelf life trials. Cod loins were stored at different temperatures

Table 1 Multiple alignment of the priming sequences in the *car*A gene and their corresponding primers (bottom). The top eight sequences were obtained from NCBI while the bottom three were sequenced in the present study

Species (accession/definition)	Forward primer (5′–′3)	LNA probe (5′–′3)	Reverse primer (5′–′3)
Ps. putida (DQ178193)	GGCTTTCAGGTAGTCGGACAG	.GCCAGTTGCTCGC.	CAACAGATCGTTACCCTGACTT
Ps. putida (DQ178194)	GGCTTTCAGGTAGTCGGACAG	.GCCAGTTGCTCGC.	CAACAGATCGTTACCCTGACTT
Ps. putida (DQ178195)	GGCTTTCAGGTAGTCGGACAG	.GCCAGTTGCTCGC.	CAACAGATCGTTACCCTGACTT
Ps. putida (DQ178196)	GGCTTTCAGGTAGTCGGACAG	.GCCAGTTGCTCGC.	CAACAGATCGTTACCCTGACTT
Ps. fluorescens (DQ178197)	GGCTTTCAGGTAGTCGGACAG	.GCCAGTTGCTCGC.	CAACAGATCGTTACCCTGACTT
Ps. fluorescens (DQ178198)	GGCTTTCAGGTAATCGGACAG	.GCCAGTTGCTCGC.	CAACAGATCGTTACCCTGACTT
Ps. fluorescens (DQ178200)	GGCTTTCAGGTAATCGGACAG	.GCCAGTTGCTCGC.	CAACAGATCGTTACCCTGACTT
Ps. fluorescens (DQ178201)	GGCTTTCAGGTAATCGGACAG	.GCCAGTTGCTCGC.	CAACAGATCGTTACCCTGACTT
Pseudomonas spp. Group I[a]	GGCTTTCAGGTAATCGGACAG	.GCCAGTTGCTCGC.	CAGCAGATCGTTACCCTGACTT
Pseudomonas spp. Group II[a]	GGCTTTCAGGTAATCGGACAG	.GCCAGTTGCTGGC.	CAGCAGATCGTTACCCTGACTT
Ps. fluorescens (Strain 31)	GGCTTTCAGGTAATCGGACAG	.GCCAGTTGCTCGC.	CAGCAAATCGTTACCCTGACTT
Oligos for PCR	GGCTTTCAGGTARTCGGACAG	.GCCAGTTGCTCGC.	CARCARATCGTTACCCTGACTT

[a] Two strains of each group were sequenced.

Table 2 List of strains tested in the qPCR assay, either from strain collections or isolated from Icelandic fish

Species	16S rRNA Similarity (%)	Accession number	Origin/ collection no.	No. of strains	SYBR response	TaqMan response
Pseudomonas fluorescens	Nd		DSMZ 6609	1	+	+
Pseudomonas fluorescens	Nd		DSMZ 50090	1	+	+
Pseudomonas fluorescens	Nd		DSMZ 50415	1	+	+
Pseudomonas fragi	Nd		DSMZ 3456	1	+	—
Pseudomonas fluorescens	Nd		NCTC 3756	1	+	+
Pseudomonas aeroginosa	Nd		ATCC 9027	1	+	Nd
Pseudomonas fluorescens	100	AY092072	Haddock	2	+	Nd
Pseudomonas fluorescens	99	AY581137	Haddock	2	+	Nd
Pseudomonas spp.	97	AY689025	Haddock	1	+	Nd
Pseudomonas putida [a]	100	AF336352	Cod	5	+	Nd
Pseudomonas fluorescens	100	AY092072	Cod	1	+	Nd
Pseudomonas libanensis	100	DQ288882	Cod	2	+	Nd
Pseudomonas tolaasii	100	EU169178	Cod	1	+	Nd
Pseudomonas spp. Group I	Nd		Cod	2	+	+
Pseudomonas spp. Group II	Nd		Cod	2	+	—
Pseudoalteromonas spp. [b]	99		Cod	4	—	—
Photobacterium phosphoreum	100	DQ099331	Cod	5	—	Nd
Acinetobacter spp.	100	AJ301674	Cod	2	—	—
Shewanella spp.	100	EU016165	Cod	18	—	—
Shewanella putrefaciens	100	AB205574	Cod	1	—	—
Shewanella hanedai	100	DQ011269	Cod	1	—	—
Stenotrophomonas maltophilia	100	EU263112	Cod	1	—	—
Pseudoalteromonas spp.	100	EU195932	Cod	6	—	—
Aeromonas sobria	100	DQ133179	Cod	7	—	—
Aeromonas salmonicida smithia	100	AB027544	Cod	5	—	Nd
Vibrio vulnificus[c]			Mussel	1	—	Nd
Vibrio fluvialis[c]			Mussel	1	—	Nd
Vibrio cholera[c]			Mussel	1	—	Nd
Vibrio alginolyticus [c]			Mussel	1	—	Nd
Vibrio parahaemolyticus[c]			Mussel	1	—	Nd

[a] These strains showed also 100% similarity to *Ps. fluorescens* and *Ps. trivialis* in the partial 16S sequencing. [b] The strains were previously phenotypically assigned as *Pseudomonas* spp. groups III–IV according to the classification of Shewan *et al.*[12] [c] *Vibrio* strains were identified using API-20E species identification strip. Nd, not determined.

