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New method for rapid and sensitive quantification of sulphide-producing bacteria in fish from arctic and temperate waters

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Abstract

The offensive, fishy, rotten H_2S-off-odours in spoiled, aerobically and cold stored fish from arctic and temperate waters are generally caused by sulphide-producing bacteria (SPB), mainly *Shewanella putrefaciens*. In the present work, a new, rapid, simple and accurate method for estimation of the SPB content in fish from these areas is described. The quantification is based on the formation rate of iron sulphide during growth of SPBs incubated at 30 °C in a liquid growth medium containing cysteine, sodium thiosulphate and iron(III)citrate as specific substrates for iron sulphide formation. The iron sulphide turns the medium grey and masks the background fluorescence in the medium when the SPB content in the assay is approximately 10^9 cfu/ml. The fluorescence change could be detected instrumentally and the colour change visually. The method was developed and evaluated in tests with *S. putrefaciens* CCUG 13452 DT as well as naturally occurring SPBs in cod, salmon, wolf fish and coal fish. A linear correlation between the SPB count and detection time was obtained over the entire range from 1 to 10^9 cfu SPB/g, corresponding to detection times 17 and 1 h, respectively. The correlation is described by the equation: log cfu/g fish $= -0.59(\pm 0.17) \times DT + 9.65(\pm 0.09)$, where DT is the detection time in hours. The model was valid for all the tested fish species and all tested naturally occurring SPBs in these species. The regression coefficients (R^2) for cod, coal fish, wolf fish and salmon were 0.99, 0.92, 0.97 and 0.97, respectively. The detection level of the method is 1 SPB per sample tube, corresponding to 16 cfu/g fish. The method could be used to predict the remaining shelf life of the fish for different markets, even when the time–temperature history during storage of the fish is unknown.

Keywords: Rapid methods; *Shewanella putrefaciens*; Sulphide-producing bacteria; Fish; Shelf life prediction; Early warning; Fluorescence

1. Introduction

Spoiled marine fish from arctic and temperate water is characterised by the development of offensive, fishy, rotten H_2S-off-odours and flavours (Gram and Huss, 1996), and most species are sensory rejected within 1–2 weeks of chilled storage. The

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off-odours are mainly related to specific spoilage organisms (SSOs), which are defined as the microbes that have contributed to the spoilage of the food at the point of sensory rejection. The most relevant SSOs in aerobically stored and vacuum-packed marine fish species are sulphide-producing bacteria (SPB), mainly *Shewanella putrefaciens* in cold water species like cod, and *Vibrio* sp. in species from tropical waters (Jørgensen and Huss, 1989; Gram et al., 1987). Some sulphide-producing *Aeromonas* and Enterobacteriaceae spp. may occasionally be present (Gram et al., 1987).

The remaining shelf life of the fish has been found to correlate closely to the SSO content (Jørgensen et al., 1988; Dalgaard et al., 2002). Knowledge of the remaining shelf life is most relevant in both fish trading and fish processing, but prediction of remaining shelf life from the content of SSOs has been little used so far. This is partly due to the time required to quantify SSOs in fish species with existing methods, which can take 24–48 h, and decisions must often be taken earlier. Other tools for shelf life prediction have, however, been developed. One is the Seafood Spoilage predictor (Dalgaard et al., 2002), which calculate the remaining shelf life of the fish from estimated SSO contents based on the time–temperature history of the fish. The method is developed for a number of species, but lack of reliable documentation of the time–temperature history of the fish during catch, processing and distribution is a limiting factor for use. Another is the quality index method (QIM), which is based on sensory observation of a number of quality parameters (Luten and Martinsdottir, 1997). The method is rapid, simple and reliable for a number of species, but requires trained personnel. A new instrumental method based on near-infrared (NIR) technology has been developed recently, but further development is required before it can be used commercially (Nilsen et al., 2002). Thus, more simple and rapid methods for SSO measurement for fish are still desired.

