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Chapter 25

Shelf-Life and Bacteriological Spoilage of American Plaice (*Hippoglossoides platessoides*)

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A study of shelf-life characteristics of whole, gutted American plaice (*Hippoglossoides platessoides*) stored under different conditions was carried out over a period of 12 days. Sensory, microbiological and chemical characteristics were determined. Sensory evaluation of cooked fillets was used to determine the end of shelf life. The main emphasis was on the isolation and identification of the spoilage microflora that had developed upon storage in air at different temperatures (-1.7, 0.6, 9.9°C and in a water-ice mixture at 0.7°C), as well as on gaining a better understanding of the behavior of the concerned spoilage organisms. A sterile fish substrate was developed to assess by sensory evaluation the ability of fish bacterial isolates to produce off-odors. Potentially spoiling bacteria were further tested for their spoilage activity using electrochemical gas sensors ("electronic nose"). Their spoilage pattern was compared to that obtained for spoiling plaice. Growth experiments of 3 different bacterial groups (pure and mixed) were conducted at temperatures ranging from 0 to 15°C and the influence of microbial interaction assessed.

Marine fish is a very perishable commodity. It has a short shelf-life which depends on its original quality and storage conditions under which it is kept. Fish quality is in turn influenced by the intrinsic quality of the fish and the treatment to which is submitted during fishing, handling and processing. Spoilage is irreversible and further processing will not improve the quality of bad raw material. It is well recognized that bacteria are the main cause of spoilage of fresh, lean fish (*43,45*). The initial microflora

is usually related to its fishing areas, whereas the selection of the flora causing spoilage depends again on the processing and storage conditions of the fish.

Many methods have been proposed or tested for measuring fish quality. Sensory methods are still the most satisfactory way of assessing the freshness of fish (*7,18,37*). Quality deterioration of fish is first characterized by the initial loss of the fresh fish flavor which is followed by the development of a neutral odor/flavor, leading to the detection of off-odors/flavors and the rejection of the fish. The microflora of aerobically stored fish consists typically of Gram-negative psychrotrophic non-fermenting rods. Under traditional ice storage, *Pseudomonas* spp. and *Shewanella putrefaciens* have been almost exclusively found (*13*). This has been found to be true for many fish species whether caught in temperate (*11,28*) or subtropical/tropical waters (*12,25,29,42*). *S. putrefaciens* has been reported as the specific spoilage bacteria of marine temperate-water fish stored aerobically in ice (*22*). However, with changing storage temperature profiles and/or atmospheric conditions, the spoilage microflora may change.

Studies on a Flatfish, American Plaice

Flatfish are widely distributed. Eleven species have been found in the Icelandic fishing grounds, among which are permanently established. American plaice (*Hippoglossoides platessoides*), commonly named plaice, is one of them. It is a demersal fish usually found along the bottom of the sea, 10 to 500 m deep (*21*). According to the Fisheries Association of Iceland (1994-1996), catches of American plaice in the Icelandic fishing grounds have increased tremendously in the recent years, from 565 tons in 1989 to 7,027 tons in 1996. Therefore, its increasing importance on the market, as well as that of other flafish species, evidences the need for a better understanding of its spoilage process.

The aim of this study was to determine the influence of different storage conditions on the shelf-life of whole, gutted American plaice as well as to isolate and identify the spoilage microflora that had developed upon storage in air at different temperatures (-1.7 ± 0.3 °C, 0.6 ± 0.2 °C on ice, 9.9 ± 0.1 °C) and in a water-ice mixture (0.7 ± 0.4 °C). Screening of bacterial strains was done by means of a numerical taxonomic study and the comparison of SDS-PAGE whole-cell protein patterns of selected strains. Spoilage potential and activity of strains were evaluated and growth experiments conducted at various temperatures.

Influence of Storage Conditions on the Shelf-life and Spoilage Indices

American plaice were caught in November 1995 using a Danish seine in the South-East of Iceland and handled according to good practices. Upon landing, the fish was iced and shipped to the laboratory; received in 65h. At that time, 4 fish were sampled for analyses and the rest of the catch was divided into each treatment and stored accordingly.

Total viable counts were evaluated for both the skin and the flesh. Two pieces of skin were aseptically cut from the dark side of each fish (2 fish =1 sample), giving a total area of 30 cm^2, and mixed in a stomacher (Stomacher Lab Blender 400, A.-J. Seward Laboratories, London, UK) with 99 mL Butterfields buffer (*51*) for 1 min. Successive 10-fold dilutions were made as required. Spread-plating of aliquots was done on Iron Agar (1% NaCl; *11*) and CFC medium (Pseudomonas Agar base supplemented with CFC supplement (Oxoid)). The other side of each fish was aseptically skinned, pieces of flesh removed and comminuted in a mixer. Twenty-five grams of minced flesh were mixed with 225 mL buffer in a stomacher for 1 min. The dilution and spread-plating procedures were done as described above. The Iron Agar (IA) plates were incubated aerobically at 15 °C for 4 days as opposed to 22 °C (2 days) for the CFC medium. After incubation, evaluation of total viable counts was done in a Darkfield Quebec Colony Counter (Spencer). H_2S-producers (black colonies on IA) were counted from all plates. The bacterial flora was isolated at day 0 (65 h from catch) and at time of sensory spoilage by randomly taking 25 colonies from flesh samples (12 from skin samples) of each treatment.

Chemical analyses were done with the rest of the flesh mince within 1 h. The pH was measured in 5 g of mince moistened with 5 mL of deionised water. Trimethylamine (TMA) content was determined (*15*). Sensory evaluation of cooked fillets (steam oven: 5-6 min at 80-90 °C) was done by the Icelandic Fisheries Laboratories panel (8 to 10 trained members) using a modified Torry scale (*48*), rating from 10 = very fresh to 3 = very spoiled, with the rejection level at 5.5. This evaluation was decisive in defining the end of shelf life of the fish.

Shelf- life studies of whole, gutted American plaice were conducted at different temperatures over a 12-day period. Table I summarizes the results of the various analyses performed at the end of the sensory shelf life of plaice. At this point, total viable counts (TVC) of skin samples from all treatments were higher than those from flesh by a difference ranging from 2.4 to 3.5 log. Levels of H_2S-producers were low for all storage conditions, except for the water-ice treatment where H_2S-producers constituted almost half of the microflora. On the other hand, the high counts obtained from CFC medium for the other treatments when compared to total viable counts demonstrated the predominance of *Pseudomonas* spp. among the microflora at the end of shelf-life.

As expected, the higher temperature treatment resulted in a faster rate of spoilage of fish. Temperature plays an important role in the process of spoilage, but other environmental factors can influence that process as well. Comparison of the fish stored on ice at 0.6°C to that kept in water-ice mixture at a similar temperature (0.7°C) exemplifies the importance of extrinsic factors on spoilage. Lower bacterial levels were seen with spoiling plaice stored in the water-ice mixture and a shelf life extension of 2 days was obtained, as well as the establishment of a different microflora at spoilage. The low levels of *Pseudomonas* spp. in plaice flesh could be due to the lower oxygen tension expected under these conditions (immersed in water) as compared to the air-ice environment, while H_2S-producing bacteria benefitted from this microaerophilic environment. Plaice stored in the water-ice treatment was meant to be compared to the ice treatment. Plaice fillets usually have an undesirable color and are often smeared by blood stains as storage on ice can cause bruises, especially during pre-rigor. The water-ice treatment was assessed to find out whether it could remedy to that problem. Storing the fish under such a treatment (0.7°C) did not influence the color nor the blood stains found on the fillets (data not shown). But since the fish was originally put on ice from catch and transported to the laboratory before it was transferred to the water-ice mixture, one could expect the damage already done to be irreversible. Perhaps storing the fish under that treatment directly after catch would result in a better raw material with less damage.

