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Seafood quality analysis: Molecular identification of dominant microbiota after ice storage on several general growth media

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ABSTRACT

This study points out the limitations of several general growth media frequently used in seafood research by a systematic identification of the microorganisms on fish samples during ice storage unable to grow on those media. Aerobic psychrotrophic count (APC), replication on various general media and total cultivable microbial community denaturing gradient gel electrophoresis (DGGE) analysis revealed that many potential spoilage microorganisms were overlooked. Those microorganisms overlooked by using only one single growth medium were identified by partial 16S rRNA gene and *gyrB* gene sequencing. Members of the genera *Shewanella*, *Vibrio*, *Aliivibrio*, *Photobacterium*, *Pseudoalteromonas* and *Psychrobacter*, including *Photobacterium phosphoreum*, *Shewanella baltica* and *Pseudomonas fluorescens* are unable to grow on PCA. APC analysis also confirmed that on plate count agar (PCA) the enumeration of the microbiota was underestimated. Although Long and Hammer agar (LH) and marine agar (MA) obtained the best quantitative (APC analysis) and qualitative (replication and DGGE analyses) results for fish quality analysis, analysts have to keep in mind that some species were also unable to grow on those media, such as *Pseudomonas fragi* and *Acinetobacter* sp.

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1. Introduction

Fresh and lightly preserved fish are very sensitive to spoilage. The main factors limiting shelf life are enzyme and microbial activities. Freshly caught fish are naturally contaminated with a diversity of microbiota which depends among other things on the environment, water temperature, area of catch and handling and processing procedures (Jay, 1986). Fish from cold and temperate waters contain mainly psychrotrophic gram-negative, strict aerobic or facultative anaerobic microorganisms of the genera *Aeromonas*, *Pseudomonas*, *Moraxella/Acinetobacter*, *Shewanella*, *Flavobacterium* or the family Vibrionaceae (Liston, 1980).

Quality control and potential shelf life of fish is currently still often estimated based on the total aerobic psychrotrophic count (APC). Standards, guidelines and specifications as part of purchase agreements of chilled fish quality accept an APC of 10^6 cfu/g for human consumption (Anonymous, 1986). When reaching an APC of 10^7-10^8 cfu/g, spoilage generally becomes organoleptically detectable (Liston, 1980). The reference medium recommended by the International Organisation for Standardisation (ISO) for the psychrotrophic enumeration of microorganisms on food (ISO 17410; International Organisation for Standardisation, 2001), including fish, is plate count agar (PCA) without addition of extra salt or minerals. However, studies have reported that an APC underestimation of 1 $\log_{10}$ cfu/g on PCA is plausible in comparison with growth media containing sodium chloride (NaCl) such as Long and Hammer agar (LH; Joffraud and Leroi, 2000; Van Spreekens, 1974). Therefore, the Nordic countries recommend the NMKL 184 (Nordic Committee of Food Analysis, 2006) method using Long and Hammer agar (Van Spreekens, 1974) for the enumeration of psychrotrophic microorganisms in seafood.

Research revealed that not the total number of microbiota on fish is responsible for fish spoilage, but rather a small fraction of the microorganisms, the "specific spoilage organisms" or SSOs (Dalgaard, 1995). The microbial activity and especially the formation of volatiles such as trimethylamine (TMA), ammonium and H_2S of SSOs such as *Shewanella* sp., *Photobacterium phosphoreum* and *Pseudomonas* sp. (Dalgaard, 1995; Koutsoumanis and Nychas, 1999; Tryfinopoulou et al., 2002; Vogel et al., 2005), contribute to the off-flavours and taste associated with spoilt seafood. A close relationship between the log number of the SSOs and the shelf life may be expected. When selecting specifically for a known SSO, the use of

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Table 1
The total aerobic psychrotrophic count (APC) (log_{10} cfu/g) on Plate Count Agar (PCA) (mean of duplicate platings) for all 11 fish samples at the time of arrival (T1) and after 7 days or 4 days (ray) of ice storage (T2).

Total aerobic psychrotrophic count (APC) (log_{10} cfu/g) on Plate Count Agar											
Sampling time	Salmon	Whiting	Ray	Pangasius	Cod	Plaice	Sole	Sea bass	Angler fish	Mackerel	Sea bream
T1	2,7	5,5	6,1	4,9	3,6	4,0	5,3	4,5	4,9	4,3	5,8
T2	4,4	6,8	6,6	5,2	4,8	6,0	6,9	6,4	7,2	6,8	6,8

a specific growth medium, such as STAA (Streptomycin-Thallous Acetate-Actidione) agar for *Brochothrix* sp., *Pseudomonas* CFC (Cetrimide Fucidin Cephalosporin) or Iron agar (IA; black colonies) for *Shewanella* sp. and other H_2S-producing bacteria, is more appropriate. Despite, many food manufacturers, government agencies, retailers, distribution quality laboratories and researchers use general media and very often PCA as quality indicator.

In this study, four different media often used in general fish quality control and research, i.e., PCA, MA (marine agar), LH and IA are compared. Since the initial microbiota is fish specific, the biggest microbial variation during ice storage will be acquired by using several different fish species. Ten marine fish species and one brackish water fish were used in this study. These eleven fish samples mimic the high variety of fish species and broad variability in individual characteristics. The aim of this study is to provide a molecular identification of the most important microorganisms present in fish during ice storage that do not grow on some general growth media frequently used, in order to evaluate the usefulness of these media for seafood analysis. Denaturing gradient gel electrophoresis (DGGE) profiles visualising the differences in the dominant microbial community on the different media (by plate swabs), are used to support and illustrate the results from the plating and replication analysis.