(0, −1.5, −2 and −4 °C) and in air or modified atmosphere. Here, the pseudomonad load was compared using the plate count method (modified CFC agar) and real-time PCR quantification previously calibrated against the plate count method as described above. A clear trend was observed between the methods (Fig. 2). On average the difference between the methods was 0.04 ± 0.6 $\log_{10}$ CFU g^{-1}. Among the samples tested, 66% of them showed 0–0.5 $\log_{10}$ difference from the plate count method, 25% between

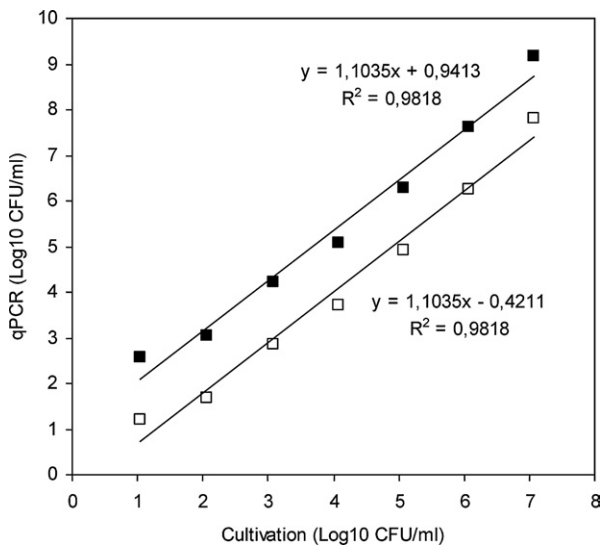

Fig. 1 Comparison of real-time PCR against plate count method for enumeration of *Pseudomonas* spp. in liquid culture with direct unmanipulated data (■) and corrected qPCR data (□).

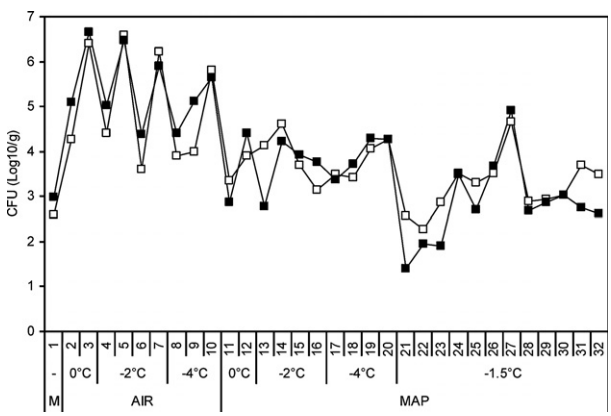

Fig. 2 Comparison between cultivation (□) and qPCR (■) for enumerating *Pseudomonas* spp in fish. Samples 1–20 were taken in shelf life trials during autumn 2006 and samples 21–32 in 2007. Sample 1 is the raw material (M) in the shelf life trials in 2006. Samples were stored at different temperatures and in air or modified atmosphere packaging.

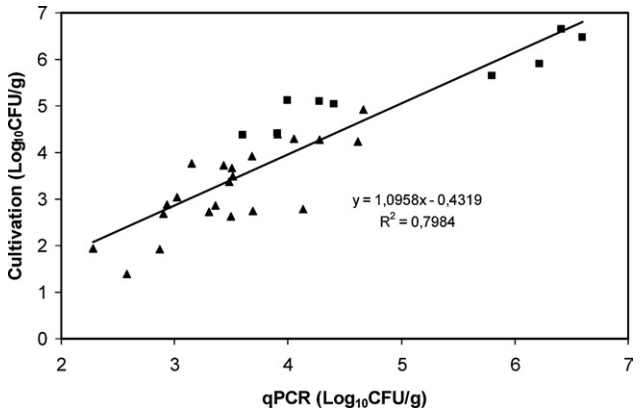

Fig. 3 Correlation curve between *Pseudomonas* spp. enumeration using cultivation (*x*-axis) and qPCR (*y*-axis) on samples obtained from shelf life trials. Samples were obtained from fish during a shelf life trial stored in air (■) and MA (▲).

0.5 and 1.0 $\log_{10}$ and 6% showed between 1.0 and 1.5 $\log_{10}$ difference. A correlation curve where cultivation data are plotted against qPCR data shows the same trend with an R^2 value of 0.7984 when all the data from air and MA storage conditions are plotted (Fig. 3). Plotting of data from air stored cod alone shows a slight shift in the slope but to a lesser extent with a separate plotting of the MA stored cod. The Pearson correlation coefficient of the total dataset was 0.891, which indicates a high degree of correlation between the methods.