The chemical indices (TMA content and pH of the flesh) employed did not provide valuable information as spoilage indices. pH was not expected to be a valuable indicator as it is known to decrease slightly during initial storage of fish on ice, later to increase and surpass its initial value as ammonia/TMA and other lower amines are produced due to bacterial activity. TMA content did not reach levels usually seen in spoiling white fish (10-15 mg N/100 g of flesh; *32*), except for the high temperature treatment (9.9°C) where 12.9 to 31.9 mg N-TMA/100 g of flesh were found after 5 days of storage. The presence of trimethylamine oxide (TMAO) was later on verified (spring trial) and found to range from 75 to 92 mg N/100 g of flesh, which would be stoichoimetrically sufficient to produce 10-15 mg N-TMA/100 g. Dyer (*9*) reported 93 mg N-TMAO/ 100 g flesh in American plaice. Dalgaard (*8*) found the reduction of TMAO in packed cod stored on ice to be proportional to the production of TMA with a correlation coefficient of -0.9551 (n=125). However, storing unpacked fish directly on ice can lead to the loss of TMAO through its leakage to melting ice. It follows that storage of plaice in the water-ice mixture surely contributed to TMAO leakage out of the flesh. This probably explains the very low TMA levels found in plaice stored in the water-ice treatment, despite the higher levels of H_2S-producing bacteria (especially *S. putrefaciens*) encountered under such storage conditions. Unfortunately, values of

Table I. Microbiological and Chemical Data Related to the Flesh and Skin of Whole Plaice at the End of Sensory Shelf-life

Parameter	*Storage conditions, °C*			
	-1.7	*0.6 (ice)*	*0.7 (water-ice)*	*9.9**
Flesh analyses				
End of sensory shelf life at day	12	10	12	4
Estimated TVC (log CFU/ g)	3.81	5.00	4.74	5.75
% H_2S-producers	6.6	2.9	43.6	6.3
Estimated *Pseud*omonas counts	3.64	5.00	3.59	5.50
TMA content (mg N / 100 g)	0.85	1.18	0.85	17
PH	6.80	6.74	6.70	6.98
Skin analyses				
Estimated TVC (log CFU/ cm^2)	7.29	8.25	7.49	8.11
% H_2S-producers	1.2	16.8	11.2	7.8
Estimated *Pseud*omonas counts	6.95	7.89	6.07	8.00

* given values were intrapolated at day 4, based on data from previous and following sampling days

TMAO content of the flesh over that storage period are not available. However, TMAO content of a batch of plaice obtained during the summer of 1997 (to be used for the preparation of a fish substrate medium) ranged from 62.5 to 70.5 mg N/100g upon receipt, but was found to have leached out of the flesh during ice storage as only 13 mg could be measured after 13 days.

Identification of Microflora of Spoiling Plaice

Preliminary bacterial identification tests were done according to Table II for all isolates. Other tests were done on some isolates to facilitate their grouping into genera. These included sensitivity to O/129 (vibriostatic agent, Oxoid), the presence of fluorescein pigment (King's medium; *24*), DNAse activity (DNAse Test agar with methyl green from Difco), gelatinase activity and penicillin sensitivity (2 μg, Oxoid). The bacterial identification scheme used was based on information gathered from different sources, such as Shewan *et al.* (*47*), Stenström and Molin (*50*) and Magnusson (*31*), among others.

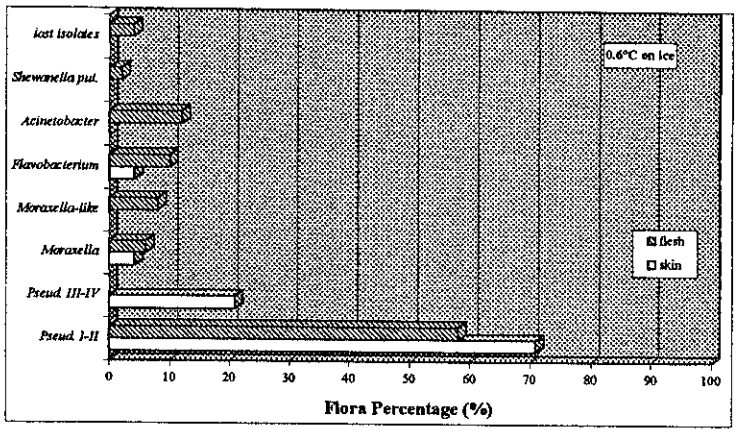
Upon storage of the fish at various temperatures and conditions, bacterial proliferation was evaluated at regular intervals. From Figure 1, it can be seen that once spoilage had occurred at 9.9°C, about 79% (n=12) of the skin and 90% (n=25) of the flesh microflora isolated belonged to the *Pseudomonas* genus. Only 2.1% *S. putrefaciens* were isolated from plaice flesh (none from the skin) after 5 days of storage, which corresponded to the low levels of H_2S-producers detected on spread-plated IA (Table I).

Table II. Preliminary Bacterial Identification Tests done on all Isolates

Test	*Description (reference)*
Gram type	Gram staining (*51*) and 3% KOH (*14*)
Form & size	Microscopic evaluation (1000 x magnification)
Catalase	3% H_2O_2
Oxidase	DrySlide (Difco)
Motility	Hanging drop: isolates grown overnight at 15°C in Iron broth (IA without agar and cysteine)
Oxidation/fermentation (glucose)	O/F medium (*19*) modified with ½ strength artificial seawater (*30*)
TMAO reduction	TMAO medium (*11*)
H_2S production	TMAO medium (*11*)

When the fish was kept on ice at 0.6°C, the skin microflora isolated at spoilage (Figure 1) dominated in *Pseudomonas* spp. (91.7%), especially group I-II (70.8%). Similarly, the flesh microflora dominated in *Pseudomonas* spp. of group I-II (58%). Despite the 16.8% level of H_2S-producers found on the skin at spoilage (Table I), no *S. putrefaciens* strains were identified among the isolated microflora. However, a low level of *S. putrefaciens* (2%) was isolated from the flesh, corresponding to the 2.9% level of H_2S-producers seen in the flesh.

When the fish was stored in the water-ice treatment, no *Pseudomonas* I-II spp. were isolated from the skin microflora at spoilage, in contrast to 70.8% *Pseudomonas* III-IV spp. Based on the differences seen between CFC and IA counts, only approximately 10% of the microflora was expected to belong to the *Pseudomonas* genus. The high percentage of *Pseudomonas* III-IV spp. obtained could indicate that CFC is more selective for *Pseudomonas* I-II species. Similarly, a lower level (10%) of



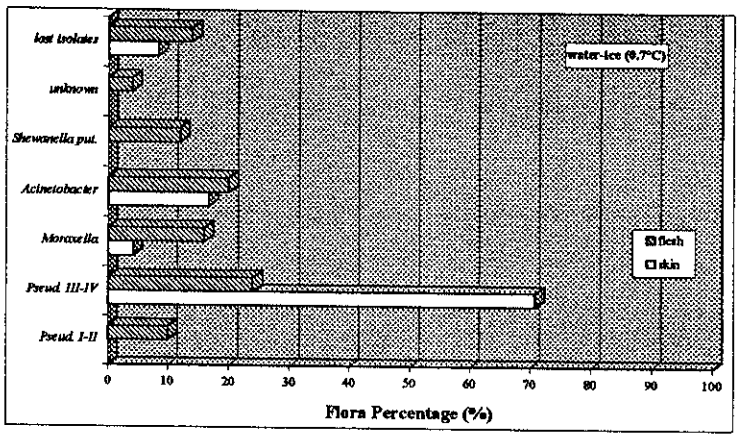


Figure 1A. Distribution (%) of the microflora found on the skin (n=12) and flesh (n=25) of plaice at the end of sensory shelf life after storage on ice or in ice-water.