The existing method for quantification of SPB in marine fish is an agar plate method using a complex growth medium supplemented with cysteine, sodium thiosulphate and ferricitrate (Gram et al., 1987). The microbes able to produce sulphide from cysteine and thiosulphate, i.e., *S. putrefaciens* as well as sulphide-producing *Vibrio*, *Aeromonas* and Enterobacterieaceae spp, appear as black colonies after 24–48 h of incubation due to precipitation of iron sulphide (Gram et al., 1987). The detection reaction is thus directly related to the spoilage property of SPBs. This is a benefit, as the risk of detecting false positives and negatives is low. However, the method has the same drawbacks as any other agar plate method: long detection time, relatively high detection level (50–100 cfu/g) and no possibility for early warning in case of high numbers of SPBs.

Other approaches for rapid methods for detection of *S. putrefaciens* have been tried. Huss et al. (1987) suggested a method based on microbial reduction of TMAO to TMA, measured as a change of colour of the redox indicator resazurin, or a change in conductivity. Good correlation between estimated contents and real contents of SPB ($r=0.99$) and relatively rapid detection (20–6 h for 10^0–10^8 SPB/g) was obtained. Fonnesbech et al. (1993) suggested an immunological method using specific antibodies for *S. putrefaciens*, and DiChristina and DeLong (1993) developed a gene probe for *S. putrefaciens*. However, to our knowledge, none of these methods have been made commercially available or reported in scientific studies later.

The objective of the present work has been to develop a rapid, simple and sensitive method for quantification of SPBs in marine fish species that also allow early warning, i.e., samples with high numbers of SPBs are detected earlier than samples with low numbers. Such methods have previously been developed for other bacteria by adding specific substrates for the relevant bacterium or spoilage property to nonselective growth media (Berg and Fiksdal, 1988; Dupont et al., 1996; Dalgaard et al., 1996; Huss et al., 1987; Gram, 1992). During incubation, the turnover of the specific substrate is detected when the target bacterium has grown to a certain number. The initial content of target bacteria could then be calculated based on the time until detection, as the initial numbers of target bacteria are inversely correlated with the detection time. In the present work, it has been investigated whether iron sulphide formation, detected as a decrease in the background fluorescence of the medium, could be used as a specific reaction for quantification of SPBs in marine fish species from arctic and temperate waters.

2. Materials and methods

2.1. Bacterial strains and fish samples

S. putrefaciens CCUG 13452 DT (Culture Collection, University of Göteborg, Sweden) was used as reference strain for SPBs. Naturally occurring SPBs from fresh fish samples of cod (*Gadus morhua*), wolf fish (*Anarhichas lupus*), coal fish (*Pollachius virens*) and salmon (*Salmo salar*) were isolated from iron agar plates. Total cultures of the same fish species were obtained from fish homogenates. The fish was provided from local producers and fish farmers.

To investigate possible interference between SPBs, fish muscle and the other bacteria, *Photobacterium phosphoreum*, *Pseudomonas fluorescens* and total culture of SPB-free cod were used. *P. phosphoreum* CCUG 16288 (Culture Collection, University of Göteborg) was selected because it is a bioluminating and TMA producing SSO in marine fish, but not a SPB. *P. fluorescens* DSMZ 50090 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was selected because it causes fluorescence, and because it is frequently present in marine fish. SPB-free cod was obtained by freezing cod samples within 3 days after catch, and storing it at −20 °C for at least 10 weeks. This treatment killed naturally occurring SPB and a few other subdominant bacteria in cod from the North Atlantic, but did not lower the total viable count significantly (Skjerdal et al., 1999; Skjerdal and Esaiassen, 2003).

2.2. Media

Iron agar (Iron Agar Lyngby, CM 964, Oxoid, Basingstoke, UK) was used as reference method for quantification of SPB and total counts in fish and pure cultures.

