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QUANTITATIVE DETECTION OF THE SPOILAGE BACTERIA *PSEUDOMONAS* SPP. AND *PHOTOBACTERIUM PHOSPHOREUM* IN FISH BY REAL-TIME PCR

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ABSTRACT

The *Pseudomonas* spp and *Photobacterium phosphoreum* have been found as the specific spoilage organisms of fish. The aim of this study was to develop rapid and accurate quantitative assay for Pseudomonas spp. and *Photobacterium phosphoreum* in redfish and Salmon fillets using real-time PCR. Because the redfish material is not free from *P. phosphoreum* and *P. fluorescens*, only real-time PCR assay for Pseudomonas spp. and *Photobacterium phosphoreum* in salmon fillets was established. Results indicated that the real-time was most effective when the AB Teq polymerase mixture and DNA extracted by MasterPure™ DNA Purification Kit were used. Real-time PCR using DNA extracted by MasterPure™ DNA Purification Kit was able to determine the Log_{10} number CFU/g of *P. phosphoreum* in salmon fillets matrix from 3.22 to 7.40 in nature salmon fillet. The detection time of this method was only 5 h. Real-time PCR using DNA prepared by MasterPure™ DNA Purification Kit could provide the Log number of *Pseudomonas* spp. in salmon matrix from 2.19 to 6.30 directly. The detection time of this method was only 5 h. In conclusion, the real-time PCR was a quick method to quantity the *P. phosphoreum* and *Pseudomonas* spp. in salmon fish.

Keywords: Real-time PCR, *Pseudomona*s spp., *Photobacterium phosphorous*

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1 INTRODUCTION

1.1 Background

The presence and growth of foodborne pathogens and spoilage organisms in foods significantly impact product safety and quality, causing economic losses to the food industry and posing health risk to consumers (Bajpai *et al*. 2008). Promoting safety and quality of food products in the food environment is therefore a primary task for food microbiologist.

While sterilization could eliminate viable microorganisms in the final products, extreme processing conditions often cause undesirable sensory changes and loss of nutritional values (Gomez-Lopez *et al*. 2010) Therefore assuring food safety and quality still largely relies on proper food manufacturing practises and monitoring the microbial quality in raw materials, food processing environment, and final products. Fish as a main marine production in China, the monitoring microorganism of it is important to keep good quality.

The *Pseudomonas spp* and *Photobacterium phosphoreum* have been identified as the specific spoilage organisms of fish (Liston 1992, Gram *et al.* 1990, Gram and Huss 1996). Most fish species can be decomposed by *Pseudomonas* spp. to some degree in interaction with other environmental bacteria present. The spoilage caused by *Pseudomonas* spp and *Photobacterium phosphoreum* can lead to severe economic losses and waste in the supply chain. Proper processing, storage and handling methods is the key to extended shelf life, and there is also a need for unbiased and independent quality monitoring tools. Therefore, to establish a fast and accurate detection method for *Pseudomonas* spp. and P. *phosphoreum* is very important.

1.2 Project Goal

The aim of this study is to determine the detection probability of newly developed rapid quantitative assays specific for *Pseudomonas* spp. and *P. phosphoreum* in fillet of salmon and redfish using real-time PCR approach.

These methods could be used in quality control systems in the fish supply chain, producers or buyers on foreign markets that need to verify product quality using unbiased instrument instead of individual sensory analysis systems that are currently used. These methods could also provide input into microbial growth prediction models where initial bacterial load is often required in order to give precise prediction on shelf life. These will be very helpful for the design of quantitative detection protocols and HACCP systems. It will also be useful in making accurate predictions for shelf life.

1.3 Project objectives

1. Determine the detection probability of the real-time qPCR method for *Pseudomonas* spp. and *P. phosphoreum* in fillet of redfish.
2. Determine the detection probability of the real-time qPCR method for *Pseudomonas* spp. and *P. phosphoreum* in fillet of salmon.
3. Validation of the applied PCR method.

2 LITERATURE REVIEW

2.1 Pseudomonas spp

Pseudomonas spp. belongs to the family Pseudomonadaceae of gammaproteobacteria. The genus is Gram-negative aerobic microorganism (Euzéby 1997). The metabolism of this genus is diverse, so they can grow at ubiquitous nature (Madigan *et al*., 2005). Many species of *pseudomonas* spp. can contaminate food (Gennari *et al*. 1992). *Pseudomonas* spp. is one kind of the predominant spoilage bacteria in chilled fish at aerobic conditions (Fitzgerald *et al*. 2003, Hozbor *et al*. 2006, Doyle 2007). *Pseudomonas* spp. is one of the contributors to the spoilage of catfish in the state of Delaware (Maull *et al*. 2012).

Some members of the *Pseudomonas* spp. are also known as opportunistic fish pathogens. *Pseudomonas* spp. was also isolated from *Orechromis niloticus* suffered from saprolegniosis. Four *Pseudomonas* species including *P. fluorescence*, *P. putida*, *P. aeruginosa* and *P. anguilliseptica* has been found in *Oreochromis niloticus*, *Mugil cephalus*, *Cyprinus carpio*, *Hypophthalmichthys molitrix* (El-Hady *et al*. 2011). *Pseudomonas aeruginosa* was isolated and stored in China. *P. fluorescens* is considered one of the primary causes of Bacterial hemorrhagic septicemia in fish (Shiose *et al*. 1974). *P. fluorescens* is a short motile Gram-negative rod with polar flagella. *P. fluorescens* widely distributed in the aquatic environment, is probably due to spread through water (Austin and Austin 1999). The fish infected by *P. fluorescens* may develop Red Skin Disease under stressful conditions such as overcrowding (Allen *et al*. 1983, Frerichs and Holliman 1991 and Azza *et al*. 2002), low temperature and injuries (Aly 1994 and Abdomenech *et al*. 1999), secondary invader of damaged fish tissue (Otte 1963) and in chronic virus infection (Roberts and Horne 1978). It can infect many fish species, including Indian major carps, black carp, common carp, goldfish, Japanese flounder, and wedge sole (Geng *et al*. 2006, Swain *et al*. 2007, Bullock 1965, Lo´pez *et al*. 2011). *P. fluorescens* affects fresh-water and salt-water fish throughout the world and causes severe economic losses and decreases fish farms efficiencies (Stoskopf 1993 and Fayed *et al*. 1997, Wang *et al*. 2009).

P. fluorescens is a psychrotrophic bacterium, which can survive for 40 days at 5°C and more than 72 days at -11°C in fish feeds (Zmysłowska and Lewandowska, 1999). It has been identified as an opportunistic pathogen causing respiratory, urinary and bloodstream infections in patients (Lenenete *et al*. 1985, Fishwick *et al*. 2005; Pappas *et al*. 2006, Gershman *et al*. 2008). Fish infected by *P. fluorescens* processed or stored at low temperature will deteriorate and may do harm to the health of human beings, and the *P. fluorescens* is frequently used as an indicator (Jay *et al*. 2003).

Although these bacteria can, in some case cause pathogenesis, the major concern on industrial scale is its spoilage potential. Most fish species are decomposed by this bacterium to some degree in interaction with other environmental bacteria present.

2.2 Photobacterium phosphoreum

Photobacterium phosphoreum was first isolated from the aquatic environment by Martinus Beijerinck. It is Gram-negative, usually motile rod, and is found in aquatic habitats in association with eukaryotes. *P. phosphoreum* is one of many organisms that produce bioluminescence in marine organisms (Thompson *et al*., 2004). *P. phosphoreum* is a typical bacterium of deep sea fishes (Herring 1993). *P. phosphoreum* is common in the marine

environment and can grow at 4 °C, and it is a psychrotrophic and halophilic histamine producer (Fujii *et al*. 1997).

P. phosphoreum is identified as specific spoiler organisms in modified atmospheres packed cod (Dalgaard *et al*. 1993, Dalgaard 1995) and cold-smoked salmon (Jorgensen LV *et al*. 2000). *P. phosphoreum* were also found in both freshly processed and stored salmon (Olofsson *et al*. 2007). *P. phosphoreum* is the most important spoilage bacterium in packed chilled fish fillet, it can use trimethylamine oxide (TMAO) as a terminal electron acceptor. Then, the TMAO is converted to trimethylamine (TMA), and the fish products appear distinctively spoiled and fishy flavour (Flodgaard *et al*. 2005).

On the other hand, P. *phosphoreum* has been reported to be the dominant histamine producer in fish stored at temperatures lower than 15°C (Lehane and Olley 2000). An extremely high level of histamine accumulation (1000 mg/kg) has been detected in fish within a week of storage at 4 °C in the presence of *P. phosphoreum* (Torido *et al*. 2012). These results suggest that P. *phosphoreum* can cause large amounts of histamine accumulation in fish meat stored and transported at low temperatures. So there is the risk of histamine food poisoning due to the possibility of histamine accumulation when fish is stored at low temperatures. *P. phosphoreum* (Kanki *et al*. 2004) have been reported as the cause of histamine food poisoning, and the histamine food poisoning will lead the allergic symptoms such as urticaria, cutaneous flushing, headache, and nausea (Taylor and Eitenmiller 1986). So to develop a fast and accurate detection method for *P. phosphoreum* at early stage will be advantageous in reducing fish loss and danger to health.

2.3 Real-Time PCR

Several methods for detection of *P. phosphoreum* and *P. fluorescens* have been developed to determine concentrations of spoilage microbes (Gram *et al*. 1987, Stanbridge and Board 1994, Dalgaard *et al*. 1996). However, the conventional methods are time-consuming and take at least 2 days and sometimes require specialized apparatus as for the detection of *P. phosphoreum* using Malthuse conductance method (Dalgaard *et al.* 1996). The PCR techniques are powerful and reliable tools for the detection of bacteria in foods (Lauri and Mariani 2009). Especially Real time quantitative PCR (qPCR) is increasingly used for quantification of microbial populations in food matrices. Because the DNA synthesized in each cycle can be labelled by fluorescent agent, and this labelled DNA can be measured by fluorescence detector connected with thermocycler, in qPCR the PCR reaction can be monitored when it occurs in real time, and data can be collected during the reaction. The reaction results are determined as threshold cycle (Ct). Ct indicates the times of amplification cycles that the fluorescence can be detected by the instrument, and it is linear relationship with the initial copies of target DNA in a wide range (Logan *et al*. 2009).

The non-specific quantification qPCR is simplest and often used. In this method the fluorophores such as ethisium bromide, SYBR green I, SYBR Gold and SYTO9 was used to label DNA. These molecules will emit a strong fluorescent signal when they are binding with the minor-groove of dsDNA and under the appropriate wavelength of light without no additional oligonucleotide design or chemical conjugation, and small changes of the template sequence will not affect them (Logan *et al*. 2009).

qPCR is rapid for the quantitative analysis can be conducted without post-processing. Because the sensitivity of qPCR is high, the enrichment times can also be shortened compared

to other methods (Martin *et al*. 2010). Traditional culture methods need 2 to 3 days in enrichment step, but qPCR need only up to 12 h (Martin *et al*. 2010). Moreover, the concentration of biogenic amines produced by spoilage microorganisms can be detected indirectly by qPCR (Martínez *et al*. 2011). The relationship between the concentration of biogenic amines in food and results of qPCR for biogenic amines-producers has been confirmed (Ladero *et al*. 2008, 2010). Actually there are many qPCR methods that can be used for different food matrix (Landete *et al*. 2011), and several qPCR assays used to detect and quantify the strains producing biogenic amines or histamine in different foods (Nannelli *et al*. 2008, Fernández *et al*. 2006, Bjornsdottir-Butler *et al*. 2011, Reynisson *et al*. 2008). However, the qPCR used to detect spoilage microorganisms is still an early period (Martínez *et al*. 2011). The main difficulty is that the many types of food containing PCR inhibitors need to be tested. To obtain an accurate quantification of the target microbes the inhibitors must be detected carefully (Edwards and Logan 2009). In addition, food samples can be used directly as template providers, however, the result of qPCR is better when the nucleic acids are extracted from the food matrix (Martínez *et al*. 2011). The effect of extraction is affected by the physical state (liquid or solid), texture, and composition (content of proteins, sugars or fat) of matrix. Now there is no single extraction method that can be used for all foods and drinks. For each kind matrix, the efficiency and repeatability of extraction method must be analysed (Demeke and Jenkins 2010).

3 MATERIALS AND METHODS

3.1 Bacterial Strains and Primers

P. fluorescens was stored in the laboratory of Matis. *P. phosphoreum* was isolated and purified by using modified Long and Hammer's medium containing 1% (w/v) NaCl agar (Van Spreekens 1974) from redfish infected by *P. phosphoreum*. The DNA of isolation was extracted using a quick extraction of DNA and identified by real-time PCR. Working cultures of *P. fluorescens* and *P. phosphoreum* were prepared by incubating in liquid culture medium over night at 22°C and 17°C respectively.

Oligos for PCR of *P. fluorescens* were as follows:

- Forward primer 5′ GGCTTTCAGGTARTCGGACAG 3′
- LNA probe 5′ GCCAGTTGCTCGC 3′
- Reverse primer 5′ CARCARATCGTTACCCTGACTT 3′.

Oligos for PCR of *P. phosphoreum* are currently undisclosed.