2. Materials and methods

2.1. Raw material, preparation and storage

Eleven fresh, gutted fish samples were taken from a supermarket's supply centre. Following fish species were selected: plaice (*Pleuronectes platessa*), common sole (*Solea solea*), European sea bass (*Dicentrarchus labrax*), gilthead sea bream (*Sparus aurata*), salmon (*Salmo salar*), whiting (*Merlangius merlangus*), mackerel (*Scomber scombrus*), pangasius (*Pangasius pangasius*), ray (*Raya* sp.), cod (*Gadus morhua*) and angler fish (*Lophius piscatorius*). Small fish were collected whole, a 200-g piece was taken from the bigger fish (cod, salmon and angler fish). Whiting and pangasius were only available as fillets. Ray, sole and angler fish had their skin removed at purchase. The samples were put in sterile stomacher bags and transported on ice to the lab. Upon arrival at the lab (T1), a piece of 10 g was aseptically excised for microbiological analysis, and the remaining fish was stored in a sterile bag on ice at 0 ± 0.5 °C. A second sample was taken at T2, namely after 7 days or 4 days (ray) of ice storage.

2.2. Cultivation and isolation of microorganisms from the fish samples

A 10-g fish sample was transferred aseptically to a stomacher bag, 90 ml of maximum recovery diluent (MRD, Oxoid) was added and the mixture was homogenised for 2 min. Samples (0.1 ml) of serial dilutions in MRD of the fish homogenates were spread on the four growth media: plate count agar (PCA, Oxoid), marine agar (MA, Difco), Long and Hammer medium (LH; Van Spreekens, 1974) and Lyngby iron agar (IA; Atlas, 2006). MA and LH contain 1% NaCl and either mimic the composition of seawater or are rich in essential compounds for an optimal enumeration of marine microorganisms. Although IA with 0.5% NaCl is specifically used for the enumeration of H_2S-producing microorganisms (black colonies), it is used often for the enumeration of the total microbiota as well (Karl and Meyer, 2007; Kyrana and Lougovois, 2002; Paarup et al., 2002; Tzikas et al., 2007). LH medium was composed of (grams per litre distilled water): proteose pepton (Oxoid) 20, gelatin (Oxoid) 40, dipotassium phosphate (K_2HPO_4) 1, NaCl 10, agar (Oxoid) 15, and ammonium ferric (III) citrate 0.25. IA was composed of (grams per litre distilled water): proteose pepton (Oxoid) 20, agar (Oxoid) 12, NaCl 5, beef extract 3, yeast extract 3, L-cysteine ($C_3H_7O_2$ SN) 0.6, iron (III) citrate ($C_6H_5FeO_7$. $5H_2O$) 0.3, sodium thiosulphate ($Na_2S_2O_3$. $5H_2O$) 0.3. Standard incubation periods and temperatures for the specific media were used, namely 3 days at 21 °C for PCA and MA and 5 days at 15 °C for LH and IA. After incubation all colonies were counted to give the aerobic psychrothropic count (APC). Duplicate plating was performed for every sample. After counting, these duplicates were used for replication analysis (2.3.) and plate swabs for DGGE analysis (2.5.), respectively. A *Student t-test* was used to determine whether the results of total count on the media significantly differed from one another.

2.3. Replication of the cultivated microorganisms on different growth media

One of the duplicates of the enumeration plates of all four media were used for replication on the other fresh media. The dilution used for replication could differ between fish species and depended on the APC of the fish sample and whether separate colonies were present. The number of colonies present on the replicated plates ranged from 9 to 166 colonies (listed between brackets in Table 2). This technique was used to detect which microorganisms were unable to grow on one of the studied media. The replications were made on the other three media using a replicator and velveteen tissues (Fisher Bioblock). The last replica was pressed onto a fresh petri dish of the original medium to exclude false negatives due to insufficient uptake onto the fabric. These replicates were incubated as indicated above.

2.4. Identification of the microbial isolates failing to grow on one or more media

2.4.1. DNA-extraction

After replication, a selection of 121 isolates with different colony morphology at T2 were selected based on their inability to grow on one of the media. These strains were purified and DNA-extraction was performed using a modified Flamm method (Flamm et al., 1984), adjusted with lysostaphine (0.5 mg/µl) and mutanolysine-lysozyme solution (1 U/µl mutanolysine, 2.5 mg/ml lysozyme) which were dissolved in HPLC water and TE-buffer (0.05 M Tris, 0.02 M EDTA, pH 8) respectively and were added to the pellet of pure culture at the start of the DNA-extraction.

2.4.2. Rep-PCR

A $(GTG)_5$ rep-fingerprinting technique was used to cluster the purified isolates based on their fingerprint. The microbial DNA

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Table 2
Percentages of colonies non-growing on the different media (PCA, MA, LH and IA) after replication at both time points (in bold), T1 (sampling at the time of arrival) and T2 (after 7 or 4 days (ray) of ice storage). X/Y: The growth medium Y from which the colonies are replicated onto growth medium X. Between brackets are the number of colonies that have been transferred by replication as mentioned. When replicated from PCA onto LH, MA or IA, the number and media indicated after the asterisk refer to the percentage of colonies not growing on the mentioned salt-containing medium. The average percentage of non-growing colonies from the total pool of fish species is given in the last row.