In the fluorescence experiments, an iron broth (IB) with the same ingredients as iron agar except for agar, was used. The IB was composed of (g/l); 2% bacto peptone (Difco, Kansas City, USA); 0.3% Lab Lemco powder (Oxoid); 0.3% bacto yeast extract (Difco); 0.03% ferric-citrate (Merck, Darmstadt, Germany); 0.03% sodium thiosulphate (Merck); 0.5% NaCl. After sterilisation at 121 °C for 15 min, sterile-filtered L-cysteine (Sigma, C-7880, St. Louis, MO) was added to 0.04%. Ferric citrate dissolved completely during sterilization. The pH was adjusted to 8.2 with 2N NaOH prior to sterilization. After sterilization and addition of L-cysteine, the pH was 7.4 ± 0.2.

2.3. Sample preparation

Cell cultures of *S. putrefaciens*, *P. phosphoreum* and *P. fluorescens* in IB were prepared in baffled shake flasks (100 ml) with 10–15 ml IB medium, incubated overnight at 20 °C with slight shaking (approximately 5 rpm, amplitude approximately 7 cm). Fourfold dilutions of the cultures, performed in saline peptone water (peptone 0.1%; NaCl, 0.85%, w/v), were used as inocula in the fluorescence assay.

Fish samples were homogenised with saline peptone water using a stomacher bag with filter (Seward Medical, London, UK) for 4 min in a stomacher (Lab Blender 400, Seward Medical). SPB-free fish samples were diluted 10-fold by homogenisation, using 5 g fish sample and 45 ml peptone water. Fish samples with naturally occurring SPB were diluted fourfold, using 10 g of fish and 30 ml saline peptone water, in order to minimise the detection time. The homogenates were used as inocula in the florescence assay, either directly or after dilutions with saline peptone water.

In experiments with SPB-free cod, *S. putrefaciens* was added a level of 0–10% of the total viable count in the muscle.

2.4. Growth experiments—fluorescence assay

Prepared samples (1.5 ml) were added to tubes with IB (4.5 ml). The solution was distributed in two clear cuvettes (4.5 ml), 3 ml sample in each, and the cuvettes incubated at 30 °C. The fluorescence was measured every 30 or 60 min by placing the cuvettes in a laboratory fluorometer (Turner Designs TD-700, Turner Designs Instruments, Sunnyvale, CA, USA), using an excitation filter with 380-nm narrow band pass and an emission filter with a 450-nm narrow band pass. The wavelengths were selected based on a scanning of pure IB and IB containing different levels of *S. putrefaciens* with a spectrometer (Perkin Elmer, Luminescence Spectrometer LS 50B, Nor-

walk, USA). The fluorometer was single-point calibrated with pure IB.

2.5. Validation of the method

The developed method was experimentally validated by testing other typical SSOs in marine fish alone and in combination with SPB, total cultures of native and SPB-free fish samples as described above and mathematically by calculating the bias and accuracy factor (Ross, 1996). The bias and the accuracy factor were calculated as:

$$\text{Bias factor} = 10^{\left(\sum \log(\text{SPB predicted}/\text{SPB observed})/n\right)} \quad (1)$$

$$\text{Accuracy factor} = 10^{\left(\sum |\log(\text{SPB predicted}/\text{SPB observed})|/n\right)} \quad (2)$$

where "SPB predicted" is the predicted number of SPB derived from the fluorescence assay, and "SPB observed" is the number of SPB determined on iron agar. The replicability of the developed model was then tested in experimental studies with new fish samples.

3. Results

3.1. Fluorescence in uninoculated IB

A hypothesis to be tested in this work was whether formation of iron sulphide during growth of SPB could mask background fluorescence in the growth medium. The fluorescence in uninoculated IB was approximately 300–400 fsu (fluorescence units per minute) higher than in distilled water in the used system during incubation at 30 °C for at least 48 h. Addition of fluorescing substrates was therefore not considered necessary.