3.2 Inoculation of matrix

Three-day old redfish (since catching) was obtained from HB Grandi and salmon slaughtered same day was obtained from Fjarðarlax and shipped to laboratory. Salmon and redfish fillets were tested for the presence of *P. fluorescens* and *P. phosphoreum* by mixing of 25 g samples with 225 mL chilled Maximum Recovery diluent (Oxoid, Hampshire, UK) respectively, and subsequent plating 100 µL this diliution on modified cephaloridine fucidin cetrimide (CFC) agar (Stanbridge and Board, 1994) and modified Long and Hammer's medium (LH) containing 1% (w/v) NaCl agar (Van Spreekens, 1974). At the same time, the DNA of 1 mL ten-fold diluted fish matrix was extracted by using the MasterPure™ DNA Purification Kit

(Epicentre® an Illumine® company), and was analysed by real-time PCR. If no *P. fluorescens* and *P. phosphoreum* are detected, the fish fillets will be used for artificial inoculation (Figure 1). Minced fish fillet (25 g) was added into 225 g of chilled Maximum Recovery diluent (Oxoid, Hampshire, UK) in a stomacher bag with lateral filter and mixed for 30 s in stomacher (Seward Limited, Norfolk, UK). The ten-fold diluted fish fillet samples (1 Ml) was added in a sterile 2 mL eppendorf tube and frozen at -20°C for later inoculation.

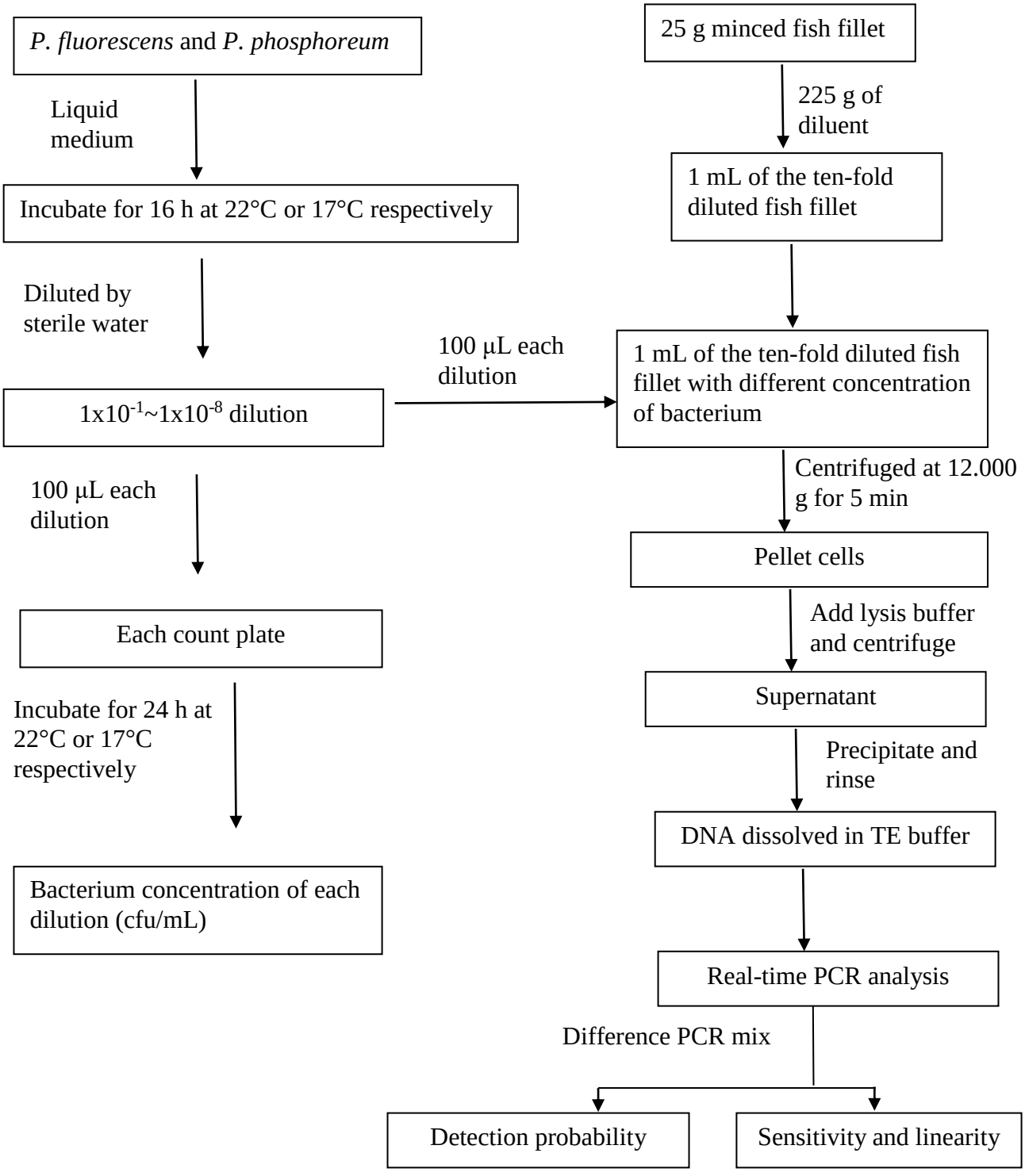

Figure 1: Flow diagram showing inoculation of *P. fluorescens* and *P. phosphoreum* and isolation of DNA for PCR analysis.

The cultured *P. fluorescens* and *P. phosphoreum* were diluted to contain an analysis range from 0-10^6 CFU/mL (1:10 diluted matrix) which corresponds to values likely to be obtained in real life situation. The precise number of CFU in the dilutions was determined by the plate count method. Each dilution (100 µL) was used for inoculation of 1 mL ten-fold diluted fish fillet matrix. Subsequently the artificially spiked fish fillets matrix was stored at -20°C until DNA isolation and PCR analysis. As a control, the un-inoculated fish fillets of each type were subjected to the same procedure. All the experiments were done in three parallel samples.

3.3 Preparation of DNA samples

3.3.1 Protocol 1 (Quick extraction of DNA)

liquid culture of *P. phosphoreum* (300 µL) was centrifuged at 12.000 g for 5 min to form a pellet. The supernatant was carefully removed and discarded. Five per cent Chelex Solution (200 µL) was added to the pellet and mixed by vortex. The suspension was incubated at 55°C for 15 min. Then the mixture was centrifuged at 11,000 g for 7 min after being boiled for 10 min and iced for 3 min. The supernatant containing DNA was ten-told diluted before real-time PCR.

3.3.2 Protocol 2 (Epicentre)

The DNA was isolated using the MasterPure™ DNA Purification Kit (Epicentre® an Illumine® company) according to the recommendations of manufacturers. 1 mL of the ten-fold diluted artificially spiked fish fillet matrix was centrifuged at 1,000 g (12,000 rpm) for 5 min to pellet the cells. The pellet was added in 300 µL of tissue and Cell Lysis Solution containing 1 µL the Proteinase K and mixed thoroughly. This mixture samples were incubated at 65°C for 15 min and mixed by vortex every 5 min. When the samples are cooled to 37°C 1 µL of 5 µg/mL RNase A was added in and mixed thoroughly. The samples were incubated at 37°C for 30 min, and placed on ice for 3-5 min. Then 175 µL of MPC Protein Precipitation Reagent was added in and mixed vigorously for 10 s by vortex. The samples were centrifuged at 10,000 g and 4°C for 10 minutes. The supernatant was transferred to a clean microcentrifuge tube and added in 500 µL of 80% isopropanol. The tube was centrifuged at 10,000 g and 4°C for 10 minutes after being inverted 30-40 times. The 80% isopropanol was poured and the DNA was rinsed twice with 70% ethanol. The DNA was dissolved in 35 µL TE buffer after the ethanol being removed. The DNA can be used for real-time PCR.

3.3.3 Protocol 3 (Hotshot genomic DNA preparation)

1 mL of the ten-fold diluted artificially spiked fish fillet matrix was centrifuged at 12.000 g for 5 min to form a pellet. The supernatant was carefully removed and discarded. 75 µL Alkaline Lysis Reagent containing 25 mmol/L NaOH and 0.2 mmol/L EDTA was added to the pellet and mixed by vortex. The mixture was incubated at 95°C for 30 min and cooled to 4°C on the ice. Then 75 µL Neutralization Buffer containing 40 mmol/L Tris-HCl was added into the mixture and mixed thoroughly. This mixture was centrifuged at 10,000 g for 10 min. The supernatant containing DNA was transferred to a clean microcentrifuge tube. The DNA can be used immediately or stored at -20°C.

3.4 Real-time PCR Analysis

All PCR reactions were done using the Mx3005p instrument and the suitable mix after test. Primers were synthesized and purified with HPLC (MWG, Ebersberg, Germany). The reaction volume was 25 µL with 400 nmol L^{-1} for primer concentration (Reynisson *et al*., 2008). The thermal profile was as follows: 95°C for 10 min followed by 40 cycles at 95°C for 30 s, 57°C for 30 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.

Real-time PCR master mixes from three different suppliers were compared: Universal master mix (Life Technologies, New York, America), referred as AB mix, Probe mix (A&A biotechnology, Gdynia, Poland) referred as Brill II mix and in-house prepared mix referred as CB mix. TaqMan PCR reactions were performed in 25 µL reaction volume with the Teg polymerase (Matis-Prokaria, Reykjavı´k, 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 in-house prepared mix, AB, and Brill II.

3.5 Determination of the detection probability

P. fluorescens and *P. phosphoreum* inoculated in fish fillets were quantified using both plating method and real-time PCR, and ten-fold diluted to 1×10^1, 1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6 CFU mL^{-1}. The DNA of each dilution was prepared as described in 3.3. Five parallel extractions were conducted for each dilution. DNA sample (5 µL) was added to PCR tubes. The cycle conditions are the same as described in 3.4. The experiment was repeated three times. Each PCR will give a positive or negative result at the concentration tested. The threshold line was set to a fluorescence value of 0.1. The detection probability was obtained by plotting the relative number of positive PCRs observed against the concentration of the cell suspension.

3.6 Sensitivity and linearity

Sensitivity and linearity of the assays was determined by analysis of tenfold serial dilutions of extracted DNA from pure cultures. The *P. fluorescens* and *P. phosphoreum* cultures were incubated in liquid culture medium over night at 22°C and 17°C respectively. 1 mL culture of each strain was used for DNA extract by using the MasterPure™ DNA Purification Kit (Epicentre® an Illumine® company) according to the protocol of manufacturers. Ten-fold serial dilutions of extract DNA 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). The copy number of DNA was calculated by using modified CFC agar plates. A decimal dilution of each strain culture was prepared in sterile solution. The dilutions were inoculated on modified CFC agar plates. The plates were incubated at 22°C or 17°C for 24 h. One colony equals to one copy. All experiments were performed in duplicate.

3.7 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 detection probability trials.

4 RESULTS

4.1 Isolation of *P. phosphoreum*

After 100 µL dilution (10^{-1}) matrix of redfish fillets containing *P. phosphoreum* was spread on modified Long and Hammer's medium containing 1% (w/v) NaCl agar (LH medium), and incubated at 17°C for 2 days, 6 clones were inoculated into medium and kept at 17°C overnight. The DNA of these 6 clones were extracted and analysed by real-time PCR. Results suggested that colonies of 1, 2, 3, 5 and 6 were positive reaction (Figure 2). One of these clones is streaked on LH medium, and the pure culture was obtained (Figure 3).

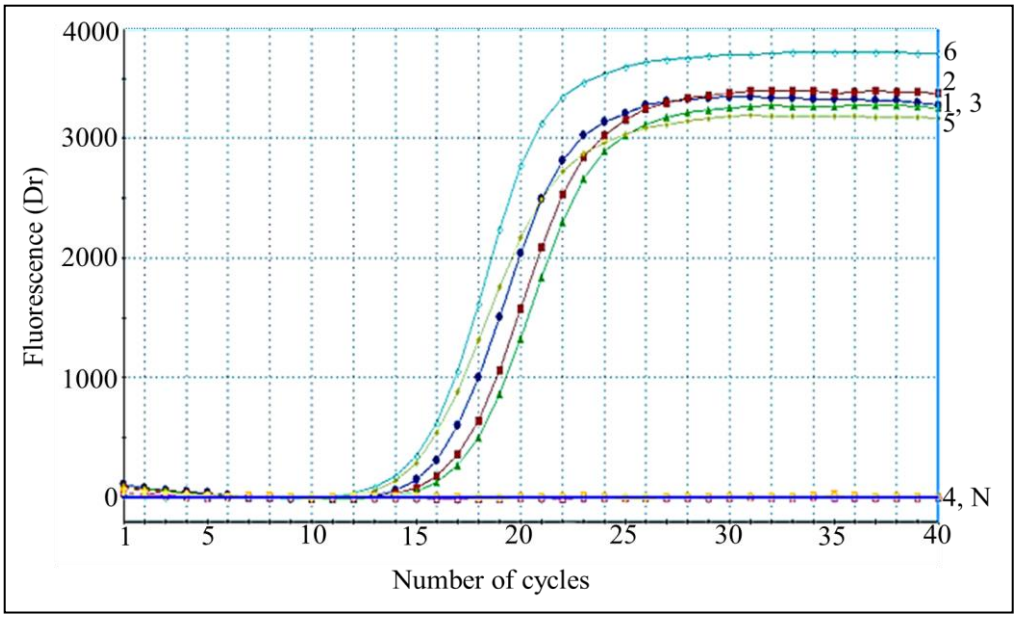

Figure 2: Real-time PCR result for the colonies of *P. phosphoreum* isolated from redfish
1-6: colonies 1-6; N: negative control.