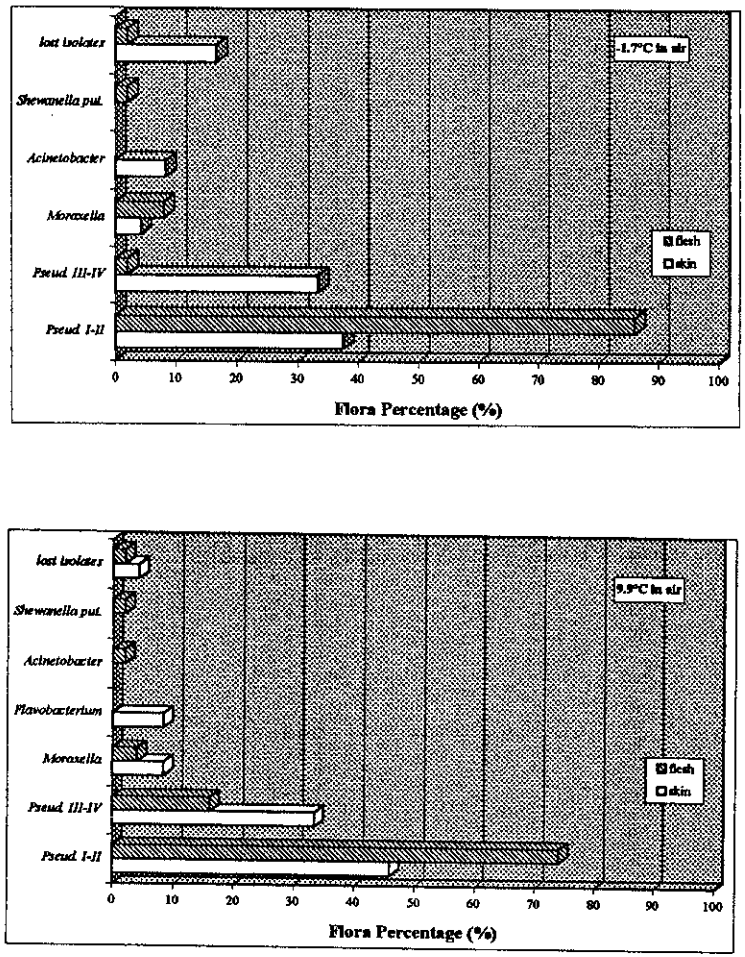


Figure 1B. Distribution (%) of the microflora found on the skin (n=12) and flesh (n=25) of plaice at the end of sensory shelf life after storage in the air at -1.7 and 9.9°C.

Pseudomonas I-II spp. was found in the flesh samples at spoilage, compared to 24% for *Pseudomonas* III-IV spp. The rest of the flesh microflora constituted of a rather high percentage of H_2S-producing bacteria (43.6%) where only 12% was identified as *S. putrefaciens*, while other bacterial species established in the flesh included *Moraxella* (16%) and *Acinetobacter* (20%), apart from some unknown and lost strains (18%). It should be mentioned that in another trial a similarly low level (12%) of *Pseudomonas* I-II spp was found in the flesh at spoilage as opposed to a high level (60%) of *S. putrefaciens*. These variations in the level of *S. putrefaciens* found can be explained by differences in the exact time of isolation of the spoilage microflora, as steep changes are known to occur in fish following incipient spoilage (*46*).

When the storage temperature was as low as -1.7°C, high levels of *Pseudomonas* spp. were found at the end of sensory shelf life, both on skin (69.8%) and flesh (88%) with group I-II dominating. *S. putrefaciens* accounted for only 2% of the isolated flesh flora, whereas 8% *Moraxella* spp. were found. Overall, *Pseudomonas* I-II spp. dominated on the skin and flesh of plaice at the end of sensory shelf life from storage temperature ranging from -1.7 to 9.9°C, while a different microflora had established during storage in the water-ice mixture mainly made up of *Pseudomonas* III-IV, *S. putrefaciens*, *Moraxella* and *Acinetobacter* spp.

Further Characterization of Strains Representing the Spoilage Flesh Microflora

After presumptive identification of the isolated bacterial strains (394), 60 representative strains out of 248 flesh isolates were selected for a numerical taxonomic study to allow for comparison of strains in order to facilitate later on the screening of strains to be assessed for spoilage potential in a sterile fish substrate. These strains were also compared by their SDS-PAGE electrophoretic whole-cell protein patterns. Numerical taxonomy has been defined by Sneath and Sokal (*49*) as "the grouping by numerical methods of taxonomic units into taxa on the basis of their characteristics". It implies studying as many aspects (traits or characters) of the biology of the organisms as possible. This generates a mass of information, but the key feature of numerical taxonomic methods is that all characters have equal importance.

The strains were chosen from all treatments tested and included *Pseudomonas* I-II spp., *S. putrefaciens*, *Cytophaga/Flavobacterium*, *Acinetobacter*, *Moraxella* and *Moraxella*-like spp. All strains were characterized by 84 tests/properties such as various biochemical assays, antibiotic sensitivity, growth inhibition due to temperature, NaCl, pH, presence of metals, food preservatives and disinfectants, as well as the utilization of various carbon energy sources. All properties were determined in duplicate for 6 strains (strain numbers 10, 20, 30, 40, 50 and 60) giving a total of 66 operational taxonomic units (OTU). Fourteen (14) of the tests, which were either positive or

negative for all strains, were not included in the numerical analysis. Of the 70 characters coded, some were subdivided, such as size range of inhibition zone caused by antibiotic inhibition where 5 levels of inhibition were determined; and acidification or alkalinization of medium containing glucose (O/F test) where fermentation, oxidation and no reaction were included.

Data were scored as positive (1) and negative (0) and analyzed using the NTSYS pc software package (*41*). Similarity between strains was calculated using the simple matching coefficient (S_{sm}) and a dendogram was constructed using the unweighted pair group method with arithmetic averages (UPGMA) algorithm. The % similarity of strains tested in duplicate should be 100% if there was no error related to the tests. Strains 20a and 20b showed 100 % similarity, 40a and 40b were 99.1 % similar, 50a and 50b 98.6%, while strains 10a and 10b, and 30a and 30b showed lower similarity, 96.4 and 95.5%, respectively. These % similarities are rather close to 100% which indicates little erroneous results among the tests performed.

Results demonstrated 3 main clusters. All strains found in cluster 1 belonged to the *Pseudomonas* group I, except for 3 strains that had been previously presumptively identified as *Acinetobacter* spp. (strain 4) and *Pseudomonas* group II ssp. (strains 5 and 36). Strain 4 was found to be non-motile and oxidase negative, while strains 5 and 36 did not produce fluorescent pigments on King's B medium. Motility and pigment production were probably undetectable for these strains at the time of the analysis. Four main sub-clusters were identified from that cluster.