3.2. Detection of S. putrefaciens, reference strain

S. putrefaciens was cultivated in IB, and the fluorescence measured during the subsequent incubation. The results are shown in Fig. 1A. After inoculation, the fluorescence changes went through three phases. First, the fluorescence remained constant for a period, then it increased 100–200 fsu during approximately 1.5 h before it decreased rapidly to nearly no fluorescence. During the latter period, the solution turned grey and eventually black due to formation of iron sulphide, i.e., the background fluorescence from the medium was masked by the black precipitate. During the fluorescence increase period, the solution remained clear, but the colour changed from yellow to bright yellow. In order to investigate whether this change was related to the iron sulphide formation, a new experiment was performed using a modified IB where the ferricitrate was replaced with ferrous sulphate, i.e., Fe^{3+} was replaced with Fe^{2+}. The results are shown in Fig. 1B. In this experiment, the fluorescence hardly increased, and the colour of the medium remained constant until the solution turned grey and the fluorescence decreased.

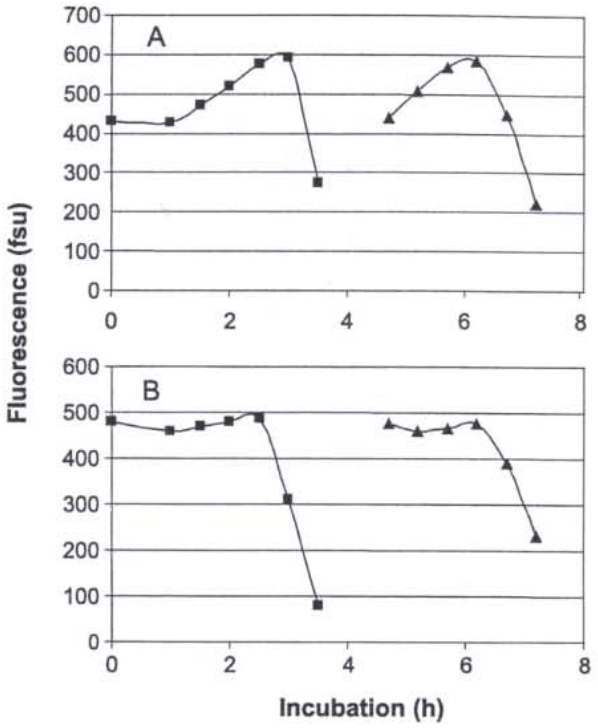

Fig. 1. Development of fluorescence in cultures of *S. putrefaciens* cultivated in standard iron broth (A) and modified iron broth where Fe(III) was replaced with Fe(II) (B). Initial counts of sulphide producing bacteria were (■) 9.7×10^6 cfu/ml and (▲) 6.1×10^5 cfu/ml.

3.3. Varying initial S. putrefaciens counts and incubation conditions

Diluted cultures of *S. putrefaciens* with cell concentrations ranging from 10^2 to 10^6 cfu/ml were inoculated together with homogenates of SPB-free cod in IB, and the fluorescence during incubation measured. All samples showed the same fluorescence pattern as in Fig. 1A, but for each 10-fold dilution, the curves were displaced by approximately 100 min (results not shown). The samples with the highest numbers of *S. putrefaciens* were detected first.

The displacement indicated an approximate doubling time of *S. putrefaciens* in the test system of 30 min. The number of SPBs when the fluorescence started to increase was estimated to be approximately 2×10^8 cfu/ml, and when it decreased to be approximately 2×10^9 cfu/ml. These numbers were confirmed experimentally with measurements of SPB using agar plates (results not shown). The content of *S. putrefaciens* in these experiments ranged from 0% to 10% of the total viable count. The time until change in fluorescence was related to the content of *S. putrefaciens*, not to the total viable count.