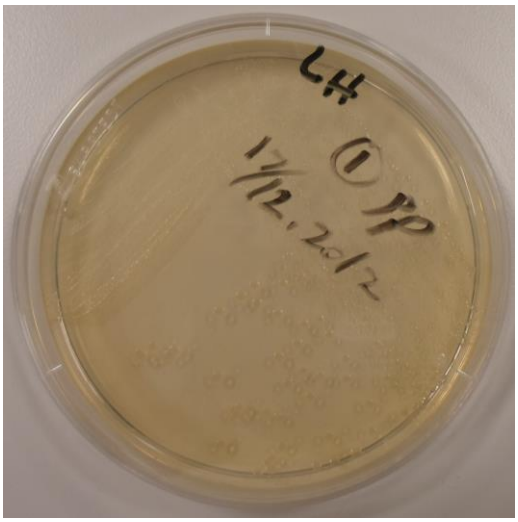

Figure 3: Pure culture of *P. phosphoreum* isolated from redfish.

4.2 Detection results of *Pseudomonas* spp. and *P. phosphoreum* in redfish

The DNA of 1 mL ten-fold diluted redfish matrix was extracted, and analysed by real-time PCR. Research results showed that there were amplified products of *P. phosphoreum* and *Pseudomonas* spp. in all the 3 DNA samples from redfish matrix (Figure 4A and Figure 4B). These results indicated that the spoiling process had initiated in the redfish fillets.

At the same time, 100 μL ten-fold diluted redfish matrix was plated on modified CFC agar (Stanbridge and Board 1994) and modified LH agar (Van Spreekens 1974) and incubated at 22°C and 17°C for 5 days respectively. Research results indicated that there were transparent bacteria colonies on the modified LH agar and pink bacteria colonies on the modified CFC agar (Figure 5A and Figure 5B). The Log number in the modified LH agar is 2.90 CFU/g, and the Log number in the modified is 1.90 CFU/g. It also indicated that there were *P. phosphoreum* and *Pseudomonas* spp. in the redfish fillets.

In order to obtain redfish free from *P. phosphoreum* and *Pseudomonas* spp., the ultraviolet ray (UV) was used to degrade the DNA of *P. phosphoreum* and *Pseudomonas* spp. in the redfish. UV irradiated the redfish matrix for 10 min, 20 min and 30 min respectively. The DNA of them was extracted using the MasterPure™ DNA Purification Kit, and analysed by Real-time PCR. Study results suggested that UV could degrade the DNA of *P. phosphoreum* and *Pseudomonas* spp. partly (Figure 6A and 6B), and the degradation rate is proportional to the irradiation time. However, the matrix treated by UV could not be recollected completely, it would affect the accuracy of real-time PCR. So it was concluded that the redfish fillets matrix could not be used for artificial inoculation.

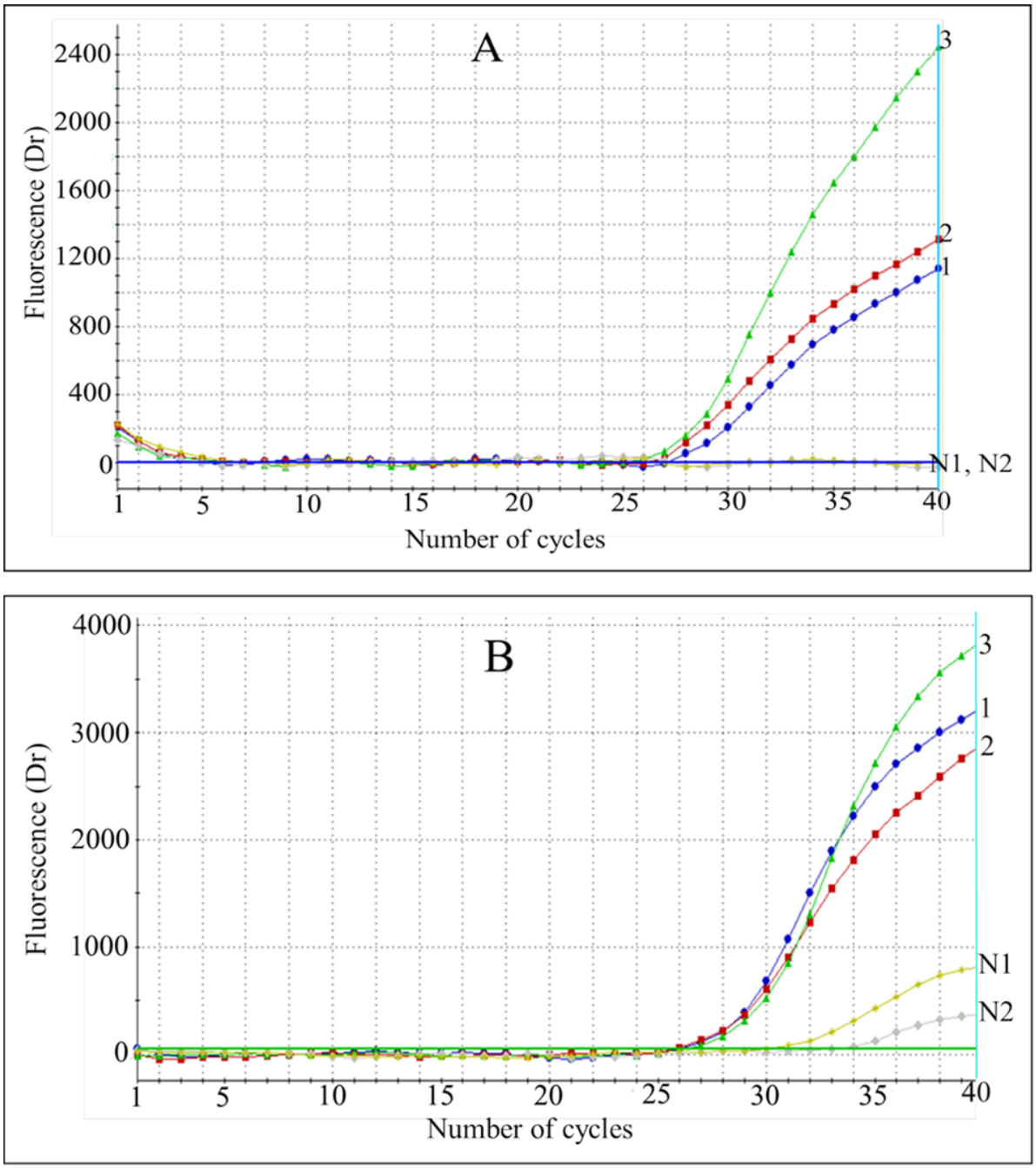

Figure 4: Real-time PCR results of *P. phosphoreum* and *Pseudomonas* spp. in redfish
A: *P. phosphoreum*; B: *Pseudomonas* spp.; 1-3: DNA samples of redfish 1-3; N1-N2: Negative control 1and 2.

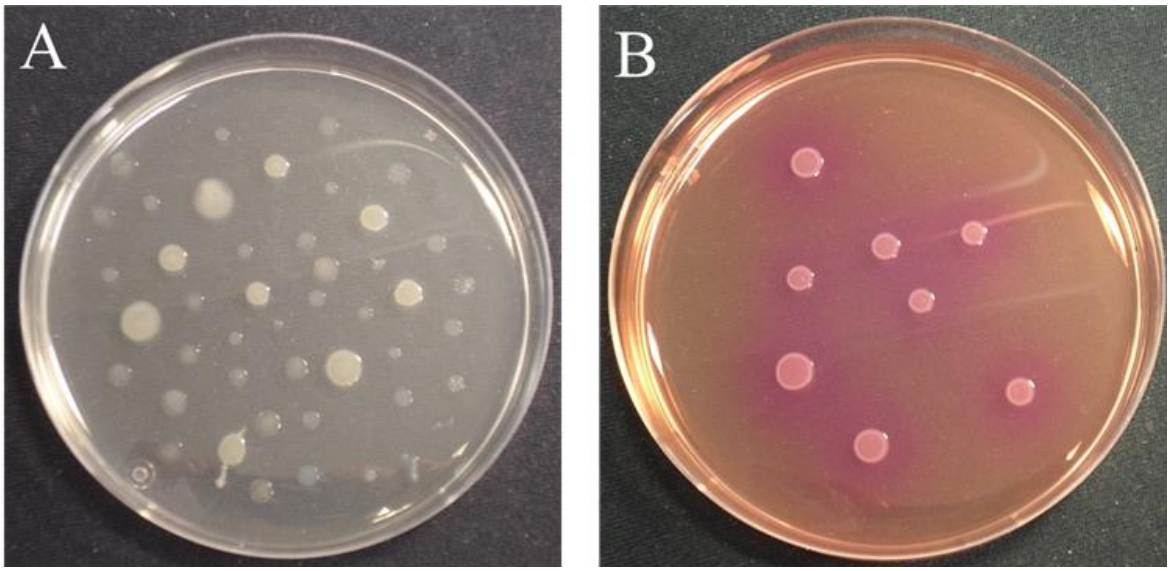

Figure 5: Culture results of redfish mixture on modified LH and modified CFC agar
A: Modified LH agar; B: Modified CFC agar

4.3 Detection results of *Pseudomonas* spp. and *P. phosphoreum* in salmon

There were no colonies on the plates of modified Long and Hammer's medium containing 1% (w/v) NaCl agar and modified cephaloridine fucidin cetrimide (CFC) agar spread with salmon matrix and incubated for 5 days (Figure 7A and 7B). In real-time PCR, the DNA of redfish containing *Pseudomonas* spp. and *P. phosphoreum* was used as positive control. The real-time PCR results of *P. phosphoreum* and *Pseudomonas* spp. for the DNA extracted from salmon matrix also showed negative reaction (Figure 8A and 8B). Therefore, it was concluded that the salmon fillets could be used for artificial inoculation.

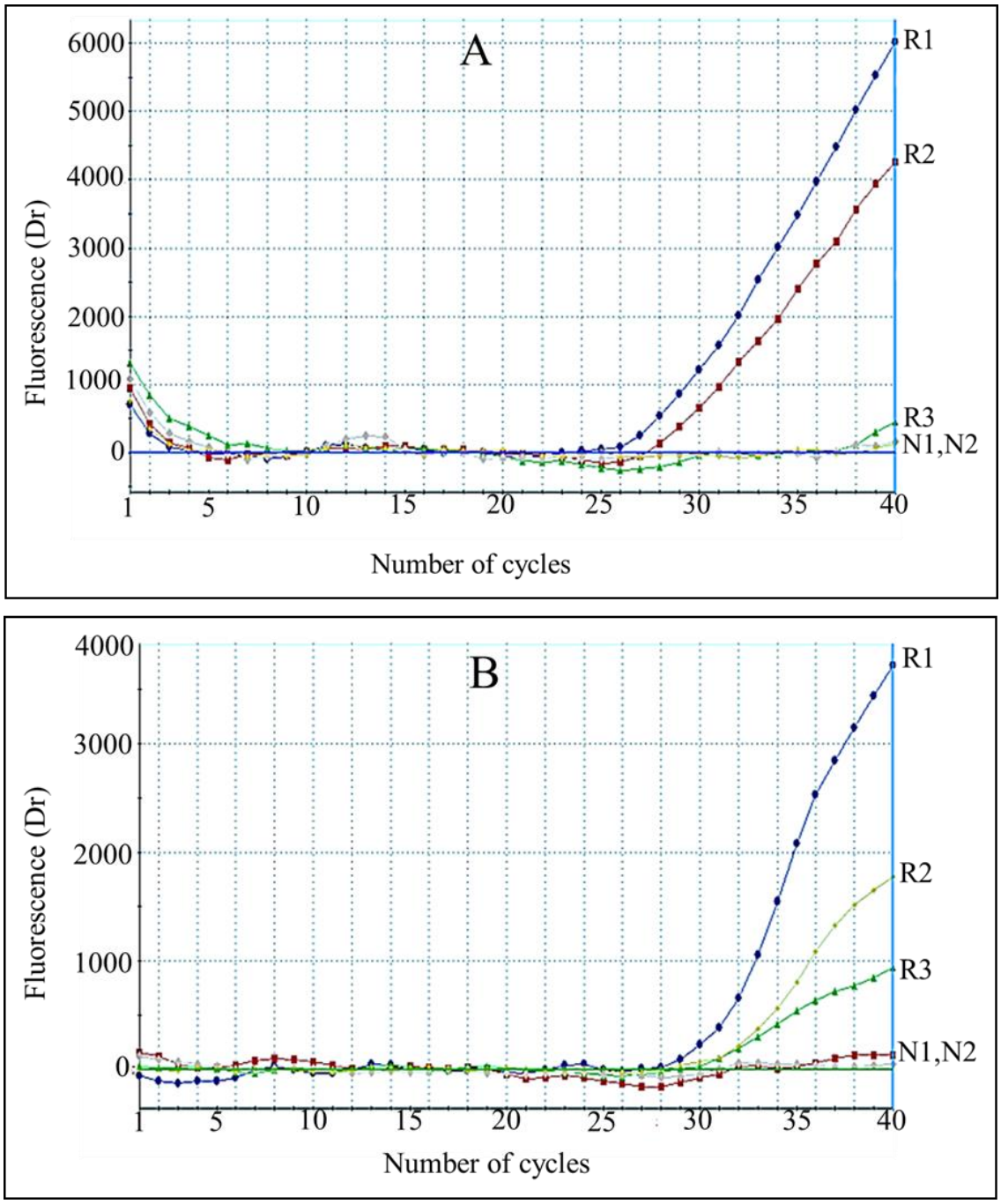

Figure 6: Real-time PCR results of *P. phosphoreum* and *Pseudomonas* spp. in redfish treated by UV A: *P. phosphoreum*; B: *Pseudomonas* spp.; R1: Redfish matrix treated by UV for 10 min; R2: Redfish matrix treated by UV for 20 min; R3: Redfish matrix treated by UV for 30 min; N1, N2: Negative control 1and 2