Discussion

This is the first study that describes a rapid quantification of *Pseudomonas* spp. in fish using real-time PCR assay. The assay was successful and confirmed on various *Pseudomonas* species targets. Most of the strains used in the present study were isolated in Iceland but the fact that the assay was effective across species boundaries within the genus and responded also to strains obtained from strain collections confirms that the method is not geographically restricted. The SYBR green system showed that *Pseudomonas* spp. belonging to the phenotypic groups I and II, according to Shewan *et al.*,[12,25] responded to the assay but not strains belonging to groups III–IV. Partial 16S sequencing also reveals that groups III–IV strains belong to the genus *Pseudoalteromonas* but not *Pseudomonas*.

The method should be applicable to estimate *Pseudomonas* spp. load in any fish species but may not necessarily reflect the spoilage status of every fish type since fish spoilage is species specific and depended on the origin of the fish, processing and storage conditions. The development of the assay was mainly directed against the fish spoilage organisms belonging to the genus *Pseudomonas* such as *Ps. fluorescens*, *putita* and *fragi*. [26] The assay responded also to *Ps. aeruginosa* (with decreased sensitivity) but specific real-time PCR detection methods for this pathogen have been developed before. [27,28]

Quantification using real-time PCR is possible over at least seven orders of magnitude without further diluting the sample after conventional sample preparation. However, samples must be serially diluted when using a conventional plate count method to enable separate growth of colonies on an agar plate. In a direct comparison between quantification by the real-time PCR assay and the standard plate count method, an average difference of 1.36 logarithmic units was observed at all concentrations. Such a difference between cultivation and real-time PCR has been reported before and can be explained in different ways.[29] Absolute quantification using real-time PCR is always dependent on the determination of the standard used. In this case the DNA concentration of the standard was determined using a spectrophotometer and the plasmid copy number was calculated from its size, which may not be in clear correlation to the number of colonies growing on an agar plate. Another factor that can influence quantification using PCR is the quality of the standard, being higher than extracted genomic bacterial DNA from fish samples as they can be contaminated with a range of substances that can influence the PCR, *e.g.* proteins, fish DNA or DNA from other bacteria, or the extraction efficiency is not sufficient. This, however, does not explain the difference between the methods because this would lead to reduced values with qPCR in comparison to cultivation, and that was not the case. Other more plausible explanations are the detection of dead cells or CFUs originating from more than one cell.

When the DNA standard was corrected and calibrated against the number of colony forming units of a serially diluted liquid culture high correlation was found between the PCR assay and plate counting of *Pseudomonas* spp. when both methods were tested on a series of samples from two cod shelf life trials.

It is possible that storage conditions of the fish can have an effect on the relationship between cultivation and qPCR enumeration. If some specific storage conditions favor the growth of *Pseudoalteromonas* spp. over that of *Pseudomonas* groups I–II, cultivation data may then be expected to be higher than qPCR data as these bacteria sometimes appear to have similar phenotypic traits on CFC medium but are distinguished in the *car*A targeted real-time PCR. This aspect however needs further investigation with larger datasets with samples from shelf life trials.

Pseudomonas spp. are environmentally opportunistic bacteria that can be expected to be found in different environments and in different quantities.[10,12,30] Therefore, the detection alone of this species is of limited value if the aim is to gain information on the contamination level, *e.g.* in fish products or fish processing environment. Because of the rapidity of the developed assay, a direct implementation of such methods to quality and processing management systems in food production is now realistic. Determination of contamination levels for *Pseudomonas* in high and low quality fish products or processing equipment by rapid testing of this species could therefore be of considerable benefit to the industry. In addition, since *Pseudomonas* spp. is a widespread environmental group this system could be adopted for different purposes than quality monitoring of food.

Predictive microbiology can be used to predict the shelf life of different food products and, at present, computer aided models have been designed for that purpose. [8] The method presented here provides the possibility of using rapid testing of bacterial load in combination with microbial models to predict the remaining shelf life of a fish product, *e.g.* during processing or shipping. Within the EU project Chill-On, this method is being investigated with the aim of implementing it into the supply chain to predict the remaining shelf life of cod.

In recent years, new diagnostic methods for various bacteria have been developed. Most of them are focused on food safety and the detection of pathogens.[31–33] The use of internal amplification controls (IAC) is now almost regarded as mandatory in these kinds of tests in order to prevent false negative results.[34] Typically, the IAC system includes the addition of DNA which is a target for the internal control priming system. Another possibility is to use comparative analysis to monitor PCR inhibition.[35] The diagnosis of pathogens usually involves a pre-enrichment step, so quantification of initial bacterial contamination is not possible. In the present study IAC was omitted since it influences the quantitative characteristics of the assay, especially when the target DNA is at low quantity. However, such system might be possible to construct with internal TaqMan PCR using other set of primers and a reporter dye with a different emission wavelength than SYBR green.

Methods for detection of *Pseudomonas* spp. have been reported previously for different purposes.[34,36] The detection of *Pseudomonas* spp. using the *car*A gene was described using classical PCR without the possibility of quantification.[21,37]

In conclusion, this study describes the development of a rapid assay for quantification of an important group of spoilage organisms in fish, *Pseudomonas* spp. This method could be of great value for the seafood industry in internal quality control systems as well as quality monitoring of products and processing facilities.

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