The above reported results were all obtained using 30 °C incubation temperature, pH 7.4 and iron concentration of approximately 1.3 mM. Lower inoculation temperatures gave the same fluorescence pattern as at 30 °C, but the detection times and displacement periods between each 10-fold dilution were longer. Incubation at 37 °C gave hardly any growth of *S. putrefaciens*, and the growth medium did not become grey. Higher and lower pH in the IB medium also gave longer detection times. Furthermore, a twofold increase in the iron concentration hardly influenced the detection time (results not shown).

3.4. Interference with fish muscle and other bacteria

The interference of fish muscle and other bacteria on the detection system was investigated. SPB-free cod were stored at 12 or 20 °C for 2 days in order to allow the bacteria in the fish to grow before the homogenised and diluted extracts were analysed. No changes in the fluorescence were observed during incubation even when undiluted extract containing visible "contamination" of fish muscle was used as inoculum (results not shown). Further experiments were performed with pure cultures of *P. phosphoreum* and *P. fluorescens*, which cause bioluminescence and fluorescence, respectively, and with SPB-free cod inoculated with these bacteria. No significant changes in fluorescence were observed in any of these experiments.

3.5. Analyses of naturally occurring SPB in fish

Cod, salmon, coal fish and wolf fish with various levels of SPBs were analysed. All samples showed an increase and a subsequent decrease of fluorescence (results not shown), similar to the patterns showed in Fig. 1A. The displacement between each 10-fold dilution could be calculated to approximately 100–110 min, i.e., close to what was observed for the reference strain *S. putrefaciens*.

In order to investigate whether the naturally occurring SPBs in the fish samples gave the same fluorescence pattern as the reference strain, black and grey colonies were isolated from IA plates and analysed in the assay. In all cases, the fluorescence patterns were as observed in Fig. 1A, and the displacement time between 10-fold dilutions were 100–110 min.

3.6. Correlation between detection time and SPB content

In order to investigate whether there was a correlation between the detection time (DT) and the real content of SPB for each fish species, the SPB content for each sample was quantified using the standard method and plotted against the observed DT (Fig. 2). A linear correlation between measured log SPB/g and DT was observed over the entire range from 1 to 10^9 cfu SPB/g and for all tested fish species. The DT was defined as the elapsed time from the start of the incubation to the time when the maximum fluorescence was reached. The equation (parameters ± 95% confidence limits) of the regression line was:

$$\log \text{cfu/g} = -0.59(\pm 0.17) \times \text{DT} + 9.65\ (\pm 0.09) \quad (3)$$

The average regression coefficient (R^2) for all samples was 0.96, and for each species 0.99 for cod,

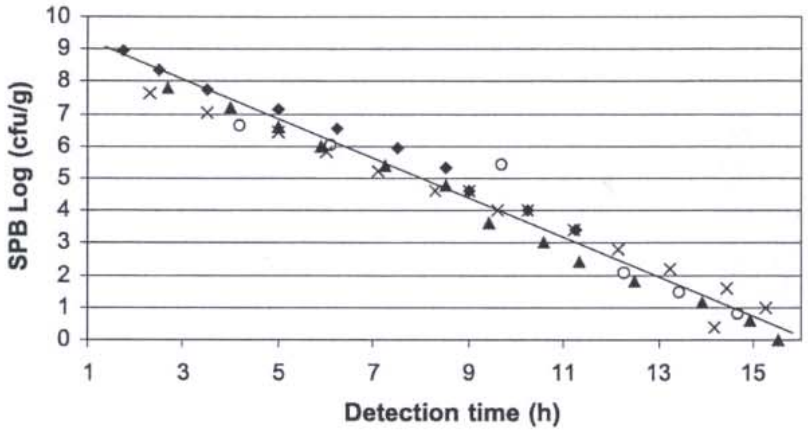

Fig. 2. Linear regression between detection time (hours) derived from fluorescence analysis and the number of sulphide-producing bacteria [log(cfu/g)] in various fish species measured on agar plates with Iron Lyngby agar. The correlation coefficients (R^2) were in cod 0.99 (♦); in coal fish 0.92 (○); in wolf fish 0.99 (▲); in salmon 0.97 (×) and 0.96 in all fish species together.