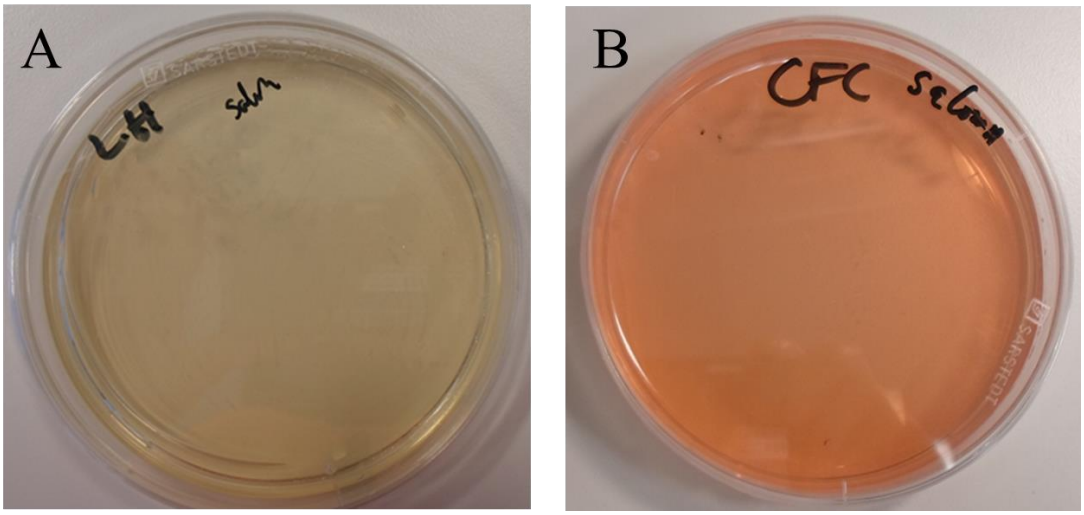

Figure 7: Culture results of salmon mixture on modified LH and modified CFC agar
A: Modified LH agar; B: Modified CFC agar.

4.4 Results of plate counts for the *P. phosphoreum* and *P. fluorescens* spiked salmon matrix

The overnight cultured *P. fluorescens* and *P. phosphoreum* were diluted in ten-fold dilution series. 100 μL sample of 10^{-4}~10^{-8} dilution was sprayed on modified CFC agar (Stanbridge and Board 1994) and modified LH agar (Van Spreekens 1974) and incubated at 22°C and 17°C for 2 days respectively The precise number of CFU in the dilutions was counted. The count result was shown in Table 1 and Table 2. For the *P. phosphoreum*, only the plates of 10^{-5} dilution could be counted. For the *P. fluorescens*, only the plates of 10^{-6} dilution could be counted. The detection limit of plate counts method for *P. phosphoreum* and *P. fluorescens* is that the concentration of cell must be at least $2.50x10^2$ CFU/g.

Table 1: Detection results of *P. phosphoreum* spiked in salmon matrix by plate counts.

Dilutions	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
CFU/ plate	>250	76±2.83	<25	<25	<25
CFU/g	More than count	7.6×10^2±28.28	Less than count	Less than count	Less than count
Log_{10} CFU/g	More than count	2.88±0.02	Less than count	Less than count	Less than count
Detection limit (Log_{10} CFU/g)	≥2.40				

Numerical values (mean ± st. dev.) obtained from triplicate assays.

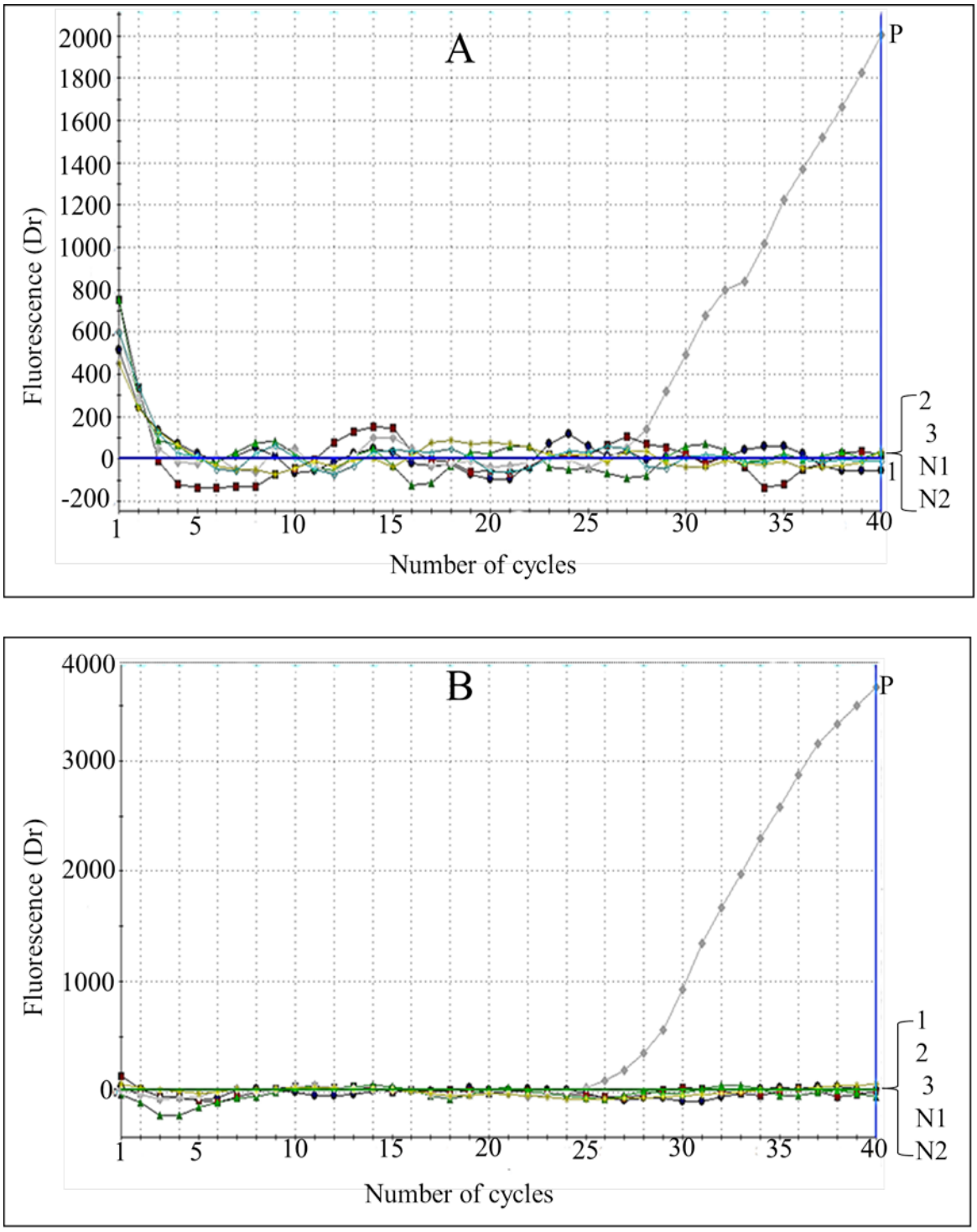

Figure 8: Real-time PCR results of *P. phosphoreum* and *Pseudomonas* spp. in salmon
A: *P. phosphoreum*; B: *Pseudomonas* spp.; 1-3: DNA samples of salmon 1-3; N1-N2: negative control 1and 2; P: positive control.

Table 2: Detection results of *P. fluorescens* spiked in salmon matrix by plate counts.

Dilutions	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
CFU/ plate	>250	>250	38±2.83	<25	<25
CFU/g	More than count	More than count	3.8×10^{2}±28.28	Less than count	Less than count
Log_{10} CFU/g	More than count	More than count	2.58±0.03	Less than count	Less than count
Detection limit (Log_{10} CFU/g)	≥2.40				

Numerical values (mean ± st. dev.) obtained from triplicate assays.

4.5 Comparison on amplification efficiency of PCR mixture from different suppliers

To obtain the better results of real-time PCR for *P. phosphoreum* and *P. fluorescens* in the salmon matrix, the in-house prepared mix, AB, and Brill II Teq polymerase mixture were tested. The ten-fold dilution series of standard were detected firstly. Amplified results were indicated in Figure 9-11. Results indicated that the amplified efficiency of three types of polymerase mixture for standard dilution series is sensitive, and the correlation coefficient (R^2) reflected a strong linear relationship. However, the negative control samples also showed amplification for in-house prepared mix. So the AB and Brill II Teq polymerase mixture were used to amplify the *P. phosphoreum* spiked in salmon matrix. Real-time PCR results (Figure 12) showed that the amplification efficiency of AB Teq polymerase mixture was better than Brill II Teq polymerase mixture. Then the amplification efficiency of AB Teq polymerase mixture for *P. fluorescens* spiked in salmon matrix was tested. Results (Figure 13) suggested that the dilution of 10^{-6} also could be detected by real-time PCR using AB Teq polymerase mixture. Since the AB Teq polymerase mixture showed the best performance, it was selected to detect *P. phosphoreum* and *P. fluorescens* in all inoculated salmon samples.

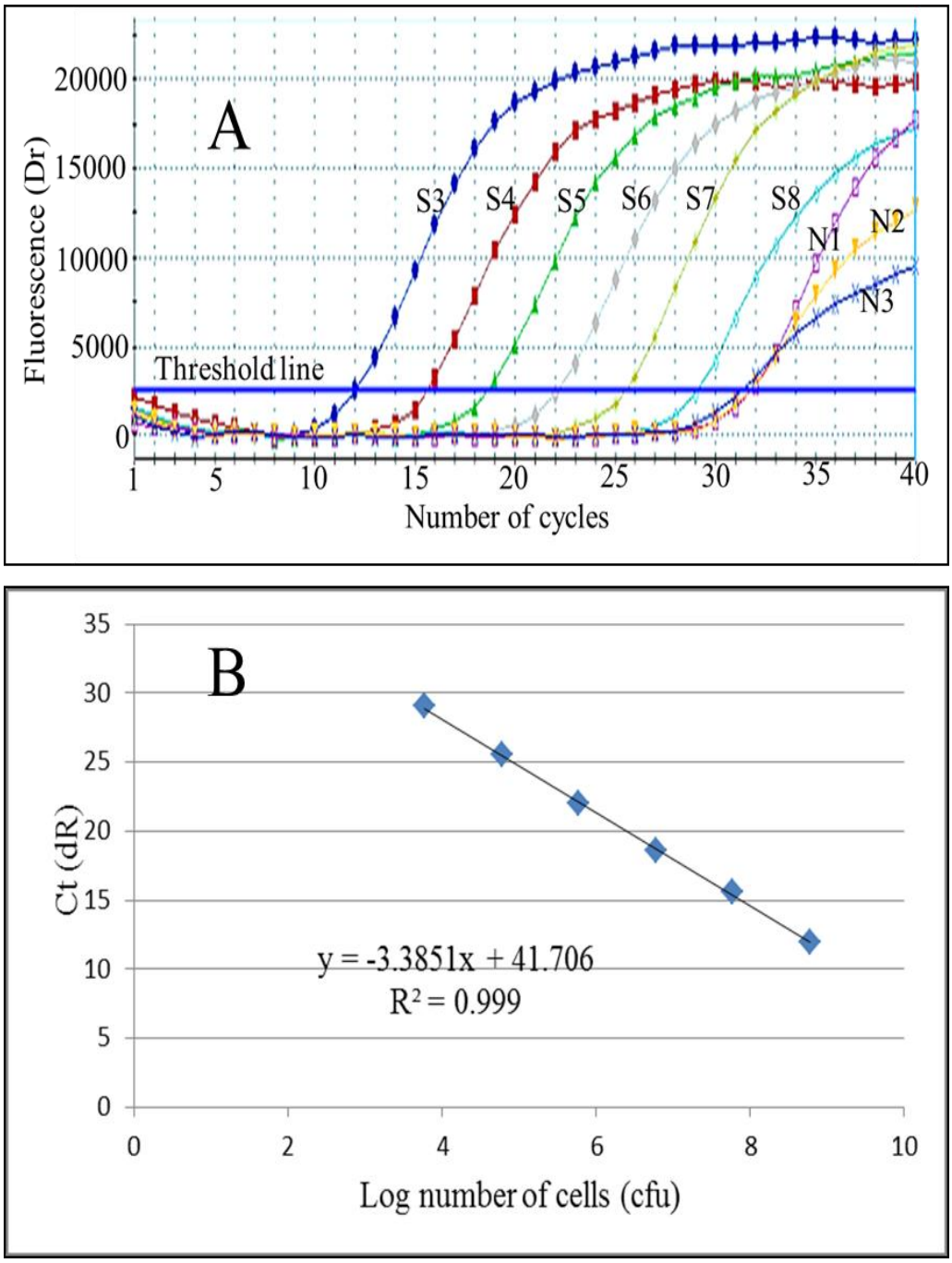

Figure 9: Real-time PCR results for the standard of *P. phosphoreum* using the Marster Mixture A: Amplyfied results; B: Standard curve; S3-S8: DNA from standard dilutions of $6x10^8$ CFU/g, $6x10^7$ CFU /g, $6x10^6$ CFU /g, $6x10^5$ CFU /g, $6x10^4$ CFU /g, $6x10^3$ CFU /g; N1-N3: negative control 1-3.