The second cluster gathered members of the *Pseudomonas* group II and 3 main sub-clusters could be identified: The third cluster was made up of *S. putrefaciens* strains. The other strains (*Moraxella* strains 10 and 21, *Moraxella*-like strain 7, *Acinetobacter* strain 55 and *Cytophaga/Flavobacterium* strains 17 and 60) showed lower similarity levels (< 82%), except for two *Cytophaga/Flavobacterium* strains (19 and 23) which had 91% similarity.

This cluster analysis grouped the different genera studied as it could have been expected. The *Pseudomonas* groups I and II clustered at 84.2% similarity and may therefore be included in the same genus, as in numerical taxonomy approximately 80% similarity (S_{sm}) levels have been used for separation of different species (*39*). The *S. putrefaciens* group clustered with some of the *Moraxella*, *Moraxella*-like, *Cytophaga/Flavobacterium* strains at the 73.4% level. Else, all the strains clustered together at a 51.2% level.

The complete nucleotide base sequence of the bacterial genome is the primary level of information of the cell and is thereby considered to be an absolute reference standard for the estimation of relationships between bacteria. A second level of information is given by the cellular proteins, and different types of electrophoresis are used to explore relationship at this level (*52*). The amino acid sequence of proteins of the bacterial cell is an indirect copy of the bacterial genome. The primary structure of proteins is related

to the molecular weight, net electrical charge and spatial conformation of the proteins. The electrophoretic separation of these macromolecules is based on one or a combination of these parameters. Gel electrophoresis of cellular proteins of a bacterial strain produces a complex banding pattern called a protein electrophoregram. When bacteria are grown under identical conditions, they can produce constant electrophoretic protein patterns, which can be used as fingerprints of the strains investigated. In one-dimensional gels, each protein band usually consists of a number of structurally different proteins having identical electrophoretic mobility. This technique is a sensitive one, mainly providing information on the similarity of strains within the same species (*52*).

The protein electrophoretic technique used in this study implied the mixture of soluble cytoplasmic proteins of a bacterial strain that had been solubilized by treatment with a denaturing agent (sodium dodecyl sulphate, SDS), then submitted to polyacrylamide gel electrophoresis (PAGE) and stained. The whole banding patterns obtained were compared among strains of the same genera. The methodology followed was based on information obtained from Ghent University, Belgium. All the 60 strains studied under the numerical taxonomic study previously described were tested here. Ten SDS-PAGE gels were prepared and run (2 gels per run) using the Mini-Protean II Electrophoresis Cell (Bio-Rad). Low molecular weight standards (phosphorylase 106 kDa, bovine serum albumin 80 kDa, ovalbumin 49.5 kDa, carbonic anhydrase 32.5 kDa, soybean trypsin inhibitor 27.5 kDa and lysozyme 18.5 kDa; Bio-Rad) were included, usually in duplicate on each gel, along with strain 22 which was repeated on each gel to verify that comparison between gels would be possible. The gels were photographed on a negative black and white film (Afga panchromatic film 25 ASA) using a yellow filter. The negatives obtained were scanned using Silverscan II, each picture inverted using the Adobe Photoshop v.4.0. The electrophoregram of each strain (except strains 7 and 17 which did not show on the gel) was copied from the pictures, pasted to a standard layer sheet and their size proportionally increased (standardized). Different layer sheets were prepared and the strains compared. In order to facilitate the comparison of the clustering obtained in the numerical taxonomic study with the electrophoregram of each strain studied; grouping of electrophoregrams of strains was done according to the clustering seen before.

Six common patterns were obtained by the electrophoretic separation of whole-cell proteins of *Pseudomonas* group I strains (data not shown). Three strains did not match any of these patterns nor those found for the other *Pseudomonas* group. Similarly, 6 common patterns were defined for the *Pseudomonas* group II spp. For the *S. putrefaciens* group, little matching was found but the patterns obtained were totally different from those seen among the *Pseudomonas* strains. Two of the 3 *Cytophaga/Flavobacterium* strains matched perfectly, while the other one had a different pattern. No similarity was found among the others strains tested. The results

obtained during this study confirmed most of the results from the numerical taxonomic study. A similarity level of at least 98% was generally required for patterns to be common. Based on the fact that visual comparison of electrophoretic protein patterns provides a reliable measure of genomic relatedness as revealed by DNA-DNA hybridization (*52*), such information should have more weight in the classification of the strains. These results have therefore been used for sorting and screening the strains to be tested for spoilage potential.

Determination of Specific Spoilage Organisms

The determination of specific spoilage organisms (SSO) requires extensive sensory, microbiological and chemical studies. The initial step involves the study and quantification of sensory, microbiological and chemical changes occurring during storage. Then, the microflora found on fish at the point of sensory rejection is isolated and these bacteria screened in a sterile fish substrate for their spoilage potential. Finally, potentially spoiling strains are tested to evaluate their spoilage activity, i.e. their growth kinetics as well as the qualitative and quantitative production of off-odors in the product of concern. All these steps were followed in the present study. The shelf life trials of plaice that were conducted have been described above and the microflora isolated at the end of sensory shelf life studied. Still, a fish substrate had to be developed in order to assess for the spoilage potential and activity of the selected strains.

Development of a Sterile Fish Substrate Medium. Different fish substrates have been mentioned in the literature by several authors. These include cooked minced fish muscle and sterile raw fish muscle tissue for sensory assessment of spoilage (*1,4,5,8*); filtered sterile muscle press juice for both sensory and chemical assessment of spoilage (*26*); and irradiated fish juice agar (*23*) among others. Since the fish substrate should be as close as possible to the fish product under investigation, sterile raw fish muscle should be the substrate of choice. But because of the small size of plaice and the small amounts of flesh available per fish, the use of sterile raw fish muscle was not feasible here. Instead, a sterile fish juice substrate was prepared from the flesh. The efficiency of filtered sterile flesh broth for detecting odor production has been demonstrated by Lerke *et al.* (*26*). Sterilization by autoclaving was also found to be a suitable method for the preparation of fish substrates (*6*).

The sterile fish juice was prepared by homogenizing 50 g of plaice (newly caught) flesh with 450 mL deionized water for 2 min, this mixture centrifuged at 12 000 RPM (Sorvall RC-5B centrifuge with GSA rotor, Du Pont Co., Wilmington, DE) for 25 minutes under cooling and the supernatant autoclaved at 121°C for 15 min. The pH of the supernatant was 6.74 prior to autoclaving and 6.75 subsequently. Based on TMAO measurements (data not shown), about 85% of TMAO was extracted from the flesh into the fish juice, but due to the dilution occurring during the preparation of the medium, only 11% of the initial amount was left after sterilization of the juice.

The dilution taking place during the extract preparation undoubtedly led to the loss of important compounds, such as amino acids. In the literature, some studies on the microbial substrates involved during fish storage have been reported. Shewan and Jones (*44*) found that the fish microflora utilized glucose, ribose, free amino acids and TMAO. Cysteine and methionine have been shown to be transformed during microbial spoilage of cod (*17*). Ringø *et al.* (*40*) reported that *S. putrefaciens* utilized a large number of amino acids, and that serine, cysteine, glutamate and aspartate were used faster than other amino acids. The free amino acid content of the raw flesh and fish juice (with and without autoclaving) during the extract preparation was evaluated by HPLC (data not shown). Only 6 and 19% of the serine and aspartate were left after extraction and sterilization, while all glutamate was lost. In fact, the level of many amino acids was decreased during the extraction, because of the ten fold dilution, whereas others were lost. Cysteine was not detected during flesh analysis, but could well become available during storage of plaice due to proteolysis. Based on the literature, these findings and the loss of TMAO occurring during the preparation of the fish substrate, the sterile fish juice was supplemented with filtered sterile solutions of the following compounds: DL - serine (Fluka), L-methionine (Sigma), L-cysteine HCl monohydrate (Sigma) and trimethylamine N-oxide dihydrate (Sigma) to reach a final concentration of 2.4 mg, 2.4 mg, 10.0 mg and 50.0 mg per 100 mL of sterile fish juice, respectively.