0.92 for coal fish, 0.99 for wolf fish and 0.97 for salmon.

3.7. Comparison of fluorescence and standard method

The estimated number of SPB based on the DT in the test system was compared to measured numbers using the standard agar plate method. The bias factor (1) and accuracy factor (2), indicating the over- and underprediction and the general deviation from the model, respectively (Ross, 1999), were calculated for each of the fish species and for the fish species all together. The results are shown in Table 1. Good correlation between the developed and reference method, without systematic over- or underprediction, was observed.

In order to investigate the replicability of the method, 21 new samples of cod, coal fish, salmon, wolf fish, haddock and red fish were analysed. The SPB content in the samples varied from 10^1 to 10^6 cfu/g. The bias and accuracy factors calculated from this data set was 0.98 and 1.07, respectively, i.e., similar to the first data set.

Table 1
Calculation of bias and accuracy factor to evaluate the method for rapid detection and enumeration of specific spoilage bacteria in selected marine fish species by comparison with independent data derived from the reference method

Fish species	Bias factor	Accuracy factor
Cod	0.92	1.08
Coal fish	1.04	1.16
Wolf fish	1.12	1.13
Salmon	1.01	1.21
Σ fish species	1.02	1.15

4. Discussion

4.1. The reliability and limitations of the method

A good correlation between the developed and reference method (Gram et al., 1987) was observed both for the reference strain of *S. putrefaciens* and naturally occurring SPBs, either tested as monocultures or as subdominant strains in polycultures in fish. Furthermore, the correlation between detection time and SPB count in the developed method was linear over the entire range from 1 to 10^9 cfu/ml sample, corresponding to 16 to about 10^9cfu/g fish, and the correlation equation was replicable. Thus, formation of iron sulphide during growth in IB, measured as changes in background medium fluorescence, seems to be a suitable detection system for quantification of the SPB count in the fish species tested, allowing early warning.

The media in the developed and reference method have essentially the same composition and specific reaction for detection, and a similar specificity of SPBs in the two methods was therefore expected. However, due to the change from solid to liquid

medium and the higher incubation temperature (30 vs. 22 °C in the reference method), the developed method needs to be evaluated for the risk of over- or underestimation of the SPB count.

The basis for quantification in agar plate methods is colony formation and the appearance of singular colonies after growth on different media. In liquid medium methods, on the other hand, the quantification basis is the turnover rate of a specific substrate in the medium caused by the total culture in the sample. As a result, liquid medium methods are more sensitive than agar plate methods, as the test bacteria are not overgrown by colonies of the dominating bacteria. The reference agar plate method for SPBs (Gram et al., 1987) detects the SPB when they represent 0.1–1% or more of the total count. The SPB content in fresh fish is rarely lower than this. However, the experiments where *S. putrefaciens* were added to SPB-free cod indicated that the sensitivity of the developed method is even lower.

Good correlation between liquid and agar medium methods could be obtained (Ross, 1999) in case only one of the bacteria in the total culture causes specific substrate turnover, or when the different bacteria contributing to the substrate turnover in the liquid medium have similar kinetics. The sulphide production in cold-stored fish from arctic and temperate waters is dominated by *S. putrefaciens* (Gram and Huss, 1996). In the present work, the incubation conditions of the fluorescence assay were therefore selected to give as rapid and correct detection of this bacterium as possible. The most rapid detection was found at incubation temperature 30 °C. This is in accordance with Dalgaard (1993), who reported the optimum temperature for growth of *S. putrefaciens* under aerobic conditions to be 30–32 °C and slightly lower for anaerobic growth. All the naturally occurring SPBs tested in the fluorescence assay gave nearly identical detection times and growth rates as the reference strain of *S. putrefaciens* when incubated at 30 °C, both in monocultures, inoculated SPB-free fish samples and untreated fish samples (results not shown). Similarly, all the tested naturally occurring SPBs grew rapidly at pH 7.4. Lag phases after inoculation of the samples, which would have led to longer detection times and more complicated models for quantification (Baranyi, 2002; Ross, 1999), was not observed for any of the tested SPBs (results not shown). Furthermore, there seemed to be no significant interference between the fish sample and the assay, indicating that fluorescence formation caused by the iron sulphide formation covered the changes in the fish (Manohar, 1971). Thus, the developed method seems to fulfil the criteria for correct quantification of SPBs in the tested fish species.