Fish species	Day of arrival (T1)				After storage on ice (T2)			
	PCA/LH	PCA/MA	PCA/IA	LH-MA-IA/PCA	PCA/LH	PCA/MA	PCA/IA	LH-MA-IA/PCA
Salmon	**33** (21)	**42** (24)	**35** (17)	**0** (7)	**85** (27)	**95** (19)	**19** (57)	**0** (21)
Whiting	**10** (52)	**36** (14)	**13** (32)	**11** (28)	**41** (46)	**43** (37)	**0** (17)	**0** (34)
Ray	**92** (145)	**83** (58)	**68** (34)	**53** (30) *(43 LH & MA)	**94** (50)	**91** (53)	**94** (31)	**0** (23)
Pangasius	**0** (58)	**2** (56)	**0** (48)	**0** (99)	**21** (47)	**38** (29)	**20** (35)	**0** (14)
Cod	**11** (72)	**37** (43)	**24** (21)	**11** (18)	**60** (20)	**94** (213)	**59** (17)	**14** (58) *(3.5 IA)
Plaice	**47** (113)	**62** (129)	**27** (49)	**20** (54) *(10 MA; 2.5 LH; 7.5 IA)	**29** (105)	**35** (86)	**10** (67)	**5** (57) *(5 MA)
Sole	**43** (101)	**48** (105)	**17** (54)	**30** (100) *(7.5 MA; 12 LH; 10.5 IA)	**58** (102)	**56** (114)	**40** (45)	**15** (52) *(15 IA)
Sea bass	**31** (67)	**46** (52)	**24** (38)	**1** (71)	**38** (39)	**38** (42)	**24** (34)	**10** (112)
Angler fish	**11** (18)	**30** (114)	**16** (51)	**52** (44) *(36 MA; 2 LH; 11 IA)	**3** (149)	**9** (166)	**1** (141)	**4** (106) *(4 MA)
Mackerel	**26** (43)	**41** (41)	**15** (26)	**22** (9) *(22 MA & IA)	**13** (76)	**17** (82)	**3** (58)	**11** (44) *(2 MA & LH)
Sea bream	**14** (21)	**27** (15)	**0** (15)	**27** (48) *(27 MA & IA)	**45** (31)	**29** (41)	**24** (29)	**2** (50) *(2 IA)
Average (%)	**29**	**41**	**22**	**21**	**44**	**50**	**27**	**6**

(50 ng/μl) was used as a template in the PCR-reaction containing 1× RedGoldstar buffer (75 mM Tris–HCl; Eurogentec) and a final concentration of 3.4 μM of $(GTG)_5$ primer, 1.5 mM Mg_2Cl (Eurogentec), 1 U RedGoldStar DNA polymerase (Eurogentec) and 0.2 mM of each deoxynucleotide triphosphate (GE Healthcare Europe GmbH) in a total reaction volume of 25 μl. The reaction was performed on a GeneAmp PCR 9700 Thermocycler (Applied Biosystems) using the amplification conditions described by Versalovic et al. (1991). PCR products were size separated in a 1.5% Seakem LE agarose gel (Lonza) in 1× TBE buffer (0.1 M Tris, 0.1 M Boric Acid, 2 mM EDTA) at 120 V for 4 h. The $(GTG)_5$ profiles were visualised under UV light after ethidium bromide staining and a digital image was captured using the G:BOX camera (Syngene). The resulting fingerprints were analysed using the Bionumerics version 6.5 software package (Applied Maths) using the EZ load 100 bp PCR Molecular Ruler (Biorad) as normalisation reference. The similarity between the fingerprints was calculated using the Pearson correlation (1% optimalisation and 1% position tolerance). The fingerprints were grouped according to their similarity by use of the UPGMA (unweighted pair group method with arithmetic averages) algorithm.

2.4.3. Identification of the microbial isolates by sequence analysis

A 1500 bp fragment of the 16S rRNA gene was amplified by PCR using the conserved primers 16F27 and 16R1522 (Brosius et al., 1978). Amplification was performed as follows: 30 cycles at 94 °C for 15 s, 57 °C for 15 s and 72 °C for 30 s followed by an elongation step at 72 °C for 8 min. Further identification was performed by *gyrB* gene sequence analysis. A 1500 bp fragment of the *gyrB* gene was amplified by PCR using the universal primers UP1 and UP2r following the protocol of Yamamoto and Harayama (1995). All PCR products were purified for sequencing with a High Pure PCR product purification kit (Roche) according to manufacturer's protocol and stored at −20 °C. The quality and quantity of the purified PCR products were verified on a 1.5% agarose gel.

Sequencing reactions were performed using the high pure PCR product as template in the PCR-reaction containing a final concentration of 30–50 ng PCR product DNA, 0.2 μM of 16F27 (16S forward primer) or UP1s and UP2rs (*gyrB*; Yamamoto and Harayama, 1995) primer, 4 μl BigDye Terminator v3.1 Cycle (Applied Biosystems) and adding HLPC water to a final volume of 10 μl. After amplification a sodium acetate/ethanol precipitation was performed. Sequencing was performed on an ABI Prism 3100 Genetic Analyser (Applied Biosystems). The partial 16S rDNA sequences were around 700 bp. The identification of phylogenetic neighbours was initially carried out by the BLAST (Altschul et al., 1997) and megaBLAST (Zhang et al., 2000) programmes against the database of type strains with validly published prokaryotic names (Chun et al., 2007). The 50 16S rDNA sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using global alignment algorithm, which was implemented at the EzTaxon server (http://www.eztaxon.org/; Chun et al., 2007). The *gyrB* sequences were assembled with Vector NTI Advance 11 (Invitrogen corp.). A tentative identification was performed by a similarity search against the FASTA websearch (http://www.ebi.ac.uk/tools/fasta33/nucleotide.html). When similarity value was low, phylogenetic trees were constructed by the Neighbour Joining (NJ) method with inclusion of selected type strains, chosen based on the results obtained from the 16S rDNA identification, by using the programme Treecon version 1.3b (Van de Peer and De Wachter, 1994). Cut-off values of 90% were used to tentatively identify the isolates. For this purpose, the sequences were aligned and cut to the same length using Clustal X version 2 (Larkin et al., 2007). The *gyrB* sequences of the type strains were submitted in the EMBL nucleotide sequence database (accession numbers FR668560 to FR668582).