Determination of Spoilage Potential of Isolated Microflora. Sensory evaluation by smelling the inoculated fish juice after storage was used as the method for determination of spoilage potential of the isolated flesh microflora. Out of the 60 fish isolates extensively studied (section 3.3), only a restricted number of strains could be assessed. This is because the olfactory system is limited, i.e. only few samples can be smelled before the nose becomes saturated and incapable of distinguishing any odors, but also due to the cost involved in sensory evaluation. The sensory panel was composed of 9 to 10 IFL trained panelists.

Twenty-eight strains were chosen from most groups defined earlier, grown overnight in Iron broth at 15°C, diluted and inoculated into 10 mL sterile fish juice (in 100 mL jars) to reach a level of 10^{3-4} CFU/mL. The strains were assessed after storage at 10°C (5 days) and 0°C (13 days). Uninoculated fish juice samples were used as controls. The results were scored numerically using a scale ranging from 0 to 4, where 0 = no off-odors, ½ = on the border, 1 = trace of off-odors, 2 = some off-odors, 3 = obvious off-odors and 4 = very smelly off-odors. Characterization of the spoilage odors detected was described when possible.

Table III lists the strains that had a higher spoilage potential, i.e. with dominating scores of 3 and/or 4, and the characteristic off-odors detected by the panelists after storage at 0°C for 13 days. Other strains tested that did not produce strong off-odors belonged to the following groups: *Acinetobacter* (55), *Moraxella* (10, 21), *Moraxella*-like (7), *Cytophaga/Flavobacterium* (17, 19, 23 and 60), *Shewanella putrefaciens* (18, 33), *Pseudomonas* I (44, 53) and *Pseudomonas* II (37, 38 and 42). The results obtained for the trial conducted at 10°C were similar (data not shown) to those described in Table III, except for few strains (18, 21, 33, 37, 38, 44, 53 and 55) that could produce stronger off-odors at this temperature. Since spoilage of iced plaice should be emphasized here, as it is the common practice for storage apart from freezing, the trial conducted at 0°C should be mostly considered.

The fruity odor, which is a synergistic flavor interaction involving ethyl esters such as acetate, butyrate and hexanoate, was mentioned by Miller III *et al.* (*34*) and has been found during the early stages of fish spoilage. This odor was attributed to *Pseudomonas fragi*, a member of the *Pseudomonas* group II, and was found to change with time into a sulphidy odor. However, according to Table 3, many strains from different clusters generated that odor, among other off-odors. Fruity, rotten and sulphydryl odors are typical of the *Pseudomonas* spoilage of iced fish. *Pseudomonas* spp. have been found to produce a number of volatile and reduce nitrate to nitrite, traits that do not fit *Ps. perolens* description. *Ps. putida* (group I) produced sulphidy, sour sink odors in a study conducted by Herbert *et al.* (*16*). *Ps. fluorescens* (group I) was found to produce major sulphide compounds (methyl mercaptan and dimethyl disulfide (DMDS)) while *S. putrefaciens* produced H_2S and dimethyl trisulphide in more (*35*) and a sweaty-foot odor was reported (*2*) as well as its capability of proteolysis. This sweaty-foot aldehydes, ketones, esters and sulphides (*10, 34,35*). *Ps. perolens* (group I) was reported to produce a musty, potato-like odor at 5, 15 and 25°C by Miller III *et al.* (*33*) and found to be an extremely proteolytic organism. A potato-like odor was only detected from strain 24 (group I) but it was found to be gelatinase positive odor resembles that of butyric acid and coincides with 2 *S. putrefaciens* strains of this study, 30 and 57.

Based on the results of the tests performed during the numerical taxonomy, strains 6, 15, 20, 24 and 27 could be *Ps. fluorescens* spp., while strain 25 could rather be a *Ps. putida* or *perolens* spp. Strains of the second cluster are believed to be *Ps. fragi* spp. In summary, the potentially spoiling strains originated from all clusters (1, 2 and 3) and corresponded to most of the electrophoretic protein patterns seen before. Finally, it should be mentioned that the significance of inoculations with pure cultures can be limited. Organisms unable to spoil a fish substrate when inoculated as a pure culture should not necessarily be called a non-spoiler. Lerke *et al.* (*27*) pointed out that such organisms may behave differently in the presence of a mixed microflora and play an important role in fish spoilage. Mixed-culture experiments may be helpful in defining

Table III. List of Strains with Higher Spoilage Potential and the Characteristic Off-odors Detected after Storage at 0°c for 13 Days

Strain	*NT*	*Total score* [1]	*Off-odor description*
58	2a	30	strong smell, sweet/fruity, fermentation, sour, S-cpds, foul
57	3	22	foul, table cloth, butyric acid, putrid, cooked turnips, H_2S
30	3	22	fishy, table cloth, fruity, butyric acid, sour, putrid, H_2S, S-cpds
6	1a	21	foul, table cloth, sweet/fruity, butyric acid, gasoline, rubber-like
16	2	20	S-cpds, sweet-/fruity, gasoline, metal-like, table cloth, putrid, rubber-like, peroxide
56	2b	20	fruity/sweet, sour, gasoline, spoiled cabbage, butyric acid
52	3	19	putrid, table cloth, fishy, TMA, spoiled cabbage, sour/sweet
25	1c	19	putrid, TMA, sweet/sour, fruity, table cloth
45	2a	17	foul, table cloth, cheesy/oxidation,. fruity, sweet/sour, gasoline, fishy
20	1b	17	H_2S, putrid, foul, cabbage, table cloth, sweet/sour
15	1d	17	S-cpds, foul, sweet/sour, cabbage, oxidation, fruity, peroxide, rubber-like, table cloth
27	1b	16	H_2S, foul, fruity, sweet/sour, fermentation, table cloth
24	1a	16	H_2S, putrid, table cloth, cooked potatoes, gasoline, fruity, foul

1: Total score = (number of panelists x score 3) + (number of panelists x score 4).

spoilers. However, one must consider the relative growth rate of isolates tested, along with their ability to grow at low temperatures and initiate/produce some chemical changes.

Determination of Spoilage Activity of Selected Bacterial Strains. It has been reported by many workers that the microflora of spoiling fish stored aerobically on ice consists almost exclusively of *Pseudomonas* spp. and *S. putrefaciens* (*13*). Herbert *et al.* (*16*) found that the most important volatile compounds of spoiling fish were H_2S, methyl mercaptan (CH_3SH) and dimethyl sulphide ($(CH_3)_2S$) which were produced by the spoilers. Other microbial products have been identified in spoiling fish, and these include volatile bases (TMA, NH_3), aldehydes, ketones, esters, hypoxanthine and other low molecular weight compounds. Therefore, volatile aroma compounds could serve as potential spoilage indices. Since off-odors and -flavors developing in fish stored in air depend on the fish species and its origin (*13*), assessing the aroma profile of a fish product could be a means for following its sensory deterioration. At the end of the fish sensory shelf life, a specific spoilage pattern would be recognized as the sum of the volatile constituents produced by the spoilers of the microflora. This specific aroma profile obtained from spoiling fish can ultimately be used in SSO determination, i.e. comparing the profile to the qualitative and quantitative production of off-odors from potentially spoiling organisms in a sterile fish substrate.