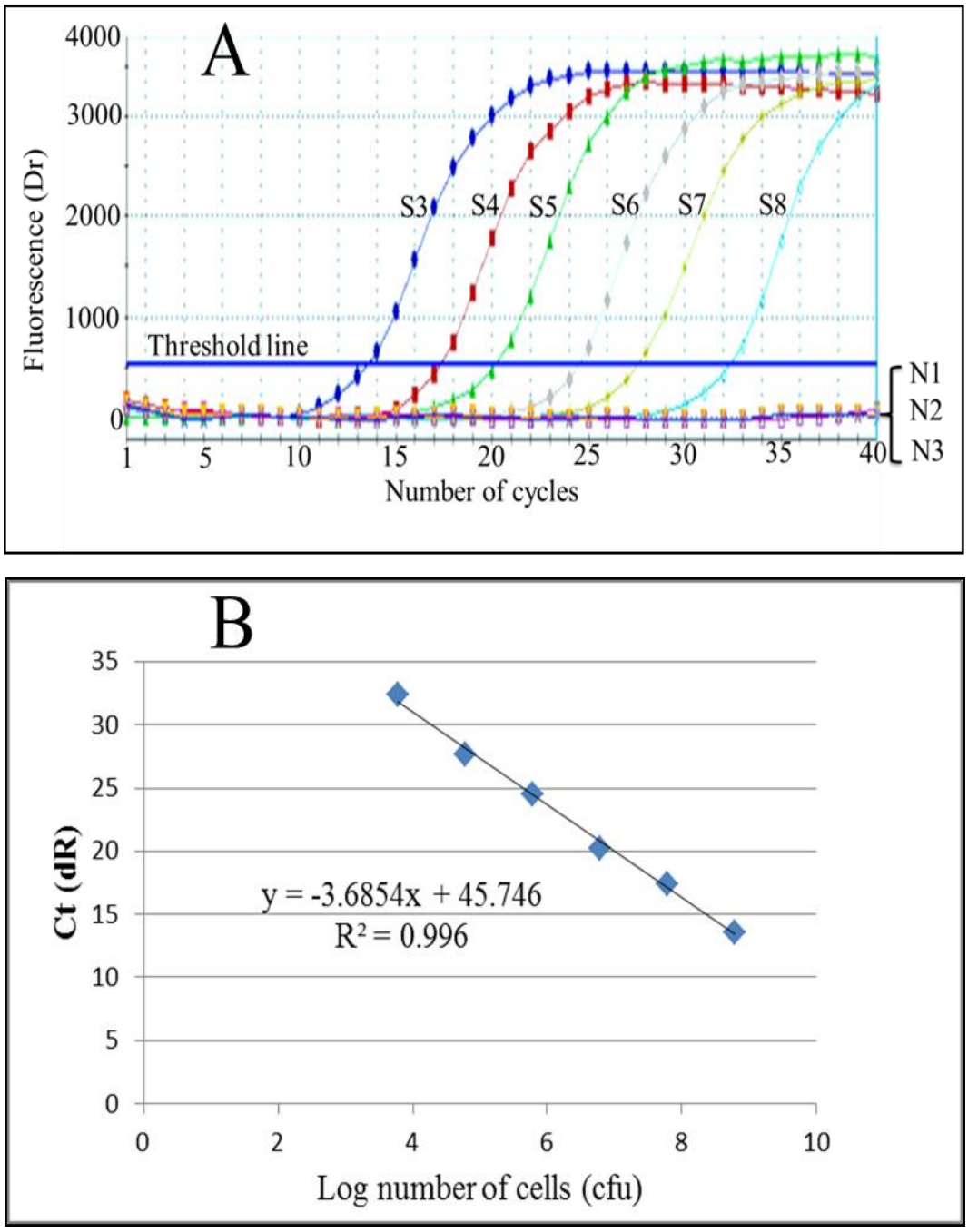

Figure 10: Real-time PCR results for the standard of *P. phosphoreum* using the AB Mixture A: Amplyfied results; B: Standard curve; S3-S8: DNA from standard dilutions of $6x10^8$ CFU/g, $6x10^7$ CFU /g, $6x10^6$ CFU /g, $6x10^5$ CFU /g, $6x10^4$ CFU /g, $6x10^3$ CFU/g; N1-N3: negative control 1-3.

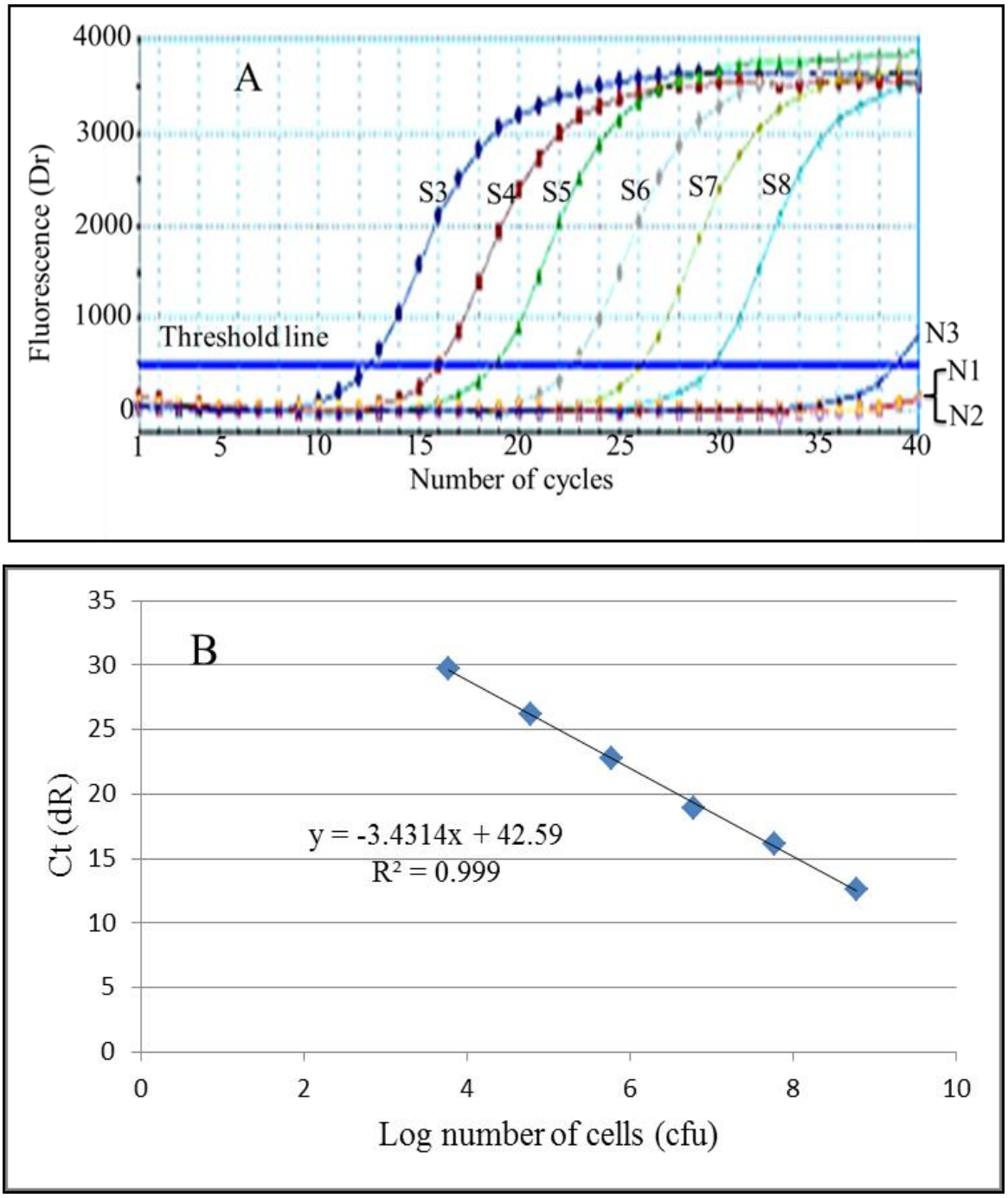

Figure 11: Real-time PCR results for the standard of *P. phosphoreum* using the Brill II Mixture A: Amplyfied results; B: Standard curve; S3-S8: DNA from standard dilutions of $6x10^8$ CFU/g, $6x10^7$ CFU /g, $6x10^6$ CFU /g, $6x10^5$ CFU /g, $6x10^4$ CFU /g, $6x10^3$ CFU /g; N1-N3: negative control 1-3.

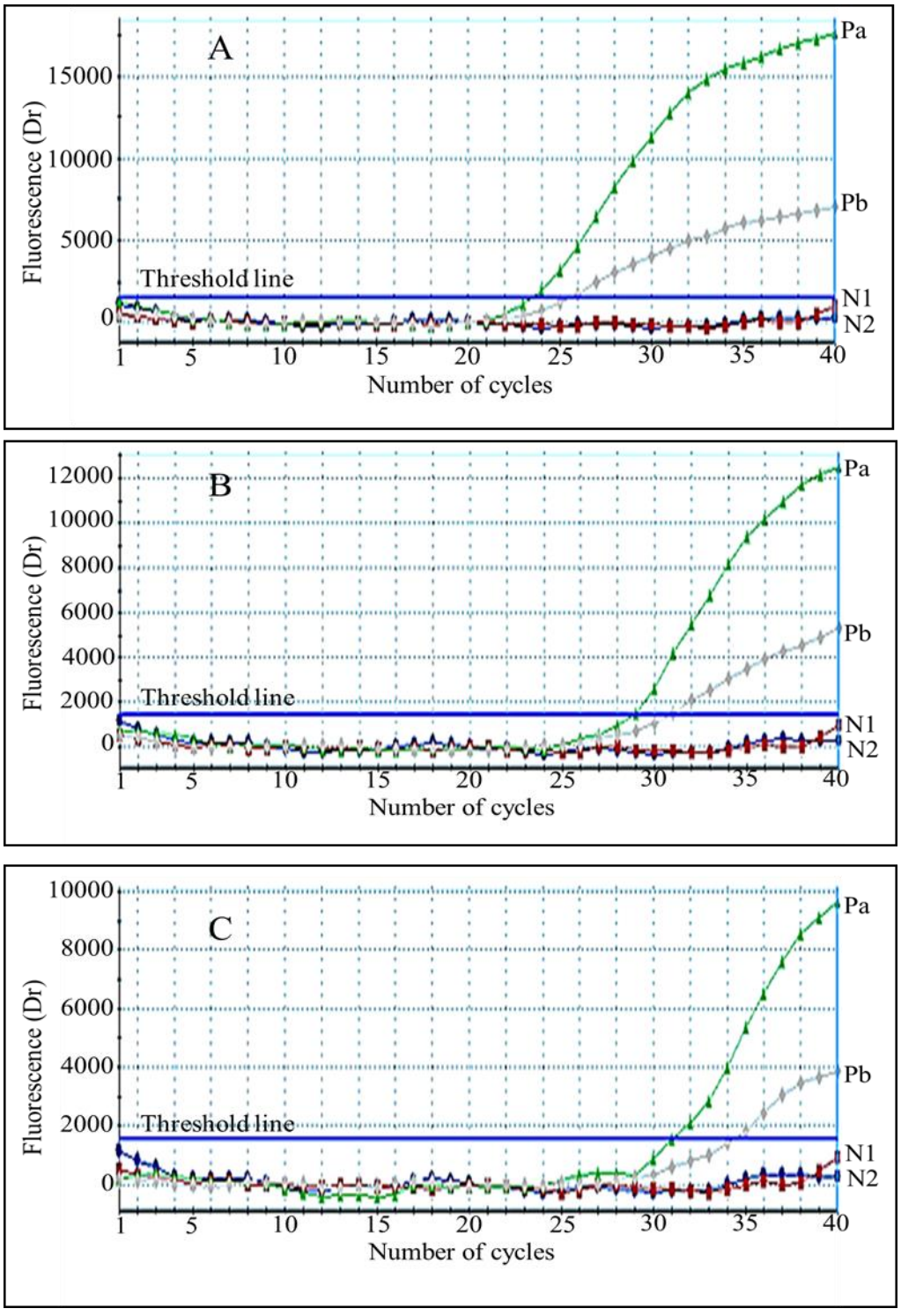

Figure 12: Real-time PCR results for the *P. phosphoreum* spiked in salmon matrix using the AB Mixture and Brill II Mixture A-C: The dilutions of *P. fluorescens* in salmon matrix from 10^{-3}~10^{-5}; Pa: Real-time PCR using the AB Mixture; Pb: Real-time PCR using the Brill II Mixture; N1-N2: negative control 1-2.