2.5. Differences in the microbial community by DGGE analysis

A complete plate swab was performed using an inoculation loop. The dilution used for the plate swab depended on the APC of the fish sample. The pellets were washed twice with 1x PBS (137 mM NaCl, 2.7 mM KCl, 0.9 mM KH_2PO_4 and 6.4 mM Na_2HPO_2 (pH 7.4)) and stored at −20 °C. A DNA-extraction was performed using the Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. This DNA, diluted in 200 μl elution buffer (QIAGEN) was used for further analysis by denaturant gradient gel electrophoresis (DGGE).

Universal primers were used for amplification of the variable 16S rRNA V3-region. The forward primer UN357f included a 40 base GC clamp at the 5′ end, the reverse primer used was UN518r (Muyzer et al., 1993). The PCR mixture was prepared as follows: each mixture (final volume, 50 μl) contained 1 μl of template DNA, 0.2 μM of each primer, 0.2 μM of each deoxynucleoside triphosphate (GE Healthcare Europe GmbH), 3.5 mM $MgCl_2$, 1 × PCR buffer (Invitrogen), 0.1% T4 gene 32P (Roche) and 1 U of Taq polymerase (Invitrogen). Template DNA was denatured for 5 min at 95 °C. In order to increase the specificity of the amplification and to reduce the formation of spurious byproducts, a "touchdown" PCR was performed as previously described by Muyzer et al. (1993). Additional 12 cycles were carried out at 55 °C, with an extension for each cycle of 1 min at 72 °C. A final extension of 7 min at 72 °C was performed.

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DGGE analysis was performed on the DCode Universal Mutation Detection system (Biorad) as described by Muyzer et al. (1993). Samples were applied to an 8% (w/v) polyacrylamide gel (acrylamide-bisacrylamide, 37.5:1) in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, Invitrogen). Optimal parallel electrophoresis experiments were performed at 60 °C by using gels containing a 40–60% urea-formamide denaturing gradient (100% corresponding to 7 mol/L urea and 40% [w/v] formamide). Electrophoresis was performed at a constant voltage of 45 V for 14 h. After separation, the gels were incubated for 20 min in 1x TAE pH 8 containing 1x Sybr Gold staining (Invitrogen), and gel images were digitised under UV illumination (G:BOX, Syngene). Bands were detected manually and viewed with Bionumerics version 6.5 software (Applied Maths).

3. Results

3.1. Aerobic psychrotrophic counts (APC) on different growth media

Averages for duplicate plate counts on PCA are presented as $\log_{10}$ cfu/g (Table 1). At T1, the APC of the fish samples ranged from 2.7 to 6.1 $\log_{10}$ cfu/g. At this point, APC was highest for the ray sample and lowest for salmon. At T2, APC increased and ranged from 4.4 to 7.2 $\log_{10}$ cfu/g. The sample of pangasius showed an increase of less than 0.5 $\log_{10}$, while those of angler fish and mackerel increased by more than 2 $\log_{10}$. The APC of the other samples increased between 1 and 2 $\log_{10}$.

When comparing PCA, MA, LH and IA at T1 (Fig. 1) and after ice storage (T2) (Fig. 2), PCA generally showed a lower APC than the salt-containing media (MA, LH and IA), but some counts were equal. Although APC between several batches of the same fish species also showed some differences (results not shown), the difference between APC on PCA and the other media was always more pronounced. Student *t*-tests showed a statistically significant difference, except between LH and MA at T1 and T2 and between PCA and IA at T1.

The highest difference in APC at T1 was observed between PCA and LH, with those on PCA being 0.3 to 1.1 $\log_{10}$ cfu/g lower than on LH (Fig. 1). Differences in APC lower than 0.5 $\log_{10}$ were interpreted as within standard plate counting error and are not shown. Five samples showed a 0.5 to 1 $\log_{10}$ lower APC on PCA than on LH, and one sample exceeded a 1 $\log_{10}$ difference. Differences between MA, LH and IA were lower than 0.5 $\log_{10}$ except for two samples (ray and plaice) where IA had a lower APC compared to MA and LH.

At T2, the difference in APC between PCA and the other media (MA, LH and IA) increased for most fish samples. APC on LH and MA was almost equal, with both being 0.5 to 1.8 $\log_{10}$ higher than on PCA (Fig. 2). Compared to T1, more samples showed a significant difference between PCA and LH/MA, and only two samples (angler fish and mackerel) had an APC difference lower than 0.5 $\log_{10}$ (not shown). Five samples (whiting, pangasius, plaice, sea bass and sea bream) showed a 0.5 to 1 $\log_{10}$ difference between PCA and LH/MA, while four samples (salmon, ray, cod and sole) showed a difference more than 1 $\log_{10}$. Five samples showed an APC difference of 0.5 $\log_{10}$ or more between LH and IA (salmon, whiting, ray, cod and sole).