Measurements of volatile compounds were done by a gas sensor instrument (FreshSense; *38*) developed at the Icelandic Fisheries Laboratories in collaboration with Element Sensor Systems (Ártorg 1, Sauðárkrókur, Iceland). This instrument, commonly called the "electronic nose", consists of a glass container (5.2 l) closed with a plastic lid, an aluminium sensor box fastened to the lid and a PC computer running a measurement program. The sensor box contained a temperature sensor and 9 different electrochemical gas sensors (CO, H_2S, NO, NO_2, SO_2 sensors from Dräger (Germany); NH_3 A7AM, H_2S, SO_2, NH_3 sensors from City Technology (Portsmouth, Britain). A converter and a microprocessor (Electronics, A/D) situated in the box were involved in the measurement reading which was sent to the PC. A fan was positioned in the container to ensure gas circulation.

Sensitivity of the gas sensors was estimated by measuring headspace of 25 mL of varying concentrations of dimethyl disulfide (DMDS) and dimethyl sulphide (DMS) diluted in water in the 5.2 l glass container. Sensitivity of the sensors was calculated as the slope of the calibration curve of varying concentration of the sulphides. The actual concentration of the components in the headspace of the samples is not known, but standards of known concentrations give an estimate of the concentration of these components. The sensors are not selective towards different compounds, but some of them have certain selectivity for specific classes of compounds. For example, the CO sensor responds well to alcohols as well as to other classes of compounds. On the other hand, the NH_3 sensors are selective for amines (*36*).

The procedure used with the gas sensor instrument for the analysis of volatile compounds in fish samples was based on a static headspace system. Each sample was put into the glass container, the lid put on and headspace of the fish sample analyzed directly in the closed glass container at room temperature. Measurements were taken every 10 seconds for 20 min. Figure 2 expresses what can be called the "spoilage pattern" proper to iced plaice (whole and gutted) at incipient and overt spoilage (days 13 and 15, respectively). The importance of the response given from the sensors is well illustrated here. CO Dräger, NH_3 A7AM (City), NO Dräger, SO_2 Dräger, NH_3 and H_2S City were the main sensors responding to the compounds found in the plaice headspace. The large increase in amine production (NH_3 A7AM and NH_3 City) passed sensory rejection was very apparent and agreed with the suddenly high total volatile base (TVB) content measured in plaice during overt spoilage as well as the high levels of H_2S-producing bacteria (data not shown).

From the 28 strains tested in section 3.4.2, 13 were found to be potential spoilers (Table III), representing most of the protein patterns defined previously. Three strains (3, 22 and 49) belonging to other patterns had been omitted and were therefore included in this study so that all common patterns were represented. The electronic nose was used to assess the spoilage activity of the selected strains, i.e. their "qualitative" and "quantitative" production of off-odors in sterile fish juice. Since the sensors used were not selective for specific compounds, the response obtained was only interpreted as a relative one providing the activity pattern for each strain tested.

The inoculation procedure followed was described in section 3.4.2. Two samples (125 mL of supplemented fish juice) were prepared for each strain and one of each incubated at 0 and 10°C for 13 and 6 days, respectively. On the sampling day, all samples were stored at 0°C until evaluated. Prior to measurement, each sample was let stand at room temperature for 30 min. Temperature is an important factor influencing the response measured. Standard conditions were therefore critical to the results obtained. The temperature was measured during headspace analysis of the samples and ranged from 13 to 16°C. Bacterial load of the inoculated fish juice samples was evaluated prior to headspace analysis for the treatment stored at 0°C, and the levels found to range from 7.09 log CFU/mL to 7.79. Lower counts did not necessarily correspond to lower responses. Figure 3 illustrates the characteristic response of the sensors to each strain tested after storage at 0°C. Strains 16 to 58 belonged to the *Pseudomonas* group II, 3 to 25 to group I and, 30, 52 and 57 were *S. putrefaciens* strains. An uninoculated fish juice sample (K) was used as a control to evaluate the normal background of the medium. The background of the fish juice primarily influenced the CO Dräger sensor which gave a response of 428 nA at 0°C. This was almost 10 times higher than that (43 nA) obtained for fresh whole plaice (data not shown). The heat-treatment (121°C for 15 min.) subjected to the raw fish juice is probably the cause of this increase. The other sensors showed a similar response whether they were evaluating the heat-treated fish juice or the fresh whole plaice.

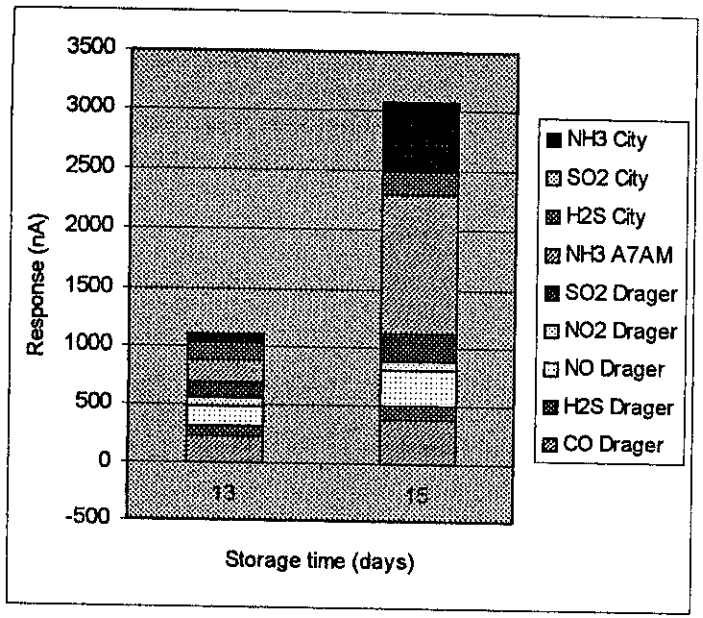


Figure 2. Specific spoilage patterns of iced plaice at incipient (d13) and overt (d15) spoilage as described by 9 different electrochemical gas sensors.

Taking into account the measured background of the fish juice (CO Dräger response of strain *minus* CO Dräger of control), the response of each sensor to each strain was corrected and compared (Table IV) to that obtained for spoiling plaice on day 13. Only values close, or contributing to some extent, to the characteristic sensor response obtained for whole spoiling plaice were considered. In that way, active spoilers could be identified. Table IV was arranged by genus to facilitate the comparison among potential spoilers.