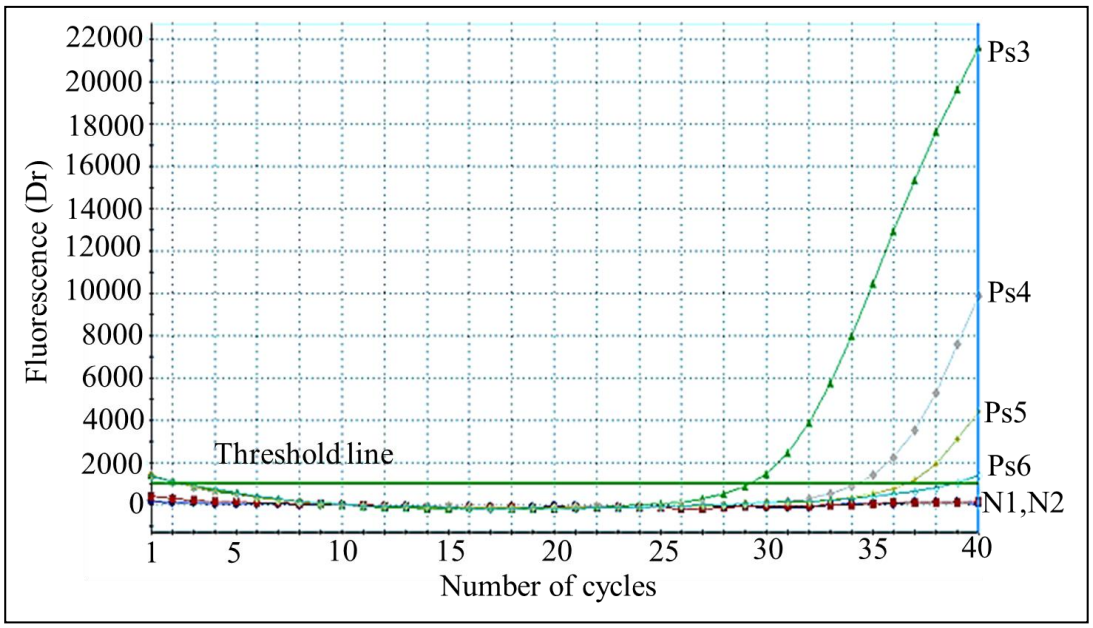

Figure 13: Real-time PCR results for the *P. fluorescens* spiked in salmon matrix using the AB Mixture Ps3-Ps6: The dilutions of *P. fluorescens* from 10^{-3}~10^{-6}; N1-N2: negative control 1-2.

4.6 Detection probability of real-time PCR for *P. phosphoreum* in salmon

4.6.1 Detection probability of P. phosphoreum for the DNA extracted by MasterPure™ DNA Purification Kit

The DNA of salmon fillets matrix spiked with *P. phosphoreum* was extracted by the MasterPure™ DNA Purification Kit according to the recommendations of manufacturers. At the same time, the DNA of salmon fillets matrix without spiked *P. phosphoreum* was isolated for real-time PCR amplification control. The DNA samples were analysed by real-time PCR using AB Teq polymerase mixture. The standard curve was constructed by analysed the standard DNA of series dilutions (ranging from $6x10^3$ CFU/g to $6x10^8$ CFU/g). Real-time PCR results showed that the spiked samples and standard samples were amplified efficiently (Figure 14). The correlation coefficient (R^2=0.996) reflected a strong linear relationship (Figure 14B). This standard curve was used for quantification of the number of *P. phosphoreum* spiked in salmon fillets matrix.

The Ct values of salmon fillets matrix spiked with series dilution of *P. phosphoreum* were listed in Table 3. For the samples of 10^{-7} dilution, the fluorescence value of 5 samples (total 11) was under threshold line (2000), and there is no Ct value for them. As the samples of 10^{-8} dilution, the fluorescence of 6 samples was under threshold line, so there is no Ct value them. The Log numbers for samples of 10^{-2}~10^{-6} dilution were calculated used Ct values according the regression equation (Table 3).

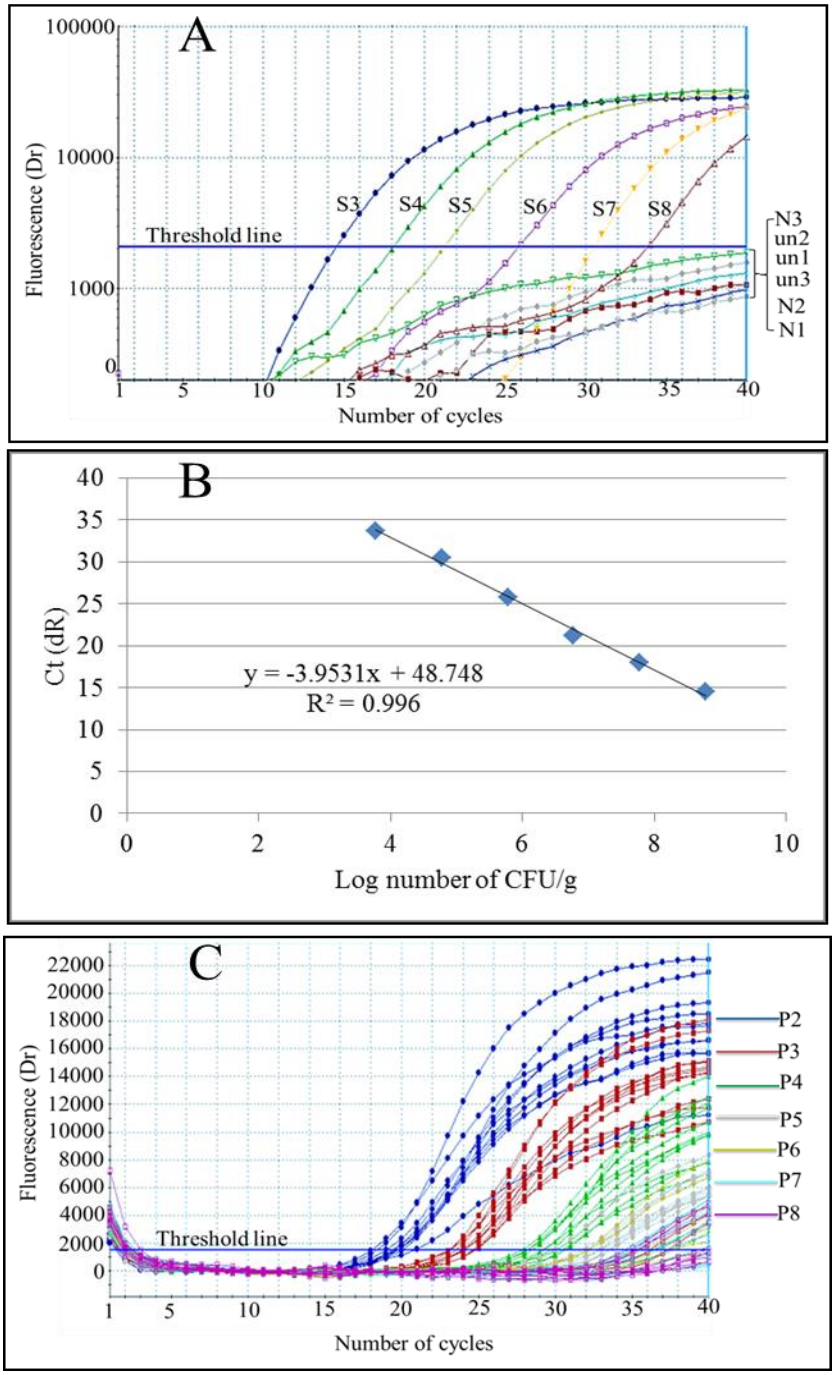

Figure 14: Real-time PCR results for the DNA of *P. phosphoreum* spiked in salmon matrix extracted by MasterPure™ DNA Purification Kit A: Amplification result for the DNA from standard dilutions of *P. phosphoreum*; B: Standard curve of *P. phosphoreum*; C: Amplification result for the DNA from *P. phosphoreum* spiked in salmon matrix; S3-S8: DNA from standard dilutions of $6x10^8$ CFU/g, $6x10^7$ CFU /g, $6x10^6$ CFU /g, $6x10^5$ CFU /g, $6x10^4$ CFU /g, $6x10^3$ CFU /g; N1-N3: Negative control 1-3; un1-un3: DNA from un-spiked salmon matrix 1-3; P2-P8: Dilutions of *P. phosphoreum* culture from 10^{-2}~10^{-8}

Table 3: Real-time PCR results for the DNA extracted from *P. phosphoreum* spiked in salmon matrix by MasterPure™ DNA Purification Kit.

Dilutions	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Ct value	20.15±0.78	24.51±0.62	29.79±0.94	33.27±1.01	36.66±2.11	-	-
PCR (Log CFU/g)	7.25±0.20	6.14±0.16	4.80±0.24	3.92±0.26	3.07±0.53	-	-
Cultivation (CFU/g)	7.40±0.20	6.29±0.16	4.96±0.24	4.08±0.26	3.22±0.53	-	-

-: No value.

4.6.2 Detection probability of P. phosphoreum for the DNA extracted by hotshot DNA extraction Kit

The DNA of salmon fillets matrix spiked with *P. phosphoreum* was extracted by the hotshot DNA extraction Kit according to the recommendations of manufacturers. At the same time, the DNA of salmon fillets matrix without spiked *P. phosphoreum* was isolated for real-time PCR amplification control. The DNA samples were analysed by real-time PCR using AB Teq polymerase mixture. The standard curve was constructed by analysed the standard DNA of series dilutions (ranging from $6x10^3$ CFU/g to $6x10^8$ CFU/g). Real-time PCR results showed that the standard samples were amplified efficiently (Figure 15A). The correlation coefficient (R^2=0.988) reflected a good linear relationship (Figure 15B). This standard curve was used for quantification of the number of *P. phosphoreum* spiked in salmon fillets matrix.

The Ct values of salmon fillets matrix spiked with series dilution of *P. phosphoreum* were listed in Table 4. Results (Figure 15C and Table 4) indicated that the amplification efficiency of real-time PCR for the DNA samples extracted by hotshot DNA extraction Kit is low. Only the samples of 10^{-2} and 10^{-3} dilution have Ct value, the fluorescence value of all other samples was under threshold line. The Log numbers for samples of 10^{-2} and 10^{-3} dilution were calculated used Ct values according the regression equation (Table 4).

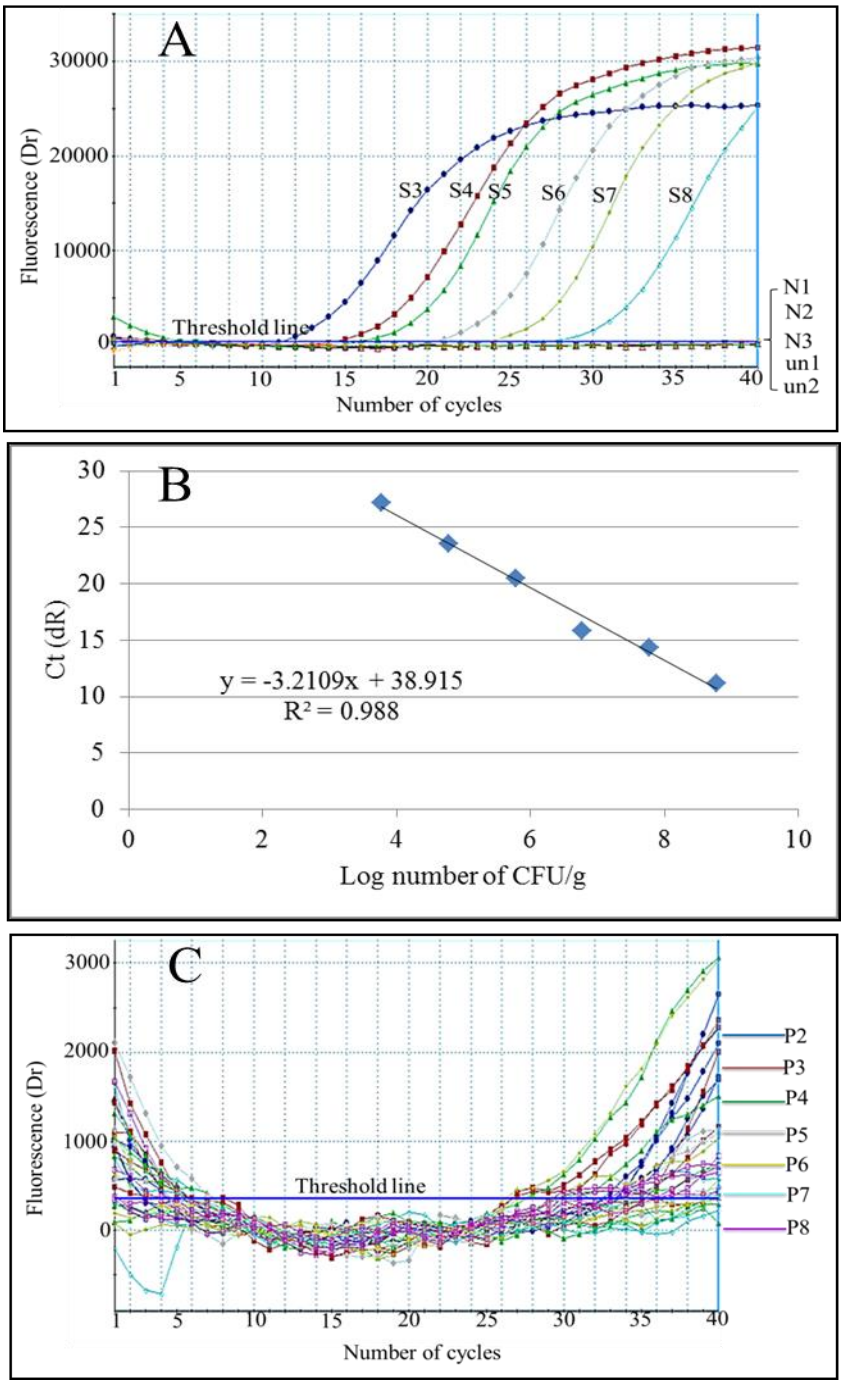

Figure 15: Real-time PCR results for the DNA of *P. phosphoreum* spiked in salmon matrix extracted by hotshot DNA extraction Kit. A: Amplification result for the DNA from standard dilutions of *P. phosphoreum*; B: Standard curve of *P. phosphoreum*; C: Amplification result for the DNA from *P. phosphoreum* spiked in salmon matrix; S3-S8: DNA from standard dilutions of $6x10^8$ CFU/g, $6x10^7$ CFU /g, $6x10^6$ CFU /g, $6x10^5$ CFU /g, $6x10^4$ CFU /g, $6x10^3$ CFU /g; N1-N3: Negative control 1-3; un1-un2: DNA from un-spiked salmon matrix 1-2; P2-P8: Dilutions of *P. phosphoreum* culture from 10^{-2}~10^{-8}