3.2. Replication of the cultivated microorganisms on different growth media

Replication showed a high percentage of colonies unable to grow on at least one medium (Table 2). On MA, LH and IA, most colonies were able to grow (results not shown).

At T1, all fish samples, excluding the pangasius sample, contained microorganisms unable to grow on all tested media. Of those samples originally incubated on MA or LH, 10–92% of the colonies, depending on the fish sample, could not grow on PCA after replication. Thirteen to 68% of the colonies from samples originally incubated on IA were unable to grow on PCA. One to 53% of the colonies from samples originally incubated on PCA could not grow on one or more of the salt-containing media. At T2, shifts were noticeable. The number of non-growing colonies replicated to PCA increased for seven fish samples (salmon, whiting, ray, pangasius, cod, sole and sea bream). As for the other four fish samples, the number of non-growing replicated colonies was equal or lower than at T1. On the other hand, the percentage of colonies taken from PCA that did not grow on the salt-containing media was mostly much lower at T2 than at T1.

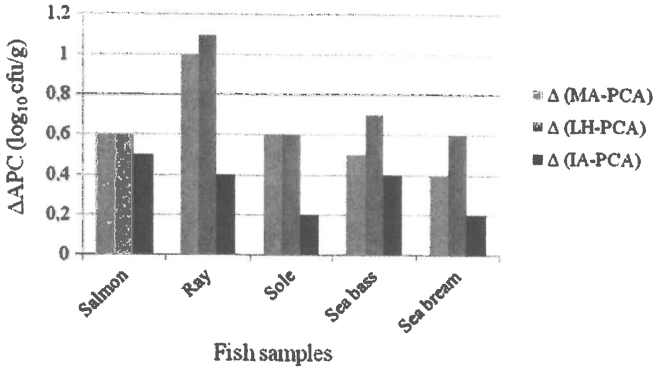

Fig. 1. Differences (Δ) in total aerobic psychrotrophic count (APC) ($\log_{10}$ cfu/g) between MA, LH, IA and PCA growth media at arrival (T1) of fish samples. Only those fish samples are shown where a higher difference than 0.5 $\log_{10}$ cfu/g between PCA and the salt-containing media (MA, LH and IA) was observed.

3.3. Identification of the microbial isolates failing to grow on one or more media

After replication, a selection of 121 isolates with different colony morphology were selected based on their inability to grow on at least one of the tested media. The colonies were collected from the plates at T2, since mainly those microorganisms present during spoilage were of interest. These isolates clustered based on their $(GTG)_5$ fingerprint (Suppl. Fig.), showed a large variety, with some large clusters present. These clusters were visually defined. PCR-reproducibility ranged from 95.3 to 99.0% similarity (Pearson correlation).

From this cluster analysis, 39 representatives were selected, with a minimum of two isolates per cluster, and identified based on

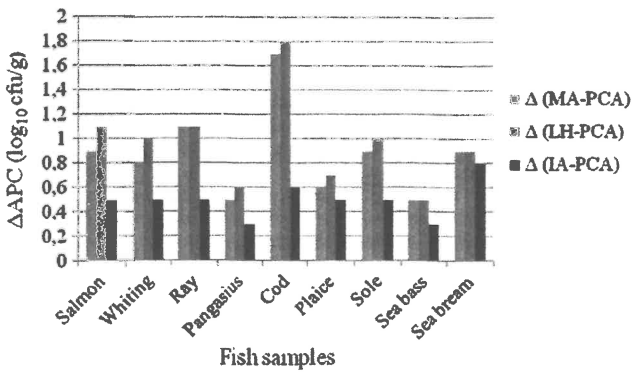

Fig. 2. Differences (Δ) in total aerobic psychrotrophic count (APC) ($\log_{10}$ cfu/g) between MA, LH, IA and PCA growth media at T2 (i.e., after 7 days or 4 days (ray) of ice storage) of fish samples. Only those fish samples are shown where a higher difference than 0.5 $\log_{10}$ cfu/g between PCA and the salt-containing media (MA, LH and IA) was observed.

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their partial 16S rRNA and *gyrB* gene sequence. Twenty-one different species were identified (Table 3). Using 16S rRNA gene analysis, the representative isolates could mostly be allocated to species complexes after BLAST search with the EzTaxon database. Further identification to species level was obtained by *gyrB* sequence analysis. However, not all *gyrB* sequences of all species were available. In these cases, 16S rRNA gene based identification on the genus and/or species complex level was used to select type strains of species not present in any accessible database of *gyrB* sequences. Phylogenetic trees were constructed with these extra type strains included together with the known *gyrB* sequences and the representative isolates (not shown).

Table 3
Identification of 121 isolates with different colony morphology from the 11 fish samples after 7 or 4 days (ray) of ice storage after purchase, which did not grow on a specific growth medium. The (range of) similarity values (*gyrB*) against the type strains of the species (exceptions are mentioned in footnotes) are listed in the second column, when the *gyrB* sequence could not be used, the 16S rRNA similarity values are used. The abundance of isolation of these species is listed in the third column by the number of isolates. The fish samples on which the microorganisms are found are in the third column.