The strains with the greatest number of responding sensors included members of *Pseudomonas* group II (22, 45, 49 and 56) especially, as well as members of group I (6 and 15). The volatile compounds produced by group I were primarily detected by CO and H_2S Dräger sensors, while those produced from group II triggered many sensors, especially SO_2 and H_2S sensors (from Dräger and City) as well as both amine sensors. On the other hand, no amines could be detected from *S. putrefaciens* strains after incubation at 0°C for 13 days. However, incubation at 10°C for 6 days (data not shown) enhanced the production of volatile compounds of certain bacteria, such as *S. putrefaciens* strains 52 and 57 and *Pseudomonas* group I strains 6, 15, 24 (apparently

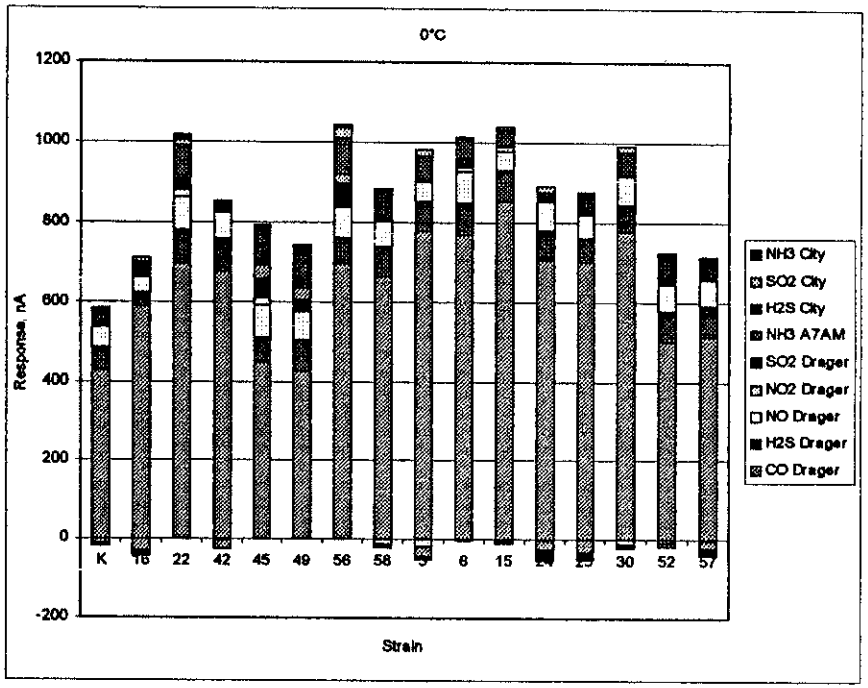


Figure 3. Characteristic response of the sensors to each strain tested after storage at 0°C. (*Pseud.* group I = 16 to 58, group II = 3 to 25; and *S. putrefaciens* = 30, 52, 57).

Ps. fluorescens) while other strains (22, 42, 45, 49, 56 and 58) belonging to the *Pseudomonas* group II (apparently *Ps. fragi*) lost some of their activity. The production of volatile compounds by strains 3, 16, 25 and 30 was not influenced by temperature (within range tested).

It is interesting to mention that no amine production could be detected at 0°C by *S. putrefaciens* strains while it was detected after storage at higher temperatures (data not shown). In fact, almost all sensors were triggered by the volatile compounds produced by *S. putrefaciens* after 6 days of storage at 10°C as opposed to a poor production at 0°C. This difference may explain the conflicts seen in the literature concerning the importance of *S. putrefaciens* in spoilage of fresh fish. Findings of high and very low levels of this organism at the end of sensory shelf life have been reported. This variation could probably be due to the initial quality of the raw material used in the experiment, the means by which the end of shelf life is determined and the isolation

Table IV. Corrected Response (nA) of Different Sensors to the Headspace of Inoculated Fish Juices (0°C) Compared to That of Spoiling Plaice after 13 Days of Ice Storage

Sample	*Drager sensors*				*City sensors*				
	CO	H_2S	NO	NO_2	SO_2	NH_3	H_2S	SO_2	NH_3
Plaice	197	18	129	57	123	48	107	9	206
3	349	18					14		
6	344	19	30	6					
15	425	22		3				7	
24	280							14	
25	275								
16								10	
22	271	24	35	13	19		38	9	23
42		22						4	
45			38	10	43	44	50	6	27
49		20			14	40	47	6	
56	269	8	28		51	33	56	23	
58		16					34	7	
30	351							11	
52		22						6	
57		18						7	

technique used. Temperature abuses on the raw material may therefore favor the proliferation of *S. putrefaciens*.

The results obtained demonstrated the importance of the various *Pseudomonas* group II spp. in the spoilage of iced American plaice as they contributed largely to the characteristic spoilage pattern defined for plaice. Still, only pure cultures were studied here and it may not totally reflect the spoilage pattern of plaice, since production of volatiles is not necessarily additive when strains are co-inoculated. Interaction of bacteria can influence the spoilage pattern of fish.

Growth Experiments on Selected Bacterial Strains. Growth experiments of bacterial strains were conducted in sterile fish juice to assess the influence of temperature on their growth rate and spoilage activities. The strains selected for this study included the most active spoilers defined in section 3.4.3, i.e. strains 22, 45, 49, 56, 6 and 15, along with other less active ones (3, 24, 30, 52, 57 and 58). Mixtures were prepared from

overnight (24 h) cultures grown in fish juice at 15°C. Table 5 lists the details concerning the strains selected. Proper dilutions were prepared for each mixture in Butterfields buffer and 100 μl pipetted into each fish juice portion (18 mL) to reach a bacterial concentration of 10^{2-4} CFU/mL. Portions to be stored at 0.5 ± 0.1°C and 5.1 ± 0.1°C were prepared in duplicate. Single portions were prepared for storage at 10.0 ± 0.0°C and 15.0 ± 0.0°C. Growth was assessed on Iron agar (IA) for all mixtures, assessing for black colonies in the presence of *S. putrefaciens*. Additionally, CFC medium was used for mixtures 4 and 7 to differentiate between *Pseudomonas* groups I (fluorescent) and II. One milliliter of inoculated fish juice was pipetted from each portion sampled (after reversing it carefully for mixing), diluted accordingly and plated using the Spiral Plater (model D; Spiral Systems Inc., Cincinnati, Ohio). The plates were incubated at 15°C for 3-4 days and counted using a Laser Bacteria Colony Counter (model 500A; Spiral System Instruments). Each portion was smelled carefully to avoid contamination and any off-odors noted.

The behavior of the bacterial mixtures were tested at 4 temperatures, from 0.5 to 15°C. From the mixtures, including only members of the same group (M-1, M-2 and M-3), it was evident that at 0.5 and 5.1°C the *Pseudomonas* group I and II strains proliferated at a similar rate, whereas *S. putrefaciens* strains grew less rapidly. However, similar growth curves were obtained at 10.0 and 15.0°C for these mixtures (results not shown). On the other hand, when *Pseudomonas* group I spp. were co-inoculated with group II spp. (Mix-4), the latter group always dominated over the former group at all temperatures tested, probably due to the production of some inhibitory compounds. In parallel, when *S. putrefaciens* strains were co-inoculated with strains of the *Pseudomonas* group I (Mix-5), growth of the former was slower at 0.5 and 5.1°C, perhaps due to inhibition or probably because of its longer generation time at these low temperatures as seen with Mix-3. Both groups (Mix-5) behaved similarly at the higher temperatures (10.0 and 15.0°C), with *S. putrefaciens* dominating towards the end of the storage period where off-odors started to be detected. The presence of *Pseudomonas* group II strains obviously affected the growth of *S. putrefaciens* strains (Mix-6) at 0.5°C, with less influence as temperature increased. When all groups were mixed (Mix-7), the dominating group (faster strains) was *Pseudomonas* II spp. at all temperatures studied. *Pseudomonas* group I strains were the next important group at the lower temperatures, while *S. putrefaciens* strains became more important at the higher temperatures.