The developed method has not been tested on tropical fish species, but as the sulphide production in these species is caused by both *Vibrio* spp. and *S. putrefaciens,* and as *Vibrio* spp. have shorter doubling times at 30 °C than *S. putrefaciens* (Easter et al., 1982; Huss, 1995), the method may overestimate the SPB content in these species. *Vibrio* sp. are very rarely found in fish from the Barents and North Atlantic due to the low water temperature, so the presence of *Vibrio* spp. is an indicator of poor hygienic conditions and/or abused storage temperature.

Based on the results, it can be concluded that the assay gives a rapid and reliable estimation of the SPB content in fish from arctic and temperate areas. However, the method could not be recommended for use in tropical fish species without further testing and possible modification.

4.2. Potential use of the method in fish processing and trading

The developed method detects SPB contents above $10^{5.5-6}$ cfu/g fish within 8 h, i.e., a normal shift period in the fish processing industry. The remaining shelf life of ice-stored cod with this content of *S. putrefaciens* is, according to Jørgensen et al. (1988), 6–8 days, and the SPB count at sensory rejection approximately 10^8 cfu/g fish. The HoReCa (hotel, restaurant and catering) segment and a number of other market segments often demand higher quality and freshness standards. However, the developed method seems to be sufficient to allow a fish producer to predict a minimum remaining shelf life, differentiated for different markets if desired, within the same day as the fish is processed.

The method has a theoretical detection level of 16 SPB/g fish, while the corresponding value for the agar plate method is 40–100 SPB/g fish, depending if the sample has been diluted 4-fold or 10-fold during homogenisation. The SPB count in cod and other marine fish species from the Barents is frequently

below 100 cfu/g fish muscle during the 5 first days of iced storage, and during the 3 first days of chilled storage at 4 °C (Skjerdal, unpublished results). When the remaining shelf life of the fish is going to be predicted based on the SPB content, the lowest possible SPB content to be used in case of negative analysis results is the detection level value. A lower detection level therefore contributes to avoid underestimation of the remaining shelf life of very fresh raw material.

4.3. Comparison with other methods, convenience and costs

The developed fluorescence method has many similarities with conductance- and capacitance-based methods, for instance, the one suggested by Huss et al. (1987), as both methods are more rapid and sensitive than the agar plate method, and both allow early warning. However, electrically based detection methods has not been used as much as they were expected to be a decade ago, partly due to the high cost of Malthus analysers and similar instruments for automated recording. In case of SPB, an additional reason may be that the methods developed in the 1980s used TMA production from TMAO as the specific reaction for detection. *P. phosphoreum,* which is an SSO in fish but not an SPB, has a high capacity to produce TMA from TMAO (Dalgaard, 1995) and could probably interfere with the detection system.

The method described in the present work has been developed without automated fluorescence recording, but such instruments are available for approximately 15,000–20,000 Euros (2003 prices). This is less than the costs for a Malthus analyser, but still too much for users having only a few samples per day. An alternative for these users is to use the colour change for detection instead of the fluorescence changes. The method presented in this paper has therefore been further developed (Lorentzen and Tryland, 2003). In this assay, a fish piece is inoculated in IB in a tube without homogenisation, incubated and the colour registered after certain incubation periods. A prototype of this assay has been tested in two fish-producing companies, with good results (Lorentzen and Tryland, 2003). With this simplified method, the SPB content could be analyzed without any other laboratory facility than an incubator for less than 10 Euros per sample.

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