Table 4: Real-time PCR results for the DNA extracted from *P. phosphoreum* spiked in salmon matrix by Hotshort DNA Purification Kit.

Dilutions	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Ct value	33.46±0.94	31.67±4.10	-	-	-	-	-
PCR (Log CFU/g)	1.70±0.29	2.26±1.28	-	-	-	-	-
Cultivation (Log CFU/g)	2.18±0.29	2.73±1.28	-	-	-	-	-

-: No value.

4.7 Detection probability of real-time PCR for *P. fluorescens* in salmon

4.7.1 Detection probability of P. fluorescens for the DNA extracted by MasterPure™ DNA Purification Kit

The DNA of salmon fillets matrix spiked with *P. fluorescens* was extracted by the MasterPure™ DNA Purification Kit according to the recommendations of manufacturers. At the same time, the DNA of salmon fillets matrix without spiked *P. fluorescens* was isolated for real-time PCR amplification control. The DNA samples were analysed by real-time PCR using AB Teq polymerase mixture. The standard curve was constructed by analysed the standard DNA of series dilutions (ranging from $2x10^4$ CFU/g to $2x10^9$ CFU/g). Real-time PCR results showed that the spiked samples and standard samples were amplified efficiently (Figure 16). The correlation coefficient (R^2=0.998) reflected a strong linear relationship (Figure 16B). This standard curve was used for quantification of the number of *P. fluorescens* spiked in salmon fillets matrix.

The Ct values of salmon fillets matrix spiked with series dilution of *P. fluorescens* were listed in Table 5. For the samples of 10^{-7} dilution, the fluorescence value of 5 samples (total 12) was under threshold line (2070), and there is no Ct value for them. As the samples of 10^{-8} dilution, the fluorescence of 7 samples was under threshold line, so there is no Ct value for them. The Log numbers for samples of 10^{-2}~10^{-6} dilution were calculated used Ct values according the regression equation (Table 5).

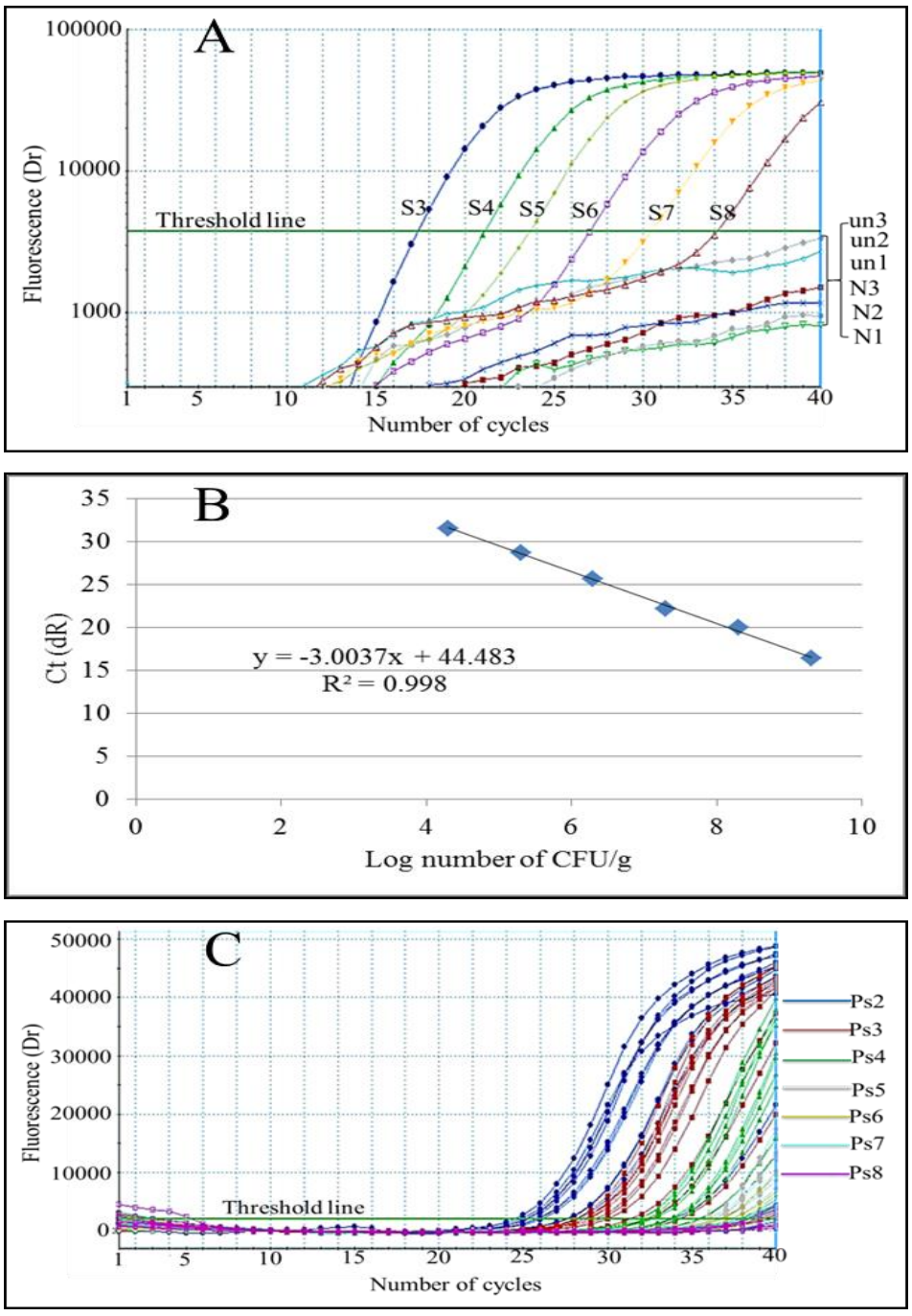

Figure 16: Real-time PCR results for the DNA of *P. fluorescens* spiked in salmon matrix extracted by MasterPure™ DNA Purification Kit A: Amplification result for the DNA from standard dilutions of *P. fluorescens*; B: Standard curve of *P. fluorescens*; C: Amplification result for the DNA from *P. fluorescens* spiked in salmon matrix; S3-S8: DNA from standard dilutions of 6x108 CFU/g, 6x107 CFU /g, 6x106 CFU /g, 6x105 CFU /g, 6x104 CFU /g, 6x103 CFU /g; N1-N3: Negative control 1-3; un1-un3: DNA from un-spiked salmon matrix 1-3; Ps2-Ps8: Dilutions of *P. fluorescens* culture from 10^{-2}~10^{-8}

Table 5: Real-time PCR results for the DNA extracted from *P. fluorescens* spiked in salmon matrix by MasterPure™ DNA Purification Kit.

Dilutions	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Ct value	26.02±1.20	27.01±0.94	33.23±1.10	36.55±0.72	38.37±0.93	-	-
PCR (Log CFU/g)	6.15±0.40	5.10±0.31	3.66±0.43	2.69±0.17	2.03±0.31	-	-
Cultivation (Log CFU/g)	6.30±0.40	5.24±0.31	3.82±0.43	2.85±0.17	2.19±0.31	-	-

-: No value.

4.7.2 Detection probability of P. fluorescens for the DNA extracted by hotshot DNA extraction Kit

The DNA of salmon fillets matrix spiked with *P. fluorescens* was extracted by the hotshot DNA extraction Kit according to the recommendations of manufacturers. At the same time, the DNA of salmon fillets matrix without spiked *P. fluorescens* was isolated for real-time PCR amplification control. The DNA samples were analysed by real-time PCR using AB Teq polymerase mixture. The standard curve was constructed by analysed the standard DNA of series dilutions (ranging from $2x10^4$ CFU/g to $2x10^9$ CFU/g). Real-time PCR results showed that the standard samples were amplified efficiently (Figure 17A). The correlation coefficient (R^2=0.981) reflected a better linear relationship (Figure 17B).

Real-time results (Figure 17C) demonstrated that the amplification efficiency of *P. fluorescens* for the DNA samples extracted by hotshot DNA extraction Kit is very low. The fluorescence value for all samples is under threshold line. Only a few samples have Ct value, and there is no repeatability among parallel samples. So the average Ct value could not be calculated, and the Log number for spiked samples also could not be obtained.

4.8 Comparison the detection efficiency of different methods

The quantification result for *P. phosphoreum* spiked in salmon fillets matrix obtained from different methods was showed in Table 6. These data illustrated that detection limit of real-time PCR using DNA prepared by MasterPure™ DNA Purification Kit is the lowest. The samples of 10^{-6} dilution could also be detected effectively by it, while real-time PCR using DNA prepared by Hotshort DNA Purification Kit only could detected the samples of 10^{-3} dilution. The detection limit of plate count is a little higher than that of real-time PCR using DNA prepared by MasterPure™ DNA Purification Kit. The samples of 10^{-5} dilution could be detected by plate count. Result in Table 6 also indicated that real-time PCR using DNA prepared by MasterPure™ DNA Purification Kit could provide the number of *P. phosphoreum* spiked in salmon matrix from 10^{-2} dilution to 10^{-6} dilution directly, while plate count could only provide the number of 10^{-5} dilution directly. For the sample of same dilution, the number obtained from real-time PCR using DNA prepared by MasterPure™ DNA Purification Kit is higher than other two type methods.