Table V. Strains Used in the Preparation of the Bacterial Mixtures

Mixture name	*Bacterial composition*	*NT-cluster*	*Bacterial identification*
M-1	3-6-15-24	1 a & d	*Ps. fluorescens*
M-2	22-45-49-56-58	2a & b	*Ps. fragi*
M-3	30-52-57	3	*S. putrefaciens*
M-4	M-1 + M-2	1 & 2	*Ps. fluorescens & Ps. fragi*
M-5	M-1 + M-3	1 & 3	*Ps. fluorescens & S. putrefaciens*
M-6	M-2 + M-3	2 & 3	*Ps. fragi & S. putrefaciens*
M-7	all strains	1, 2 & 3	*Ps. fluor., Ps. fragi & S. put.*

Table VI summarizes the growth experimental data obtained for the different mixtures studied. At 0.5°C, *Pseudomonas* II spp. were responsible for the early off-odor detection, as demonstrated by Mix-2, 6 and 7, while *Pseudomonas* group I and *S. putrefaciens* spp. were considered as late spoilers. With increasing temperature, both *Pseudomonas* II and *S. putrefaciens* ssp. were involved in early spoilage (Mix-2, 3, 5, 6, 7). Interestingly, co-inoculation of both *Pseudomonas* groups (I and II) did not lead to early off-odor detection, as opposed to co-inoculating *Pseudomonas* group II spp. with *S. putrefaciens* strains or mixing all the groups.

This brings to conclude that *Pseudomonas* groups I and II spp. had similar growth behavior when grown alone in fish juice within the temperature range 0.5 to 15.0°C, while growth of *S. putrefaciens* was not as efficient at 0.5°C, but similar to the other ones at higher temperatures. Co-inoculation of these bacterial groups resulted in different behaviors. *Pseudomonas* group II spp. inhibited the growth of *Pseudomonas* group I spp. (all temperatures) and *S. putrefaciens* (0.5°C), and was found to dominate over the other groups when all mixed within the temperature range tested. These findings agree with the composition of the microflora established at spoilage on plaice stored within the temperature range -1.7 to 9.9°C where *Pseudomonas* II spp. were the dominating group. However, rapid growth was not necessarily concomitant with early off-odor detection, i.e. *Pseudomonas* II spp. produced off-odors well before *Pseudomonas* I spp. did, even though similar growth was observed. *S. putrefaciens* spp. were only found to be early spoilers at the higher temperatures.

Table VI. Off-odor Detection Time, Odor Description and TVC for Each Mixture Inoculated into Sterile Fish Juice and Incubated at Various Storage Temperatures

Mixture	*Temp. (°C)*	*Detection time (h)*	*Odor description*	*TVC (CFU/ mL)*
M-1	0.5	276.5	sulphidy	1×10^8
M-2		**227.5**	**Fruity**	1×10^8
M-3		276.5	fishy, NH_3, TMA	1×10^8
M-4		276.5	Fruity	1×10^8
M-5		276.5	dog food (S-cpd)	1×10^8
M-6		**227.5**	**Sulphidy**	4×10^7
M-7		**253.5**	**Fruity**	6×10^7
M-1	5.1	133	Sulphidy	6×10^7
M-2		133	Sweet	5×10^7
M-3		133	fishy, NH_3	2×10^8
M-4		133	sweet, sulphidy	1×10^8
M-5		133	Sulphidy	1×10^8
M-6		133	Sweet	1×10^8
M-7		133	Sulphidy	1×10^8
M-1	10.0	96	Sulphidy	2×10^8
M-2		**69**	**Fruity, sweet**	7×10^7
M-3		**69**	**fishy (a little)**	2×10^8
M-4		96	Sulphidy	2×10^8
M-5		**69**	**Sulphidy**	6×10^7
M-6		**69**	**Fruity, fishy**	1×10^8
M-7		**69**	**Fruity, fishy**	1×10^8
M-1	15.0	58.5	Sulphidy	1×10^8
M-2		**34.5**	**Sweet**	2×10^7
M-3		**34.5**	**Burnt match**	4×10^7
M-4		58.5	Sulphidy	1×10^8
M-5		**44**	**fishy (a little)**	2×10^8
M-6		**44**	**fishy (a little)**	1×10^8
M-7		**44**	**Sulphidy**	1×10^8

Conclusion

Pseudomonas I-II spp. were found to dominate the microflora of spoiling American plaice in the temperature range of -1.7 to 9.9°C under aerobic conditions, while *S. putrefaciens* contributed to 2 to 7% of the total flesh microflora at the end of sensory shelf life. Under microaerophilic storage conditions (water-ice mixture), a different microflora had established in the flesh of spoiling plaice, including mainly *S. putrefaciens*, *Pseudomonas* III-IV, *Acinetobacter* and *Moraxella* spp. TMA was not a good spoilage index. TVB is a possible chemical index of fish spoilage, but was neither found to be an apparent one as it increased by small amounts in plaice stored on ice and in the water-ice mixture till sensory rejection (spring trial, data not shown).

Comparative results of bacterial classification were obtained by the numerical taxonomic and SDS-PAGE whole-cell protein patterns studies which facilitated the screening of the bacterial isolates prior to the determination of the spoilage organisms. Members of the *Pseudomonas* groups I and II, and *S. putrefaciens* genus were found to produce off-odors in a sterile fish substrate. *Pseudomonas* II spp., viz. *Ps. fragi*, were found to be the main potential and active bacterial group involved in the spoilage of American plaice, especially at chill temperatures. They proliferated at a faster rate when co-inoculated with other bacterial groups and hindered their growth, especially at the lower temperatures. *Pseudomonas* I spp. (*Ps. fluorescens*) were found to be late off-odor producers, mainly active at lower temperatures. *S. putrefaciens* grew poorly at the lower temperatures and showed little spoilage activity, as opposed to enhanced growth and activity at higher temperatures. Under temperature abuse, it is likely that *S. putrefaciens* will proliferate and produce additional volatile compounds adding to the spoilage pattern of plaice.

Other groups of bacteria isolated from the microflora at the end of sensory shelf life of plaice, such as *Cytophaga/Flavobacterium*, *Acinetobacter*, *Moraxella* and *Moraxella*-like spp., were not found to be potential spoilers when inoculated alone. These organisms may behave differently in presence of a mixed microflora, but the scope of this work did not allow to study all possible combinations of bacterial mixtures. Only the behavior of the potentially spoiling bacteria when mixed to other potential groups was evaluated. The role of the non-evident spoilers among the members of the spoilage microflora is of course unclear, but it is possible that proteolysis may be one of them. For instance, Castell and Mapplebeck (*3*) reported the proteolytic action of some strains of *Flavobacteria* in a study conducted on fish spoilage.

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Chapter 26

The Effect of Ozone on the Microbiological Quality of Cod *(Gadus morhua)* and Mackerel *(Scomber scombrus)*

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The effects of ozone on the quality and storage life of cod and mackerel samples were studied using microbiological (total viable count, histamine-forming bacteria, *Pseudomonas* spp., coliforms, faecal coliforms and *Escherichia coli)* and sensory analysis during storage. The results showed that fish samples treated with ozone and ozone + UV light did not appear to significantly enhance their microbiological and sensory quality. The microbiological quality of ozonated refrigerated seawater (RSW) used to treat cod and mackerel samples was also studied during storage, but differences between control and ozone and ozone + UV light treatments were not significant (P>0.05).

The rapid deterioration of fish has always been, and still is, one of the main factors that has limited the consumption of this important food product. Therefore, it is of particular importance for fish processors or product manufacturers to extend the storage life of fish in any possible way such as ozonation.

Ozone (O_3) is an allotrope of oxygen, highly toxic and reactive. Ozone kills microorganisms by oxidizing and destroying their cell walls or membranes. It has the advantage of being able to kill resistant microorganisms such as spores and viruses at relatively low concentrations without requiring a long contact time (*17*). In humans,