Identification	Similarity values (%)	# isolates	From fish sample	Not growing on
Photobacterium				
P. phosphoreum	99.6	4	salmon, cod, whiting	PCA
P. iliopiscicarium	99.1	3	sole, plaice, whiting	PCA
Shewanella				
S. frigidimarina	94.6–99.2[a]	47	all fish	PCA
S. vesiculosa	98.5–99.5	5	angler fish, plaice, sole, salmon, pangasius	PCA
S. baltica	98.7–99.1	3	angler fish, cod, mackerel	PCA
S. glacialipiscicola	98.7	1	plaice	PCA
Vibrio/Aliivibrio				
V. litoralis	97.0 (16S)	2	mackerel, sole	PCA
A. logei	96.2	1	salmon	PCA
Pseudoalteromonas				
Psa. nigrifaciens	99.8	33	all fish except cod	PCA
Psychrobacter				
Psb. cibarius-like[b]	/	1	mackerel	PCA
Psb. proteolyticus	94.0–95.2	2	angler fish	PCA
Psb. fozii	/[c]	1	angler fish	LH
Psb. maritimus	/[d]	1	angler fish	LH
Psb. cibarius	/[e]	1	mackerel	MA, LH & IA
Pseudomonas				
Ps. fragi	92.2–93.3	5	angler fish, mackerel, sea bass	MA, LH & IA
Ps. fluorescens	89.2	1	gilthead sea bream	PCA
Acinetobacter				
Acinetobacter sp.	80.8[f]	1	mackerel	MA, LH & IA
Flavobacterium				
F. hydatis	97.7–98.3 (16S)	4	salmon, plaice, cod	MA
F. hercynium	96.6 (16S)	2	angler fish, gilthead sea bream	MA
Janthinobacterium				
J. lividum	95.6	1	cod	MA
Brochothrix				
B. thermosphacta	99.6 (16S)	2	gilthead sea bream, sole	IA

[a] The similarity values could not be calculated based on a type strain, but were calculated based on an environment isolate (NCIMB 400). Phylogenetic tree analysis confirmed the identification.

[b] The isolate was identified based on *gyrB* gene sequence and phylogenetic tree analysis. FASTA results gave 96.2% similarity with the *Psb. immobilis* type strain, however, phylogenetic tree analysis with inclusion of extra type strains confirmed a higher similarity with *Psb. cibarius*. Sequence comparison between both isolates and the type strain showed several mismatches with the sequence of the *Psb. cibarius*-like isolate.

[c] The isolate showed 93.6% similarity with Psb. luti type strain, but phylogenetic tree analysis gave a higher similarity with *Psb. fozii*.

[d] FASTA results gave a similarity value of 93.6% with the *Psb. luti* type strain, phylogenetic tree analysis gave a higher similarity with the *Psb. maritimus* type strain.

[e] FASTA results gave a similarity value of 97.4% with the *Psb. immobilis* type strain, phylogenetic tree analysis gave a higher similarity with the *Psb. cibarius* type strain.

[f] The *Acinetobacter* isolate showed 80.8% similarity with the *Acetinobacter johnsonii* type strain.

Members of the genera *Photobacterium*, *Shewanella*, *Vibrio/Aliivibrio*, *Pseudoalteromonas*, *Psychrobacter* and *Pseudomonas* were unable to grow on PCA, but tended to grow very well on fish during ice storage as evidenced from their isolation on the other growth media at T2 (Table 3). In particular, *Shewanella frigidimarina* and *Pseudoalteromonas nigrifaciens* were abundantly present in this study, with 47 and 33 isolates found, respectively. On the other hand, some microorganisms could only grow on PCA, i.e. some species of the genera *Psychrobacter*, *Pseudomonas* and *Acinetobacter*. Within the genus *Psychrobacter*, *Psb. fozii* and *Psb. maritimus* could not grow on LH, and *Psb. cibarius* could not grow on either of the salt-containing media. *Psb. proteolyticus* and an isolate identified as *Psb. cibarius* by *gyrB* sequence analysis (*Psb. cibarius*-like species) were, however, unable to grow on PCA. Within the genus *Pseudomonas* two species were identified. *Ps. fragi* was unable to grow on salt-containing media, while *Ps. fluorescens* was unable to grow on PCA. Some species of the genus *Flavobacterium* and *Janthinobacterium* were unable to grow on MA. *Brochothrix thermospacta* was unable to grow on IA.

3.4. Differences in the microbial community on the different growth media by DGGE analysis

The DGGE analysis of the plate swabs of mackerel from the different growth media at T1 and T2 is shown in Fig. 3A. A clear difference in band patterns is observed between PCA and the other media, indicating a different microbiota on PCA at both time points. In comparison to MA, LH and IA, PCA exhibited only a very small number of bands especially at T1. At T2 the number of bands for PCA highly increased but the pattern observed from PCA is still quite different from the other media. The DGGE pattern from the salt-containing media showed some variation between both time points; nevertheless, the difference was rather small compared to the difference on PCA. A similar clearly distinct pattern of PCA from the other media was also observed for several other fish samples such as plaice, salmon, gilthead sea bream, whiting, sea bass, sole and cod (not shown). However, not all fish samples showed such a clear difference in DGGE pattern between PCA and the other media. DGGE analysis of the brackish water fish pangasius (Fig. 3B) showed that besides a few bands, the pattern obtained from PCA plate swabs was more similar to the salt-containing media at T1, indicating that the dominant microbial flora on all media was more or less the same. At T2, a shift in DGGE pattern was noticed where the differences between PCA and the salt-containing media became much larger. Rather subtle differences in dominant microbiota were also observed for the fish samples of angler fish and ray (not shown).