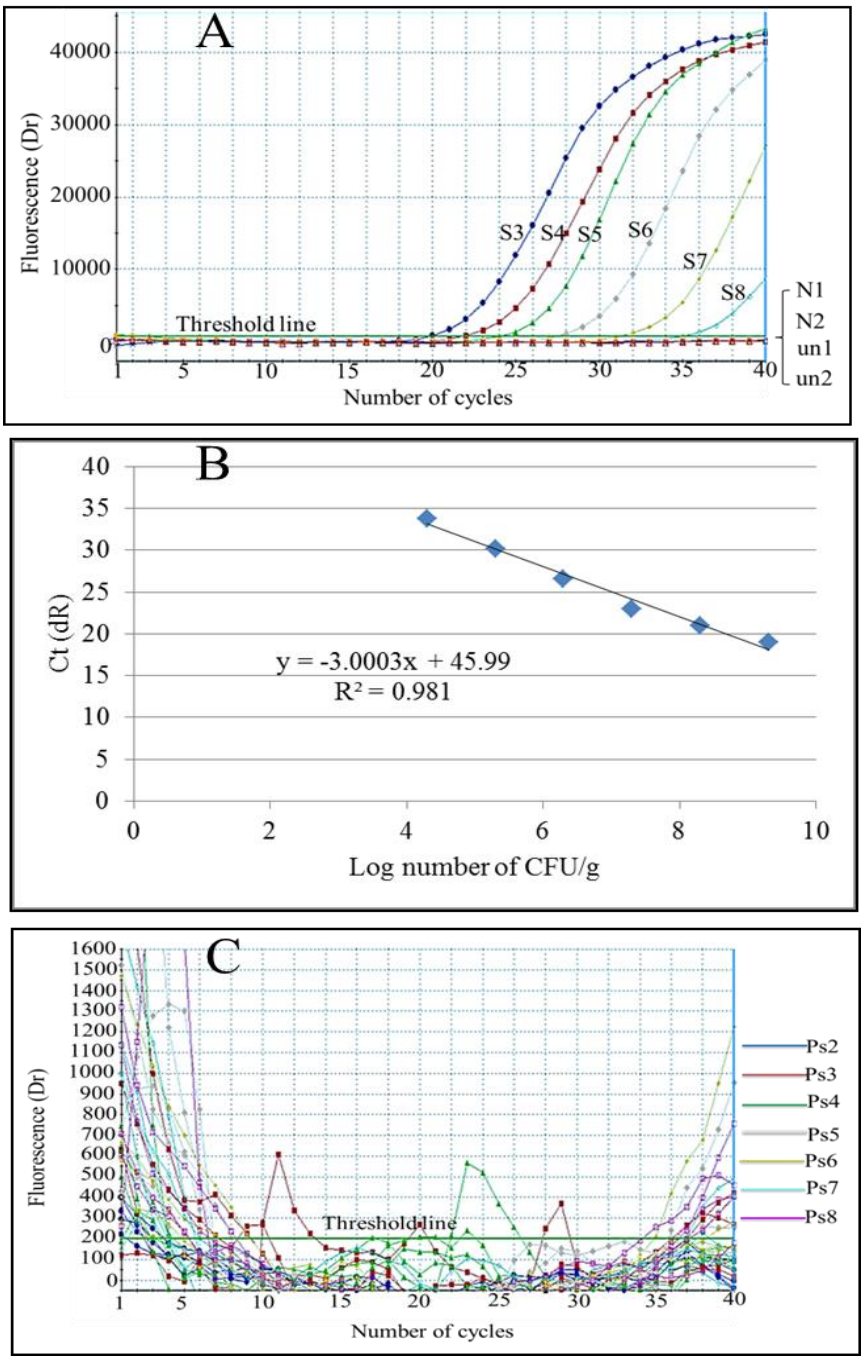

Figure 17: Real-time PCR results for the DNA of *P. fluorescens* spiked in salmon matrix extracted by hotshot DNA extraction Kit A: Amplification result for the DNA from standard dilutions of *P. fluorescens*; B: Standard curve of *P. fluorescens*; C: Amplification result for the DNA from *P. fluorescens* spiked in salmon matrix; S3-S8: DNA from standard dilutions of 6x108 CFU/g, 6x107 CFU /g, 6x106 CFU /g, 6x105 CFU /g, 6x104 CFU /g, 6x103 CFU /g; N1-N2: Negative control 1-2; un1-un2: DNA from un-spiked salmon matrix 1-2; Ps2-Ps8: Dilutions of *P. fluorescens* culture from 10^{-2}~10^{-8}

Table 6: Detected results of *P. phosphoreum* spiked in salmon by different methods.

Sample	dilution	Log10 number (CFU/g)		
		Real-time PCR[A]*	Real-time PCR[B]*	Plate counts
1	10^{-2}	7.40±0.20	2.18±0.29	-
2	10^{-3}	6.29±0.16	2.73±1.28	-
3	10^{-4}	4.96±0.24	-	-
4	10^{-5}	4.08±0.26	-	2.88±0.02
5	10^{-6}	3.22±0.53	-	-
6	10^{-7}	-	-	-
7	10^{-8}	-	-	-

*A: DNA was extracted by MasterPure™ DNA Purification Kit; B: DNA was extracted by Hotshort DNA Purification Kit; -: No value.

The quantification result for *P. fluorescens* spiked in salmon fillets matrix obtained from different methods was showed in Table 7. These data illustrated that the detection limit of real-time PCR using DNA prepared by Hotshort DNA Purification Kit is very high, it could not offer the number of *P. fluorescens* spiked in salmon matrix even if for the 10^{-2} dilution. The detection limit of real-time PCR using DNA prepared by MasterPure™ DNA Purification Kit and plate count is almost same. The samples of 10^{-6} dilution could be detected by the two types of method. However, the that real-time PCR using DNA prepared by MasterPure™ DNA Purification Kit could provide the number of *P. fluorescens* spiked in salmon matrix from 10^{-2} dilution to 10^{-6} dilution directly, while plate count could only provide the number of 10^{-6} dilution directly.

Table 7: Detected results of *P. fluorescens* spiked in salmon by different methods.

Sample	dilution	Log_{10} number (CFU)		
		Real-time PCR[A]*	Real-time PCR[B]*	Plate counts
1	10^{-2}	6.30±0.40	-	-
2	10^{-3}	5.24±0.31	-	-
3	10^{-4}	3.82±0.43	-	-
4	10^{-5}	2.85±0.17	-	-
5	10^{-6}	2.19±0.31	-	2.58±0.03
6	10^{-7}	-	-	-
7	10^{-8}	-	-	-

*A: DNA was extracted by MasterPure™ DNA Purification Kit; B: DNA was extracted by Hotshort DNA Purification Kit; -: No value.

5 DISCUSSION

Photobacter. *phosphoreum* is a specific spoilage organism (SSO) of fish (Liston, 1992; Gram *et al.* 1990). Recently a real-time PCR assay for the *P. phosphoreum* pure cultured was developed including a TaqMan® probe in the PCR set-up (Reynisson E, unpublished). But for the naturally contaminated fish, the amplification efficiency and detection limit are often affected by PCR inhibitors such as proteins, fat and DNA of fish (Rådström *et al*. 2003). The PCR effect of inhibitors on different DNA polymerases and their buffer systems is different (Abu Al-Soud *et al.* 1998, Wolffs 2004). In this study, the amplification efficiency of a in-house prepared mix, AB (Applied Biosystems), and Brilliant II Teq polymerase mixture (Agilent) for *P. phosphoreum* spiked in salmon fish were tested. Results indicated that AB Teq polymerase mixture showed higher resistance to inhibitors of salmon fish. Quantification *P. phosphoreum* spiked in salmon fish using real-time PCR with AB Teq polymerase mixture is possible at least in 5 orders of magnitude without further diluting the sample after conventional sample preparation, while the samples must be serially diluted using a conventional plate count method to enable obtain countable colonies on an agar plate. Comparing quantification between the real-time PCR assay and the standard plate count method, an average difference of 1.2 logarithmic units was observed. The difference between cultivation and real-time PCR has also been reported before in other bacteria quantification (Mavrodi *et al*. 2007). This maybe owing to its higher amplification efficiency for the *P. phosphoreum* spiked in salmon matrix, or the ability not only detected the live cells but also died cells. However, it is not possible to quantify *P. phosphorous* from a sample containing complicated microflora using a plating method since no selective culture medium is available. Comparing quantification between real-time PCR assay using DNA prepared by MasterPure™ DNA Purification Kit and the real-time PCR assay using DNA extracted by Hotshort DNA Purification method, the amplification efficiency and sensitivity of the assay using DNA prepared by MasterPure™ DNA Purification Kit is obviously higher. There are more inhibitors in DNA solution prepared by Hotshort DNA purification method since there is no precipitation step for proteins, and there is no wash step of ethanol.

Pseudomonas spp. is also a specific spoilage organism (SSO) of marine fish (Gram and Huss 1996). Quantification of *Pseudomonas* spp. spiked in salmon fish by real-time PCR with AB Teq polymerase mixture was also tested. Compared to the *P. phosphoreum* assay, the resistance of AB Teq polymerase mixture to inhibitors is weak. This may be affected by inhibitors from *P. phosphoreum* spiked in the salmon at the same time. Here the real-time PCR assay and the standard plate count method showedcomparable quantification results. Quantification the *P. phosphoreum* spiked in salmon fish using real-time PCR with AB Teq polymerase mixture is possible at least 5 orders of magnitude without further diluting the sample after conventional sample preparation, while the samples must be serially diluted used a conventional plate count method to enable obtain countable colonies on an agar plate. On another hand, the real-time PCR only need 5 hours to quantity these spoilage bacteria from sample to results while conventional plate count method need at least 5 days or are not readily available as for *P. phosphoreum*. Comparison of quantification between the real-time PCR assay using DNA prepared by MasterPure™ DNA Purification Kit and Hotshort DNA purification method, amplification efficiency using DNA prepared by MasterPure™ DNA Purification Kit is also higher than that of PCR assay using DNA extracted by Hotshort DNA purification method.

The real-time PCR using DNA extracted by MasterPure™ DNA Purification Kit was a quick and efficiency method to quantity the *P. phosphorous* and *Pseudomonas* spp. in salmon fish. It might be used to check the fish quality when they are imported to the processed factory in China.

6 CONCLUSION

Research results indicated that the real-time PCR using DNA extracted by MasterPure™ DNA Purification Kit was a quick and efficiency method to quantity *P. P. phosphoreum* in salmon fish. It was able to determine the Log_{10} number CFU/g of *P. phosphoreum* in salmon fillets matrix from 3.22 to 7.40, while there is no plate count method was available to provide measuring *P. phosphoreum* in naturally contaminated salmon fillet. The detection time for real-time PCR using DNA extracted by MasterPure™ DNA Purification Kit was only 5 hours. At last, the real-time PCR using DNA prepared by MasterPure™ DNA Purification Kit could provide the log_{10} number of *P. P. phosphoreum* in salmon matrix from 3.22 to 7.40 directly.

As for the *Pseudomonas* spp. assay, the detection time for real-time PCR using DNA extracted by MasterPure™ DNA Purification Kit was only 5 h, while that for plate count was 3 days. At last, the real-time PCR using DNA prepared by MasterPure™ DNA Purification Kit could provide the Log number of *Pseudomonas* spp. in salmon matrix from 2.19 to 6.30 directly, while plate count could only provide the number from 2.4 to 3.4 directly.

In conclusion, the rapid assay for quantification of important group of spoilage organisms in salmon, *P. P. phosphoreum* and *Pseudomonas* spp could be of great value for the quality monitoring of products.

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APPENDIX

1. Modified Long and Hammer's medium (LH)

Reagent	Amount for 1000 mL
Proteose peptone	20.0 g
Gelatin	40.0 g
K_2HPO4	1.0 g
NaCl	10.0 g
Agar	15.0 g
Distilled water	990.0 mL
Ammonium Ferric (III) citrate (added prior to pouring)	0.25g

The pH is adjusted to 7.0 prior to autoclaving (121 ℃, 15 min.). Ammonium Ferric (III) citrate is prepared as a sterile solution (0.25 g dissolved into 10 mL distilled water, and 1 mL added to 100 mL medium prior to pouring).

2. Modified cephaloridine fucidin cetrimide (CFC) agar

Reagent	Amount for 1000 mL
Gelatin peptone	16.0 g
Casein hydrolysate	10.0 g
Potassium sulphate	10.0 g
Magnesium chloride	1.4 g
Agar	11.0 g
L-acginine HCl	10.0 g
1% phenol red solution	1.0 mL
Glyclrol	5.0 mL
Distilled water	1000 mL
Cetrimide (SR0103)	10.0 mg
Fucidin (SR0103)	10.0 mg
Cephalosporin (SR0103)	50.0 mg

To Prepare the Agar Base: Suspend 24.2 g of the agar base, in 500 mL of distilled water. Add 5 mL of glycerol. Bring to the boil to dissolve completely, sterilise by autoclaving at 121°C for 15 minutes. Allow the medium to cool to 50°C.

To Prepare Pseudomonas CFC Agar: To 500 mL of agar base cooled to 50°C add the contents of 1 vial of Pseudomonas CFC Supplement (SR0103) rehydrated as directed. Mix well and pour into sterile Petri dishes. pH 7.25 at 25°C.

3. BD Difco™ Marine Broth 2216

Reagent	Amount for 1000 mL
Peptone	5.0 g
Yeast Extract	1.0 g
Ferric Citrate	0.1 g
Sodium Chloride	19.45 g
Magnesium Chloride	8.8 g
Sodium Sulfate	3.24 g
Calcium Chloride	1.8 g
Potassium Chloride	0.55 g
Sodium Bicarbonate	0.16 g
Potassium Bromide	80 mg
Strontium Chloride	34 mg
Boric Acid	22 mg
Sodium Silicate	4 mg
Sodium Fluoride	2.4 mg
Ammonium Nitrate	1.6 mg
Disodium Phosphate	8 mg
Distilled water	1000 mL

Suspend 37.4 g of the Difco Marine Broth 2216 powder in 1 L of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121ºC for 15 minutes.

4. BD™ Tryptic Soy Broth (TSB)

Reagent	Amount for 1000 mL
Bacto™ Tryptone (Pancreatic Digest of Casein)	17.0 g
Bacto Soytone (Peptic Digest of Soybean Meal)	3.0 g
Glucose (=Dextrose)	2.5 g
Sodium Chloride	5.0 g
Dipotassium Hydrogen Phosphate	2.5 g
Distilled water	1000 mL

Suspend 30.0 g of the Tryptic Soy Broth (TSB) powder in 1 L of purified water. Mix thoroughly. Warm slightly to completely dissolve the powder. Autoclave at 121ºC for 15 minutes. The pH of this broth will be 7.3 ± 0.2. Adjusted and/or supplemented as required to meet performance criteria.

5. Buffers for HotSHOT genomic DNA preparation

Alkaline Lysis Reagent		
Reagent	Final Conc.	Amount for 200 mL
NaOH	25 mmol/L	200 mg
EDTA	0.2 mmol/L	14.88 mg

Neutralization Buffer		
Reagent	Final Conc.	Amount for 200 mL
Tris-HCl	40 mmol/L	1.3 g

Add ddH_2O to a final volume of 200 mL. pH of Alkaline Lysis Reagent will be 12. pH of Neutralization Buffer will be 5. There is no need to adjust pH for these solutions.