4. Discussion

4.1. Quantitative differences between the media

Most samples of fish species examined, except three (salmon, pangasius and cod), reached an aerobic psychrotrophic count of 6 $\log_{10}$ cfu/g on PCA at T2. At the same time, an APC of 7 $\log_{10}$ cfu/g was counted on the salt-containing media indicating that these fish are definitely no longer suitable for purchase. On the other hand, some fish samples still showed a quite low APC at T2 (cod, salmon and pangasius). However, large fish tend to spoil more slowly than small

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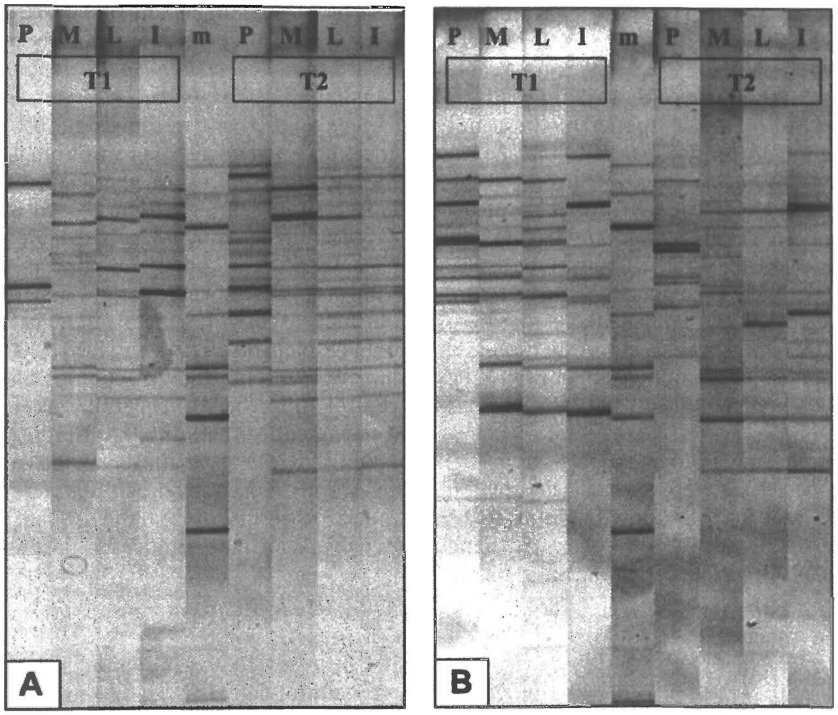

Fig. 3. DGGE - V3 profile of a plates wab of all media P (PCA), M (MA), L (LH), I (IA) from mackerel (A) and pangasius (B) at the day of arrival (T1) and after 7 days of ice storage (T2). An internal reference marker m was used for the comparison of different gels.

fish, and also aquacultured fish may have a longer shelf life, when caught in ideal conditions than similar fish from wild catch (Huss, 1995). On the contrary, elasmobranch fish such as ray tend to spoil very quickly after catch, but this is always assumed to be mainly due to organoleptic changes (fast emission of ammonia) (Múgica et al., 2008). In this study, the APC of ray was already quite high (6 $\log_{10}$ cfu/g on PCA or 7 $\log_{10}$ cfu/g on salt-containing media) at T1.

The APC results confirm earlier publications indicating differences up to one log or more between PCA and LH or MA for marine fish species (Joffraud and Leroi, 2000; Kudaka et al., 2010; Van Spreekens, 1974). In this study, several fish samples (salmon, plaice, sole, sea bass and sea bream) have a difference of at least 1 log between PCA and LH/MA. The differences were noticed at T1 as well as at T2, indicating that fish quality analysis on PCA in fish auctions or supply centres misses many microorganisms and underestimates the microbial quality. A shift was also noticed during storage: in some cases, the number of microorganisms not growing on PCA increased, while in other fish samples those growing on PCA increased. This results in the differences in APC noticed at each sampling point. The microbial growth during storage is mainly fish specific, depending on intrinsic and extrinsic characteristics of the fish and the handling procedures.

4.2. Qualitative differences concerning the growth of microorganisms between the media

Replication showed many isolates that were unable to grow on one of the media, mostly on PCA. Many bacterial species on fresh fish are only able to grow on LH, MA or IA, but also a considerable part (21%) is unable to grow on one of those salt-containing media. After ice storage, however, the number of isolates not growing on PCA has increased to approximately 50% of the total number of isolates, while those unable to grow on LH, MA or IA have decreased from 21 to 6%. This indicates that the microorganisms important for spoilage are mainly species needing those salt-containing media in order to be detected. The inability of microorganisms to grow could be caused by the absence of sodium chloride in PCA as previously noted in several studies (Boskou and Debevere, 1996; Liston, 1980; Vallé et al., 1998). The choice of growth medium may therefore be even more important during ice storage or at the end of shelf life than for (fresh) fish immediately after catch.

Identification of the non-growing colonies on one of the tested media at T2 shows that some currently known marine specific spoilage organisms (SSOs) are unable to grow on PCA, such as *Shewanella*. Especially *S. putrefaciens* and *S. baltica* are dominant microorganisms of ice-stored marine fish and are known as typical SSOs of marine fish species (Jorgensen and Huss, 1989; Koutsoumanis and Nychas, 1999; Vogel et al., 2005). All *Shewanella* species found, could be responsible for sensory spoilage since they are all capable of TMAO reduction and are important producers of H_2S. Although most of these species are described as nonhalophilic, this feature is strain specific and most species grow preferentially on 2% NaCl (Bowman et al., 1997; Satomi et al., 2007; Vogel et al., 2005). The *Shewanella* isolates in this study were all unable to grow on PCA.

Further, species of the genera *Photobacterium* including *P. phosphoreum*, *Vibrio* and *Aliivibrio*, were not detected on PCA. *P. phosphoreum* is also a known SSO of fish. Fish in MAP packaging is especially sensitive to spoilage due to this microorganism (Dalgaard et al., 1997). The genera *Photobacterium*, *Vibrio* and *Aliivibrio* are closely related, and they all require a high sodium content and other ions for growth; most species need at least 1% NaCl (Baumann and Schubert, 1984; Farmer and Hickman-Brenner, 2006). In this study *P. phosphoreum*, *P. iliopiscarium* and *V. litoralis* were able to grow on IA, a medium with 0.5% NaCl, but not on PCA.

Two less known genera in fish spoilage, *Pseudoalteromonas* (*Psa*) and *Psychrobacter* (*Psb*), were also unable to grow on PCA. Species of the genus *Pseudoalteromonas* were not detectable on PCA; they all require NaCl for growth. *Pseudoalteromonas* is a heat labile common marine microorganism. Species of this genus appear to be

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growing during storage of several fish and fishery products (Koutsoumanis and Nychas, 2000; Paarup et al., 2002; Romero et al., 2002; Rudi et al., 2004). *Psa. nigrifaciens* makes up a large part of the microbiota for these fish samples after ice storage in this study (Table 3). In this study also several species of the genus *Psychrobacter* were found. Some of the species (*Psb. proteolyticus* and *Psb. cibarius*-like strains) are unable to grow on PCA, while others can only grow on PCA and not on the salt-containing media (*Psb. fozii*, *Psb. maritimus* and *Psb. cibarius* strain). Except for *Psb. cibarius* reported to be stimulated by NaCl to grow (Jung et al., 2005), the growth data of this study corresponds with literature data (Bozal et al., 2003; Jung et al., 2005; Romanenko et al., 2004; Yoon et al., 2003). The genus *Psychrobacter* is currently not associated with major SSOs; *Psb. immobilis* is a minor spoiler producing a musty off-odour commonly found on chilled fish (Gennari et al., 1999).

Some typical food microorganisms such as *Flavobacterium* sp., *Pseudomonas* sp. and *Acinetobacter* sp. were less able to grow on salt-containing media, specifically on MA growth seems to be inhibited. These genera have simple nutritional requirements and grow best on media without NaCl (Bernardet et al., 1996; Moore et al., 2006; Towner, 2006).

Brochothrix thermosphacta, a known SSO, especially for MAP-stored fish (Rudi et al., 2004), was unable to grow on IA.

DGGE analysis using the 16S rRNA V3-region has recently been used for several studies concerning the microbial flora of fish and other food (Ercolini, 2004; Hovda et al., 2007a, 2007b). Next to the advantages of using DGGE for bacterial community studies, analysts have to keep in mind some potential biases of the technique. For instance, by running pure strains, it was noticed that several important genera isolated in this study, including *Shewanella*, *Pseudomonas*, *Photobacterium*, *Psychrobacter* and *Pseudoalteromonas*, showed multiple bands (data not shown). The multiple bands can originate from the presence of multiple gene copy numbers with small sequence differences in the genome of the microorganism. This makes a simple interpretation of diversity conclusions impossible (de Araujo and Schneider, 2008). The number of bands cannot be correlated to the diversity, so a conclusion about a possible diversity difference between the media cannot be made. Other biases linked to DGGE are the potential co-migration of bands despite sequence variation, this means that one band in the DGGE pattern may visualise more than one species (Vallaeys et al., 1997). DGGE analysis is also limited to the detection of the most dominant bacterial populations in the community (Muyzer et al., 1993). Additionally not all bacterial species are detectable by using 16S - V3 universal primers due to selective amplification of genes by PCR (Ercolini et al., 2003). In this study, DGGE analysis was therefore only used to confirm the APC and replication results. A comparison was made between the band patterns of cultivable microorganisms on the different growth media in order to have some insight in the similarity of species composition on these media. For most fish samples, the band pattern of PCA plate swabs is very different from those of the salt-containing media. The patterns of the salt-containing media, in contrast, resemble each other.

The APC analysis and replication technique results are in concordance with the differences in DGGE profiles of the fish samples. For mackerel, the differences in APC on the different media are not significant, but identification of the colonies not growing on PCA shows that species from the genera *Shewanella*, *Vibrio*, *Pseudoalteromonas* and *Psychrobacter* are present. On the other hand, species that only grow on PCA are also present. This explains the difference in DGGE pattern (no matching bands) of mackerel between PCA and the other media. For pangasius, the APC differences are small and DGGE band patterns on the different media are quite similar at T1, but at T2 differences in APC and DGGE pattern similarity increases possibly because genera such as *Shewanella* and *Pseudoalteromonas* are found which only grow on salt-containing media.

Several methods were used in this study to observe the differences between microorganisms from fish growing on four different media. All methods show that many microorganisms will be overlooked if only one medium is used in fish quality research, and especially when only PCA is used. Members of the genera *Shewanella*, *Vibrio*, *Aliivibrio*, *Photobacterium*, *Pseudoalteromonas* and *Psychrobacter* were not able to grow on PCA. These results indicate that PCA, now used as the general reference medium for the enumeration of microbiota on all food and feed (ISO 17410, 2001), has important limits concerning the detection of microbiota on fish for quality control through APC, especially after prolonged ice storage. LH (recommended by NMKL 184; Nordic Committee of Food Analysis, 2006) and MA gave the best APC analysis results. With a few exceptions, most microorganisms were capable of growing on these media. DGGE analysis also showed that most of the patterns of these two media are quite similar for most fish samples.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in online version at doi:10.1016/j.fm.2011.03.009.

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